CHAPTER – 4:

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1. COLLECTION AND IDENTIFICATION OF THE PLANT

Diplazium esculentum, especially the young frond portion of the plant was collected from different areas of North Bengal University campus, and also from the adjoining regions of Darjeeling, West Bengal, India. These were identified by Prof. A. P. Das, Plant Taxonomy Laboratory, Department of Botany, University of North Bengal and three voucher specimens (Accession No. 9601, 9602 and 9603) were submitted to his herbarium centre.

4.2. PREPARATION OF THE PLANT MATERIAL

4.2.1. Unboiled and Boiled aqueous preparations of the plant material (Crude *D. esculentum*; CDE and Boiled *D. esculentum*; BDE)

Young frond of *D. esculentum* was washed carefully by tap water, then cut into small pieces, and divided in to two parts. The first part was finely mixed by a mixer and dried in an incubator at 40°C until completely dried. This dried plant material (CDE) was then kept at 4°C for future use. CDE has been used in some of the *in vivo* and few of the *ex vivo* experiments to compare the toxic effects (if any), between the CDE and BDE.

The second part of *D. esculentum* (100 g) was boiled with 1000 ml of distilled water for 30 min. The boiled plant material was then finely mixed using a mixer and dried in an incubator at 60°C until completely dried. This dried plant material (BDE) was then kept at 4°C for future use. BDE has been used in all of the *in vivo* and few of the *ex vivo* experiments, to investigate the effect of heat resistant toxic compound, if any, that may escape heat during cooking, and thereby create possible harmful effects upon ingestion.

4.2.2. Preparation of the methanolic plant extract

Samples were prepared according to a previously described method (Hazra et al., 2008). Briefly, the young fronds of *D. esculentum* were dried at room temperature for 7 days and finely powdered, and used for extraction. The powder (100 g) was mixed with 500 ml methanol:water in a ratio of 7:3 using a shaker for 15 h; then the mixture was centrifuged at 2850× *g* and the

supernatant was decanted. The pellet was mixed again with 500 ml methanol-water and the entire process was repeated once again, i.e., the extraction procedure was done twice. The supernatants, collected from the two phases, were mixed in a round-bottom flask and concentrated under the reduced pressure in a rotary evaporator. The concentrated extract was then lyophilized. The residue was kept at –20°C for future use. Double-distilled water (MilliQ grade) was used as the solvent for the lyophilized extract in all the experiments. This preparation has been used in most of the *in vitro*/*ex vivo* experiments.

4.3. ANIMALS AND CARE

Both male and female Swiss albino mice (*Mus musculus*) (25 ± 2 g of body weight (b.wt.)) of 6–8 weeks of age were used for all the studies. Animals were housed in polypropylene cages, with dust free paddy husk as bedding material. They were maintained in the animal house, Department of Zoology, University of North Bengal with food and water *ad libitum* under a constant 12 h dark/light cycle at an environmental temperature of 25 ± 2°C. Guinea pigs (250 g b.w.) were used for obtaining the complement for plaque-forming cell (PFC) assay. Sheep RBC (sRBC) was collected from sheep, maintained in the departmental animal house, and used for sensitizing the mouse. All the experiments were performed after obtaining the approval from the Institutional Animal Ethical Committee (IAEC) (Registration No. 840/ac/04/CPCSEA).

4.4. STUDY OF THE IMMUNOMODULATORY ACTIVITY OF *D. ESCULENTUM*

4.4.1. *In vivo* experiments

4.4.1.1. Dosage

One hundred twenty (120) Swiss albino mice were divided in to five sets (S 1–5) and each set was sub-divided in to four groups (G 1–4). Therefore, each group contained six mice. All the animals were fed with CDE and / or BDE orally with the help of a syringe specially designed by us. Group 1 (G1) of all the sets were considered as control where 0.4 ml of distilled water was given. Mice of Group 2 (G2), Group 3 (G3) and Group 4 (G4) were fed with 0.4 ml of CDE and BDE at the dose of 80, 160 and 320 mg/kg b.wt., respectively. In this way, all groups of S1 were treated daily for 15 d, S2 daily for 45 d, S3 daily for 90 d, S4 daily for 135 d and S5 daily for 180 d.

It is assumed that the average amount of *D. esculentum* consumed by a 60 kg weighed individual is about 20 g/d. Keeping this ratio in mind, we have formulated the different doses for an average adult mouse of 25 g, like 80 mg/kg body weight, i.e. 2 mg/mouse/d; 160 mg/kg body weight, i.e. 4 mg/mouse/d and 320 mg/kg body weight, i.e. 8 mg/mouse/d.

4.4.1.2. Experimental design for in vivo experiments

The *in vivo* experiments were divided in to two parts. At first, some of the CDE and BDE-treated mice were sensitized with sRBC (0.1 ml, 25% suspension in PBS) through lateral tail vein in the following manner: Set 1 mice were sensitized on the 11th day of the experiment whereas, Set 2, Set 3, Set 4 and Set 5 mice were sensitized on 41st day, 86th day, 131st day and 176th day of experiment, respectively. The day of sensitization in each case was designated as day ' 0 '. These sensitized mice were used for PFC assay and hemaggutination antibody (HA) titer assay.

Second part of the *in vivo* experiments included the measurement of the body weight, relative spleen weight, counting of the splenocytes, and counting of peritoneal macrophages of the CDE and BDE mice. Some of the CDE and BDE treated mice from each group were sacrificed after proper anesthesia (Chloroform and ether in 2:1 ratio) 24 h after the last dose, and the body weight, relative spleen weight and counting of the splenocytes were determined. The remaining CDE and BDE treated mice were used to perform the peritoneal macrophage counting assay.

4.4.1.3. PFC assay

The PFC assay was performed according to the previously described method (Raisuddin et al., 1991) with slight modifications. On the 4th day of sensitization with sRBC, single cell suspension from the spleen of these sensitized mice was prepared in PBS and cells were adjusted at a concentration of 2 x 10^6 cells/ml. For PFC assay, 0.1 ml of this suspension was mixed with 0.05 ml of guinea pigs complement and 0.05 ml of 25% sRBC (prepared in PBS) to prepare the final mixture. Cuningham chambers were prepared using glass slide and bi-gummed tape (Scotch Brand, St. Paul, MN). The chambers were loaded with a known volume of assay mixture, sealed with paraffin and petroleum jelly (1:1) and incubated at 37°C for 4 h. After incubation, the

plaques were counted under a phase contrast microscope and expressed as PFC per 10^6 spleen cells.

4.4.1.4. Collection of serum HA titer assay

The serum was collected from the blood of the sensitized mouse on the 4th day of sensitization with sRBC. Blood was collected from tail vein as a standard method. The mouse was sacrificed after proper anesthesia (Chloroform and ether in 2:1 ratio) and blood was collected from the heart also. The collected serum was divided in to two parts. First part was stored at -20°C for future use. These serum samples were further used to determine the concentration of immunoglobulin M (IgM). Second part was kept for 45 min at 56°C in a water bath for the inactivation of complement activity. Eight clear Khan tubes were taken in a rack and marked. In the first tube, 0.1 ml of the serum and 0.9 ml of PBS was added. 0.5 ml of PBS was taken to the subsequent tubes. Then 0.5 ml of the mixed solution from the first tube was added to the second tube, and 0.5 ml of the mixture from the second tube was added to the third tube. In this way, eight such dilutions (double fold) were prepared. From the last tube, 0.5 ml of solution was thrown away thus yielding a serial dilution of 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640 and 1/1280. This is known as double-fold dilution. Now, 0.1 ml of 10% sRBC (prepared in PBS) was added to each tube and the entire set up was incubated at 37°C for 12 h in a humidified atmosphere. After incubation, visible hemagglutination was observed and noted down.

4.4.1.5. Measurement of the body weight, relative spleen weight and counting of the splenocytes

Mouse was sacrificed after proper anesthesia (Chloroform and ether in 2:1 ratio) 24 h after the last dose of CDE and BDE. Body weight was measured and blood was collected from the heart and serum was separated and stored at -20°C for future use. These serum samples were further used to determine the concentration of different cytokines. The relative spleen weight (spleen weight/100 g of body weight) was also recorded. Single cell suspension of the spleen, prepared in RPMI-1640 medium, was counted using hemocytometer.

4.4.1.6. Counting of peritoneal macrophages

Freund's incomplete adjuvant (0.5 ml) was injected in the peritoneum of the CDE and BDE treated mouse 24 h prior to the experiment. Two ml of PBS was injected in the peritoneum on the next day and the peritoneal exudate cells were collected from the mouse under proper anesthesia. Cells were washed two times with PBS. The pellet was then resuspended in PBS, taken in a glass petridish and incubated for 45 min at 37°C. After incubation, the supernatant was removed and the petridish was washed with chilled PBS to collect the macrophages and centrifuged at 1000 rpm for 5 min. The pellet was resuspended in PBS and a small amount of this cell suspension was mixed with equal volume of neutral red and charged on the hemocytometer to count the number of the live macrophages under a microscope.

4.4.2. *Ex vivo* experiments

4.4.2.1. Effect of CDE and BDE on primary cultured splenocytes

Effect of CDE and BDE on mouse splenocytes were determined according to the previously described method (Yeap et al., 2010), with slight modifications. Splenocyte suspension was prepared in RPMI-1640 medium (containing 50 U/ml penicillin, 50 U/ml streptomycin and 50 U/ml nystatin). The cell number was adjusted to 2×10^6 cells per ml and 1 ml of the cell suspension with 10% of FBS was added in six-well culture plates. Five microliters of concanavalin A (5 μg/ml) and 100 μl of different concentrations (0–200 μg/ml) of CDE and BDE (suspended in RPMI 1640) were added to this and the whole set up was incubated for 24, 48 and 72 h at 37°C in an incubator having 5% CO_2 and 90% humidity. After the incubation period, the cultures were harvested and washed once at 1000 rpm for 5 min. The cell pellet was then resuspended in 0.5 ml of RPMI-1640 medium. Then, 10 μl of cell suspension was mixed with equal volume of 0.4% trypan blue and was counted by using hemocytometer under the phase contrast microscope. Only the viable cells were counted.

4.4.2.2. MTT splenocyte proliferation assay

MTT proliferation assay was carried out according to the previously described method (Mosmann, 1983) with slight modifications. Mouse splenocyte cell suspension was prepared (conc. 2×10^6 cells/ml) in RPMI 1640 medium (containing 50 U/ml penicillin, 50 U/ml

streptomycin and 50 U/ml nystatin). Then in each well of a 96 well microtiter plate, 100 µl of cell suspension was added with 10% of FBS and 100 µl of different concentrations (0–200 µg/ml) of CDE and BDE (suspended in RPMI 1640). The plate was then incubated for 24 h in 37°C incubator having 5% CO_2 and 90% humidity. After the incubation period, 20 µl of MTT solution (5 mg/ml, dissolved in PBS; pH 7.0) was added to each well. The plate was covered and incubated for 4 h at 37°C in an incubator. After incubation, 150 µl of the suspension from each well was taken out without disturbing the bottom layer, and 150 µl of dimethyl sulphoxide (DMSO) was added to each well and mixed thoroughly. Finally, the optical density (O.D.) was taken in a microplate reader at 540 nm.

4.4.2.3. Hemolytic assay

Hemolytic effects of CDE and BDE on mouse erythrocytes were evaluated by using washed erythrocytes (RBCs). A previously described method was followed for the preparation of mouse erythrocytes (Malagoli, 2007). Blood sample from Swiss albino mouse was collected (each weighing 25 ± 2 g) in citrated tubes. The cells were then washed three times with 20mM Tris–HCl containing 144mM NaCl (pH 7.4) and a 2% erythrocyte suspension was prepared. The hemolytic activities of CDE and BDE were tested according to a previously described method under *in vitro* conditions in 96-well plates (Malagoli, 2007). Each well received 100 µl of 0.85% NaCl solution containing 10mM $CaCl_2$. The first well served as negative control containing only solvent. One hundred microliters of CDE and BDE of various concentrations (0–50 µg/ml, suspended in 0.85% NaCl solution containing 10mM $CaCl_2$) were added from the second well. The last well was served as positive control containing 100 µl of 0.1% Triton X-100 in 0.85% saline. Each well then received 100 µl of a 2% suspension of mouse erythrocytes in 0.85% saline containing 10mM $CaCl_2$. Cells were centrifuged after 30 min incubation at room temperature and the supernatant was collected to measure the absorbance of the liberated hemoglobin at 540 nm. The average value was calculated from six assays.

4.4.2.4. Primary culture of splenocytes for cytokine estimation

Spleen was aseptically removed from mouse and cell suspension was prepared in minimum essential medium (MEM), containing penicillin-streptomycin (50 U/ml) and nystatin (50 U/ml).

The cell number was adjusted at 2×10^6 cells per ml and 1 ml of the cell suspension was added in each well of a six-well culture plate. Each well was then supplemented with 10% goat serum (Chaudhuri & Chakravarty, 1983). Five microlitres of concanavalin A (5 μg/ml) was also added to stimulate cytokine production. Finally, 100 μl of different concentrations (0–200 μg/ml) of CDE and BDE (suspended in MEM) were added to each well. The whole set up was then incubated for 48 h at 37°C in an incubator having 5% CO_2 and 90% humidity. Supernatants of cell cultures were collected after 48 h and used for cytokine estimation.

4.4.3. Estimation of IgM and Th1 and Th2 cytokine concentration

Previously collected serum samples and splenocyte culture supernatants were used to determine the concentrations of Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) cytokines by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (RayBiotech, Inc., USA). Serum samples from sRBC sensitized mouse were used to determine the concentration of IgM. Briefly, a 96-well flat bottom plates were coated with the captured antibody specific to each cytokine and IgM. One hundred microliters of serially diluted specific standards for each cytokine and IgM and 100 μl of the serum/cell culture supernatants (samples) were pipetted into the wells. The specific cytokine and IgM present in the sample were bound to the wells by the immobilized antibody. The wells were washed and biotinylated anti-mouse detection antibody specific for each cytokine and IgM were added. After washing away the unbound biotinylated antibody, HRP-conjugated streptavidin was pipetted to the wells. The wells were washed again and TMB substrate solution was added to the wells and the color was developed in proportion to the amount of specific cytokines and IgM bound. Finally, the stop solution was added which changed the color from blue to yellow and the intensity of the color was measured at 450 nm in a microplate reader.

4.5. EFFECT OF *D. ESCULENTUM* ON THE REPRODUCTIVE FUNCTIONS OF MALE SWISS ALBINO MOUSE

4.5.1. Dosage

One hundred twenty (120) male Swiss albino mice were divided in to five sets (S 1-5) and each set was sub-divided in to four groups (G 1-4). Therefore, each group contained six mice. Group 1

(G1) of all the sets were considered as control where 0.4 ml of distilled water was given orally. Group 2 (G2), Group 3 (G3) and Group 4 (G4) of all the sets were fed with 0.4 ml of CDE and BDE at the dose of 80 mg/kg b.wt., 160 mg/kg b.wt., and 320 mg/kg b.wt., respectively with the help of a syringe specially designed for this purpose. In this way, all groups of S1 (G1S1 to G4S1) were treated daily for 15 days, S2 (G1S2 to G4S2) daily for 45 days, S3 (G1S3 to G4S3) daily for 90 days and S4 (G1S4 to G4S4) daily for 135 days, and S5 (G1S5 to G4S5) daily for 180 days.

4.5.2. Collection of serum sample and preparation of the sperm suspension

Mouse from each group was sacrificed after proper anesthesia (chloroform and ether in 2:1 ratio) 24 h after the last dose. Blood was collected from the heart and serum was separated. The serum samples were used for the determination of total protein content. Caudal epididymis of mouse from each group was separated and minced using a pair of small scissors to release the sperm into 10 ml warmed (37°C) physiological saline. The sperm suspension was used for hypo-osmotic swelling test (HOST) and MTT reduction assay.

4.5.3. Hypo-osmotic swelling test (HOST)

Hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane, based on coiled and swollen tails. This was performed by incubating 30 µl of semen with 300 µl of 100 mOsm hypo-osmotic solution (9 g of fructose + 4.9 g of sodium citrate per liter of distilled water) at 37°C for 60 min. After incubation, 0.2 ml of the mixture was spread with a cover slip on a pre-warmed slide. A total of 200 spermatozoa were counted in different fields using a phase-contrast microscope. Sperms with swollen or coiled tails were recorded (Buckett et al., 1997; Revell and Mrode, 1994).

4.5.4. MTT reduction assay of sperm

The sperm suspension was placed in an incubator at 37° C for 10 minutes prior to perform the viability test. The MTT (3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide) assay was performed according to the previously described method (Mosmann, 1983). Briefly, the sperm suspension was diluted using phosphate buffered saline (PBS) and the number was adjusted at 30×10^6 spermatozoa/ml. Twenty four wells of a 96-well microplate were used to

assess the sperm viability of S1 mice (G1S1, G2S1, G3S1, G4S1). One hundred microliters of sperm suspension from G1S1 mouse was placed in first six wells of first column of the microplate. Similarly, 100 µl of sperm suspension from G2S1, G3S1, and G4S1 mice was placed in the six wells of the second, third and fourth columns of the microplate, respectively. Therefore, a total of 24 wells of the microplate were occupied with the sperm suspension of all the groups of S1 mice. Ten microlitres of MTT stock solution (5 mg/ml, dissolved in PBS; pH 7.0) was added to each of these 24 wells and mixed properly and the rates of MTT reduction (measured as optical density) were then recorded immediately (first reading) using a microplate reader (Bio-Rad, USA). The plate was then incubated at 37°C for 1 h and the optical density was recorded (second reading) again. The MTT reduction rate (change in optical density) was determined by calculating the difference between the first and second reading of the microplate reader, for each group of mouse. MTT reduction rates of the spermatozoa of S2, S3, S4 and S5 mice were evaluated in the similar way as mentioned above.

4.5.5. Tissue biochemistry

4.5.5.1. Testis

Total protein and cholesterol contents were determined in testicular tissue, and the samples were prepared according to a previously described method (Hammami et al., 2008). Briefly, a part of testis (about 0.5 g) was crushed in 2 ml of 0.9% normal saline. The homogenate was centrifuged at 13000 × g for 10 min. The supernatant was collected and used for the determination of total protein and cholesterol contents using commercially available standard biochemical assay kits (Crest Biosystems, Goa, India). Glycogen content in testis was determined by a previously described method (Montogomery, 1957).

The sample was prepared according to a previously described method (Hammami et al., 2008), with little modification for the determination of acid phosphatase activity. Briefly, 0.5 g of testicular tissue was homogenized in 2 ml of citric acid buffer (0.1M citric acid, 0.2M Na_2HPO_4, pH 6.2, supplemented with 0.4% Triton X-100 solution) and centrifuged at 18000 × g at 4ºC for 30 min. The supernatant was used to determine the acid phosphatase activity using

commercially available standard biochemical assay kits (Crest Biosystems, Goa, India). Acid phosphatase activity was expressed as μM/min/g of tissue.

4.5.5.2. Epididymis

Alpha-glucosidase activity in epididymis was measured according to a previously described method (Hammami et al., 2008) with little modification. Briefly, the caudal epididymis of mouse was cut, homogenized in citric acid buffer (0.1M citric acid, 0.2M Na_2HPO_4, pH 6.2, supplemented with 0.4% Triton X-100 solution) and centrifuged at 18000 × g at 4ºC for 30 min. The alpha-glucosidase activity was measured according to a previously described colorimetric method (Wang et al., 1999). The reaction system contained 1.2 ml buffer (69 mM citric acid, pH 6.8), 0.2 ml paranitrophosphateglycerol (PNPG, 23 mM) and 0.2 ml supernatant. The reaction medium was incubated at 37ºC for 4 h and 0.25 ml Na_2CO_3 (0.1 M) was added to stop the reaction. The absorbance was measured at 400 nm in a spectrophotometer and the PNP content was estimated in reference to the PNP standard curve. The α-glucosidase activity was expressed as μmol/min/g of tissue. The supernatant was also used to determine the total protein contents in epididymis using commercially available standard biochemical assay kits (Crest Biosystems, Goa, India). The concentration of sialic acid in the epididymis was estimated according to the standard thiobarbituric acid method (Aminoff, 1961).

4.5.5.3. Prostate and seminal vesicle

Sample was prepared from prostate and seminal vesicles according to a previously described method (Hammami et al., 2008). Briefly, 0.2 g of tissues were homogenized in 2 ml of 0.33% perchloric acid at 4ºC and centrifuged at 2500 × g for 10 min. Then, 1 ml of the supernatant was added to 0.5 ml K_2CO_3 (0.75M). The reaction mixture was then centrifuged at 2500 × g for 10 min and supernatant was used for the determination of citric acid content of prostate and fructose content of seminal vesicles. The fructose content was determined according to a previously described protocol (Anderson et al., 1979), with little modifications. Briefly, 100 μl of supernatant was added to distilled water to give a total volume of 0.5 ml. The reaction tube was then placed in a boiling water bath for 7 min, and centrifuged at 10000 × g for 20 min to remove the precipitated material. 0.3 ml of the supernatant was added to 1.5 mM of NADH and sorbitol

dehydrogenase preparation in sodium phosphate (0.1 M, pH 6.8) to give a final reaction mixture volume of 1 ml. The concentration of fructose was determined by comparing the initial rate of decrease in absorbance at 340 nm with that of fructose standard. The concentration of citric acid in prostate was determined according to the World Health Organization semen analysis manual (WHO, 1999).

4.5.6. Histological analysis

A part of the testis was put into Bouin's fluid for fixation, and subsequently embedded in paraffin for the histological sections followed by the microscopic examination in accordance with the routine laboratory procedure. Paraffin sections of 4–5 μm were prepared and stained with haematoxylin and eosin for histological examination. Histomorphometric analysis was performed by KLONK Image Measurement Light software (Version: 13.2.2.12).

4.5.7. Male fecundity/fertility test

Male mice treated for different durations (15, 45, 90, 135 and 180 days) were used for the fertility test. After the end of each treatment, each male was allowed to mate with two fertile females and they were left together for 15 days. This period is sufficient to cover the mouse estrous cycle which takes 4–5 days. After the mating test, each female was observed for delivery (19–21 days following the mating test) as a criterion of successful insemination. After the delivery, the entire litters, number of live pups and any clinical signs and mortalities were recorded. Fecundity was also calculated. Fecundity represents the ratio of the number of male parent of at least one viable pup to the total number of male mice exposed for mating ×100. Pups were followed up until adulthood.

4.6. EFFECT OF *D. ESCULENTUM* ON THE CHOLINERGIC NERVOUS SYSTEM OF MOUSE

4.6.1. *In vivo* experiments

4.6.1.1. Dosage

Forty eight (48) Swiss albino mice were divided in to two sets (S1 and S2) and each set was sub-divided in to four groups (G 1-4). Therefore, each group contained six mice. Group 1 (G1) of

both the sets were considered as control where 0.4 ml of distilled water was given orally. Group 2 (G2), Group 3 (G3) and Group 4 (G4) of both the sets were fed with 0.4 ml of CDE and BDE at the dose of 80 mg/kg b.w., 160 mg/kg b.w., and 320 mg/kg b.w., respectively with the help of a syringe specially designed for this purpose. In this way, all groups of S1 (G1S1 to G4S1) and S2 (G1S2 to G4S2) were treated daily for 30 days.

4.6.1.2. Preparation of enzyme source for in vivo assay

CDE and BDE treated mice from each group were sacrificed 24 h after the last dose using proper anesthesia (chloroform and ether in 2:1 ratio). The enzyme, acetylcholinesterase (AChE) was extracted according to a previously described method (Ashraf et al., 2011), with a little modification. A small portion of liver (0.5 g) of mouse from each group was cut, washed with 50 mM Tris-HCl buffer (pH 7.4) and homogenized manually in 10 ml extraction buffer (50 mM Tris-HCl buffer, pH 7.4; 1mM $MgCl_2$; 1mM $CaCl_2$; 0.32M sucrose) in a tissue homogenizer. The sample was homogenized continuously for 15 sec. at a time for 2 consecutive occasions, with an interval of 10 sec. in between. The tube was placed in ice bucket to avoid heating during the homogenization procedure. After homogenization, the contents were filtered through double layers of Whatman filter paper No. 1 and centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant was used as a source of cholinesterase enzyme. Enzyme source was made fresh everyday and used within 4 hours. Protein was determined by Bradford method and 40-60 µg protein (10 µl) was used per assay.

4.6.1.3. Determination of acetylcholinesterase activity

The AChE activity was determined according to a standard protocol (Ellman et al., 1961), with little modifications. A 0.4 ml aliquot of the supernatant (enzyme source) was added to a test tube containing 2.6 ml of phosphate buffer (pH 8.0, 0.1 M). One hundred microlitre of 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB) reagent (0.01 M) was added to it and the absorbance was measured at 412 nm. After that, 20 µl of acetylthiocholine iodide substrate (0.075 M) was added to the test tube. After 15 min incubation, absorbance was measured at 412 nm. Changes in the absorbance were recorded and the rate of change in absorbance per min. was calculated as follows (Ellman et al., 1961):

$$R = 5.74\ (10^{-4}) \times (\Delta A / C_0)$$

Where, R = rate, in moles substrate hydrolyzed per min per g of tissue;

ΔA = change in absorbance per min;

C_0 = original concentration of tissue (mg/ml).

5.74 (10^{-4}) = factor for dilution, extinction coefficient, and changes in units;

4.6.2. *Ex vivo* experiments

For *ex vivo* experiments, 70% methanolic extract of *D. esculentum* (MDE) has been used.

4.6.2.1. Determination of acetylcholinesterase inhibitory activity

Enzyme activity was measured by a previously described method (Ellman et al., 1961), with brief modifications. The method for preparation of the enzyme source was similar to the enzyme preparation step as mentioned in the *in vivo* experiments. The reaction mixture (200 µl) consisted of 160 µl of 50 mM Tris HCl buffer, pH 7.4, with or without the MDE (30 µl) followed by the addition of 10 µl enzyme (40-60 µg protein) from fresh chicken liver homogenate in 96-well plates. The contents were mixed and pre-incubated for 10 min at 25°C. Plates were preread at 412 nm using a plate reader (BioRad, Hercules, USA). The reaction was initiated by the addition of 10 µl of 1 mM DTNB and 3 mM substrate acetylthiocholine iodide. Absorbance was measured at 412 nm within 4-7 min after 15 min incubation. Control experiments were carried out to correct for non-enzymatic hydrolysis by adding enzyme after the addition of DTNB. Absorbance values were subtracted from the control and data presented as percent inhibition of enzyme activity. Experiments were carried out with their respective controls for six times.

4.6.2.2. Determination of NADH oxidase (NOX) inhibitory activity

NOX inhibitory activity was determined according to a previously described method (Ashraf et al, 2011). The reaction mixture (200 µl) consisted of 160 µl 50 mM Tris HCl buffer, pH 7.4 containing 1 mM EDTA disodium salt, with or without MDE (30 µl) followed by the addition of 10 µl enzyme (40-60 µg protein) from fresh chicken liver homogenate. The contents were mixed and pre-incubated for 10 min at 25°C. The reaction was initiated by the addition of 10 µl of 3

mM nicotinamide adenine dinucleotide (reduced). Absorbance was measured at 340 nm using a 96-well plate reader (BioRad, Hercules, USA) after 45 min incubation at 25°C. All the experiments were carried out with the respective controls. Results are mean of six independent determinations.

4.7. ASSESSMENT OF THE ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITIES OF *DIPLAZIUM ESCULENTUM*

The 70% methanolic extract of *D. esculentum* (MDE) has been used to assess the antioxidant and free radical scavenging activities.

4.7.1. Antioxidant activity in linoleic acid system

4.7.1.1. Ferric thiocyanate (FTC) method

The method of ferric thiocyanate was followed from a previously described procedure (Kikuzaki & Nakatani, 1993) with little modifications (Mitsuda et al., 1967; Osawa & Namiki, 1981). FTC method was used to determine the amount of peroxide at the initial state of lipid peroxidation. The peroxide reacts with ferrous chloride ($FeCl_2$) to form a reddish ferric chloride ($FeCl_3$) pigment. In this method, the concentration of peroxide decreases as the antioxidant activity increases. Four ml of MDE was placed in 4 ml of absolute ethanol. Then, 4.1 ml of 2.52% linoleic acid in absolute ethanol, 8 ml of 0.05 M phosphate buffer (pH 7.0) and 3.9 ml of water were placed in a screw capped vial and then placed in an oven at 40°C in the dark. A small volume (0.1 ml) of this solution was taken and 9.7 ml of 75% ethanol and 0.1 ml of 30% ammonium thiocyanate was added in to it. Exactly 3 min after the addition of 0.1 ml of 0.02 M ferrous chloride in 3.5% HCl to the reaction mixture, the absorbance was measured at 500 nm every 24 h until the absorbance of the control reached maximum. The control and standard were subjected to the same procedures as the sample, except that for the control where only the solvent was added, and for the standard, 4 mg sample was replaced with 4 mg of α-tocopherol.

4.7.1.2. Thiobarbituric acid (TBA) method

TBA value of *D.esculentum* plant extract was determined according to a previously described method (Ottolenghi, 1959). The formation malondialdehyde is the basis for the well-known TBA

method used for evaluating the extent of lipid peroxidation. Malondialdehyde binds TBA to form a red complex at low pH and high temperature (100°C) that can be measured at 532 nm. The increased amount of the red pigment formed correlates with the oxidative rancidity of the lipid. Two ml of 20% trichloroacetic acid and 2 ml of 1 % (w/v) TBA aqueous solution were added to 1 ml of sample solution prepared as in the FTC procedure, incubated in a similar manner. The mixture was then placed in a boiling water bath for 10 min. It was centrifuged after cooling, at 3000 rpm for 20 min and the absorbance of the supernatant was measured at 532 nm. Antioxidant activity was recorded based on the absorbance of the final day of the FTC assay. Both methods (FTC and TBA) described antioxidant activity by percent inhibition:

% inhibition = [(Absorbance of control on day maximum − Absorbance of sample on the same day)/Absorbance of control on the same day] × 100

All data, about total antioxidant activity, are the average of six replicate analyses.

4.7.2. Total antioxidant activity by ABTS method

The antioxidant activity of *D. esculentum* extract was assayed depending on the ability to scavenge $ABTS^{\bullet+}$ radical cation compared to trolox standard (Re et al., 1999). The $ABTS^{\bullet+}$ radical cation was pregenerated by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and incubating for 12-16 h in dark at room temperature until the reaction was complete and the absorbance was stable. The absorbance of the $ABTS^{\bullet+}$ was equilibrated to 0.70 (±0.02) by diluting with water at room temperature. Then 1 ml of $ABTS^{\bullet+}$ was mixed with 10 µl of MDE (Conc. 0.05-10 mg/ml) and the absorbance was measured at 734 nm after 6 min. All experiments were repeated six times. The percentage inhibition of the absorbance was calculated and plotted as a function of the concentration of standard and MDE to determine the trolox equivalent antioxidant capacity (TEAC) which was calculated from dividing the gradient of the plot for the sample by the gradient of the plot for trolox.

4.7.3. Scavenging activity of DPPH radical

The ability of *D. esculentum* extract to scavenge 2,2-diphenyl-1-picryl-hydrazyl (DPPH) was determined using the previously reported procedure (Shimada et al., 1992). Briefly, 0.1 mM solution of DPPH in ethanol was prepared. Then, 1 ml of this solution was added to 3 ml of

MDE at different concentrations (0-200 µg/ml). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. The absorbance was measured at 517 nm in a spectrophotometer. Lower absorbance of the reaction mixture indicated the higher free radical scavenging activity. Alpha-tocopherol (α-tocopherol) was used as positive control.

4.7.4. Free radical scavenging activity of *D. esculentum*

4.7.4.1. Hydroxyl radical scavenging activity

This was assayed according to a standard method with slight modification (Elizabeth & Rao, 1990). This assay is based on quantification of the degradation product of 2-deoxyribose by condensation with TBA. Hydroxyl radical was generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (Fenton reaction). The reaction mixture contained, in a final volume of 1 ml, 2-deoxy-2-ribose (2.8 mM), KH_2PO_4-KOH buffer (20 mM, pH 7.4), $FeCl_3$ (100 µM), EDTA (100 µM), H_2O_2 (1.0 mM), ascorbic acid (100 µM) and various concentrations (0-200 µg/ml) of MDE or reference compound. After incubation for 1 h at 37°C, 0.5 ml of the reaction mixture was added to 1 ml 2.8% TCA, then 1 ml of 1% aqueous TBA was added, and incubated at 90°C for 15 min to develop the colour. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. All tests were performed six times. Mannitol, a classical OH• scavenger, was used as a positive control. Percentage inhibition was evaluated by comparing the test and blank solutions.

4.7.4.2. Superoxide radical scavenging activity

This activity was determined based on the reduction of NBT according to a previously reported method (Fontana et al, 2001). The non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide radicals that reduce nitro blue tetrazolium (NBT) into a purple-coloured formazan. The 1 ml reaction mixture contained phosphate buffer (20 mM, pH 7.4), NADH (73 µM), NBT (50 µM), PMS (15 µM) and various concentrations (0-20 µg/ml) of MDE. The absorbance was taken at 562 nm against an appropriate blank solution after incubation for 5 min at ambient temperature. All tests were performed six times. Quercetin was used as positive control.

4.7.4.3. Nitric oxide radical scavenging activity

At physiological pH, nitric oxide generated from aqueous sodium nitroprusside (SNP) solution interacts with oxygen to produce nitrite ions, which may be quantified by the Griess Illisvoy reaction (Garratt, 1964). The reaction mixture contained 10 mM SNP, phosphate buffered saline (pH 7.4) and various concentrations (0-70 µg/ml) of MDE in a final volume of 3 ml. After incubation for 150 min at 25°C, 1 ml sulfanilamide (0.33% in 20% glacial acetic acid) was added to 0.5 ml of the incubated solution and allowed to stand for 5 min. Then 1 ml naphthylethylenediamine dihydrochloride (NED) (0.1% w/v) was added and the mixture was incubated for 30 min at 25°C. The pink chromophore generated during diazotization of nitrite ions with sulfanilamide and subsequent coupling with NED was measured spectrophotometrically at 540 nm against a blank sample. All tests were performed six times. Curcumin was used as a standard.

4.7.4.4. Hydrogen peroxide scavenging activity

This activity was determined according to a previously described method (Long et al., 1999) with minor changes. An aliquot of 50 mM H_2O_2 and various concentrations (0-2 mg/ml) of MDE were mixed (1:1 v/v) and incubated for 30 min at room temperature. Then, 90 µl of the H_2O_2-sample solution was mixed with 10 µl of HPLC-grade methanol and 0.9 ml FOX reagent was added (prepared in advance by mixing 9 volumes of 1 mM xylenol orange and 2.56 mM ammonium ferrous sulfate in 0.25 M H_2SO_4). The reaction mixture was then vortexed and incubated at room temperature for 30 min. The absorbance of the ferric-xylenol orange complex was measured at 560 nm. All tests were performed six times. Sodium pyruvate was used as the reference compound (Floriano-Sánchez et al., 2006).

4.7.4.5. Peroxynitrite scavenging activity

Peroxynitrite ($ONOO^-$) was synthesized by a previously described method (Beckman et al., 1994). An acidic solution (0.6 M HCl) of 5 ml H_2O_2 (0.7 M) was mixed with 5 ml of 0.6 M KNO_2 on an ice bath for 1 second and 5 ml of ice-cold 1.2 M NaOH was added. Excess H_2O_2 was removed by treatment with granular MnO_2 prewashed with 1.2 M NaOH and the reaction mixture was left overnight at -20°C. peroxynitrite solution was collected from the top of the

frozen mixture and the concentration was measured spectrophotometrically at 302 nm (ε = 1670 M^{-1} cm^{-1}).

The Evans Blue bleaching assay was used to measure the peroxynitrite scavenging assay (Bailly et al., 2000), with slight modification. The reaction mixture contained 50 mM phosphate buffer (pH 7.4), 0.1 mM DTPA, 90 mM NaCl, 5 mM KCl, 12.5 µM Evans Blue, various doses of MDE (0-200 µg/ml) and 1 mM peroxynitrite in a final volume of 1 ml. After incubation at 25°C for 30 min the absorbance was measured at 611 nm. The percentage scavenging of $ONOO^-$ was calculated by comparing the results of the test and blank samples. All tests were performed six times. Gallic acid was used as the reference compound.

4.7.4.6. Singlet oxygen scavenging activity

The production of singlet oxygen ($^1O_2$) was determined by monitoring *N, N*-dimethyl-4-nitrosoaniline (RNO) bleaching, using a previously reported spectrophotometric method (Pedraza-Chaverrí et al., 2004). Singlet oxygen was generated by a reaction between NaOCl and H_2O_2, and the bleaching of RNO was monitored at 440 nm. The reaction mixture contained 45 mM phosphate buffer (pH 7.1), 50 mM NaOCl, 50 mM H_2O_2 , 50 mM histidine, 10 µM RNO and various concentrations (0-200 µg/ml) of MDE in a final volume of 2 ml. It was incubated at 30°C for 40 min and the decrease in RNO absorbance was measured at 440 nm. The scavenging activity of sample was compared with that of lipoic acid, used as a reference compound. All tests were performed six times.

4.7.4.7. Hypochlorous acid scavenging activity

Hypochlorous acid was prepared immediately before the experiment by adjusting the pH of a 10% (v/v) solution of NaOCl to 6.2 with 0.6 M H_2SO_4, and the concentration of HOCl was determined by measuring the absorbance at 235 nm using the molar extinction coefficient of 100 M^{-1} cm^{-1}. The assay was carried out according to a previously described method (Aruoma & Halliwell, 1987) with minor changes. The scavenging activity was evaluated by measuring the decrease in absorbance of catalase at 404 nm. The reaction mixture contained in a final volume of 1 ml, 50 mM phosphate buffer (pH 6.8), catalase (7.2 µM), HOCl (8.4 mM) and increasing concentrations (0-100 µg/ml) of MDE. The assay mixture was incubated at 25°C for 20 min and

the absorbance was measured against an appropriate blank. All tests were performed six times. Ascorbic acid, a potent HOCl scavenger, was used as a reference compound (Pedraza-Chaverrí et al., 2007).

4.7.4.8. Fe^{2+} chelation

The ferrous ion chelating activity was evaluated by a standard method (Haro-Vicente et al., 2006) with minor changes. The reaction was carried out in HEPES buffer (20 mM, pH 7.2). Briefly, various concentrations (0-120 µg/ml) of MDE were added to 12.5 µM ferrous sulfate solution and the reaction was initiated by the addition of ferrozine (75 µM). The mixture was shaken vigorously and incubated for 20 min at room temperature, and then the absorbance was measured at 562 nm. All the tests were performed six times. EDTA was used as a positive control.

4.7.4.9. Reducing power

The Fe^{3+}-reducing power of the extract was determined by a previously described method (Oyaizu, 1986) with slight modification. Different concentrations (0-1 mg/ml) of MDE (0.5 ml) were mixed with 0.5 ml phosphate buffer (0.2 M, pH 6.6) and 0.5 ml potassium hexacyanoferrate (0.1 %), followed by incubation at 50°C in a water bath for 20 min. After incubation, 0.5 ml of TCA (10%) was added to terminate the reaction. The upper portion of the solution (1 ml) was mixed with 1 ml distilled water, and 0.1 ml $FeCl_3$ solution (0.01%) was added. The reaction mixture was left over for 10 min at room temperature and the absorbance was measured at 700 nm against an appropriate blank solution. All the tests were performed six times. A higher absorbance of the reaction mixture indicated greater reducing power. Ascorbic acid was used as a positive control.

4.7.4.10. Lipid peroxidation inhibition assay

This assay was carried out according to a previously described method (Kizil et al., 2008), with slight modification. Brain homogenate was prepared by centrifuging Swiss albino mice brain (20 ± 2 g b.w.) with 50 mM phosphate buffer (pH 7.4) and 120 mM KCl, at 3000 rpm for 10 min. A 100 µl aliquot of the supernatant homogenate was mixed with MDE of various concentrations

(0-25 µg/ml), followed by addition of 0.1 mM $FeSO_4$ and 0.1 mM ascorbic acid, and incubated for 1 h at 37°C. Then 500 µl of 28% TCA was used to stop the reaction and 380 µl of 2% TBA was added with heating at 95°C for 30 min, to generate the colour. Then the samples were cooled on ice, centrifuged at 8000 rpm for 2 min and the absorbance of the supernatant was taken at 532 nm. All the tests were performed six times. Trolox was used as the standard.

4.7.4.11. Determination of total phenolic content

Total phenolic content was determined using Folin-Ciocalteu (FC) reagent according to a previously described method (Singleton & Rossi, 1965) with a slight modification. Briefly, MDE (0.1 ml) was mixed with 0.75 ml of FC reagent (previously diluted 1000-fold with distilled water) and incubated for 5 min at 22°C, then 0.75 ml of 0.06% Na_2CO_3 solution was added. After incubation at 22°C for 90 min, the absorbance was measured at 725 nm. All the tests were performed six times. The phenolic content was evaluated using gallic acid as a standard. The result was expressed as mg of gallic acid equivalent phenolic content present in 1 g sample plant material.

4.7.4.12. Determination of total flavonoid content

The total flavonoid content was determined with aluninium chloride ($AlCl_3$) according to a known method (Zhishen et al., 1999) using quercetin as a standard. The MDE (0.1 ml) was added to 0.3 ml distilled water followed by $NaNO_2$ (0.03 ml, 5%). After 5 min at 25°C, $AlCl_3$ (0.03 ml, 10%) was added. After further 5 min, the reaction mixture was treated with 0.2 ml of 1 mM NaOH. Finally, the reaction mixture was diluted to 1 ml with water and the absorbance was measured at 510 nm. All the tests were performed six times. The flavonoid content was evaluated using quercetin as a standard. The result was expressed as mg of quercetin equivalent flavonoid present in 1 g sample plant material.

4.8. PHYTOCHEMICAL ANALYSIS OF *D. ESCULENTUM*

Qualitative analysis of phytochemicals was carried out for *D. esculentum*, to identify the presence of different phyto-constituents.

4.8.1. Terpenoids

0.5 gm of CDE was dissolved in 5 ml of methanol and filtered. Filtrate was mixed with 2 ml of chloroform, and concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A reddish brown colouration of the interface was formed to show positive results for the presence of terpenoids (Harborne, 1973).

4.8.2. Glycosides

0.5 gm of CDE was dissolved in 5 ml of methanol and filtered. Filter was mixed with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. On addition of 1 ml of concentrated H_2SO_4 to this, a brown ring in the interface indicated a deoxyribose characteristic of cardenolides, a plant glycoside (Dey et al., 2012).

4.8.3. Alkaloids

0.5 g of CDE was defatted with 5% ethyl ether for 15 min. The defatted sample was extracted for 20 min with 5 ml of aqueous HCl on a boiling water bath. The resulting mixture was centrifuged for 10 min at 3000 rpm and divided in to two parts. One ml (First part) of the filtrate was treated with few drops of Mayer's reagent. One milliliter (second part) of the filtrate was treated with with Dragendroff's reagent. Turbidity was observed in both the cases (Harborne, 1973; Trease & Evans, 1996).

4.8.4. Steroids

0.5 g of CDE was dissolved in 5 ml of methanol. 1 ml of the extract was treated with 0.5 ml of acetic acid anhydride and cooled in ice. This was mixed with 0.5 ml of chloroform. One milliliter of concentrated sulphuric acid was then added carefully by means of a pipette. At the separation level of the two liquids, a reddish-brown ring was formed, as an indication of the presence of steroids (Harborne, 1998).

4.8.5. Tannins

About 0.5 g of the CDE was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added. A blue-black precipitation indicated the presence of tannins (Segelman et al., 1969).

4.8.6. Phlobatannins

The CDE was boiled with 1% aqueous hydrochloric acid (HCl) to observe the deposition of red precipitate (Harborne, 1973).

4.8.7. Saponins

0.5 g of CDE was shaken with water in a test tube and it was warmed in a water bath. The persistent of froth indicates the presence of saponins (Kapoor et al., 1969).

4.8.8. Flavonoids

A portion of the CDE was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of diluted ammonia solution. A yellow coloration was observed, indicating a positive test for flavonoids (Edeoga et al., 2005).

4.8.9. Phenols (Ferric chloride test)

Two ml of the MDE was treated with three drops of ferric chloride solution. Formation of bluish black colored solution indicates the presence of phenols (Dey et al., 2012).

4.9. ACUTE, SUB-ACUTE, SUB-CHRONIC AND CHRONIC TOXICITY STUDY OF *D. ESCULENTUM* AS WELL AS ITS EFFECT ON SOME MAJOR ORGANS OF MOUSE

4.9.1. Acute toxicity study

Single-dose study was performed for 15 days following the safety assessment guidelines (Schilter et al., 2003). Both the adult (6-8 weeks old) male and female Swiss albino mice weighing 25 ± 2 g of b.w. were used for the acute toxicity study. Dosages were based on b.w. of the animal (expressed as mg BDE equivalent per g body weight of the animal). Mice were

randomly divided into four groups (Group – A: untreated control, Group – B: – 1 g/kg b.w., Group C – 2 g/kg b.w., Group D – 4 g/kg b.w.), consisting of six animals (n = 6) in each group. BDE was administered orally (using a round-pointed polypropylene microtip fitted onto a graded disposable syringe) as a single bolus dose (800 µl BDE). Control mice received only distilled water (800 µl). Prior to the dosage, mice were fasted overnight to eliminate feed from gastro-intestinal tract. All the mice were thoroughly observed for the onset of any toxic signs immediately and also on each day during 14 days of observation period to record any delayed toxic effects. Survival, feed intake (from day 7 to 15), and body weight (day 0 and every four days) were monitored. Mice were sacrificed under mild chloroform and ether (2:1) anesthesia on day 15, and selected vital organs including liver, kidney, testis, ovary, adrenals, spleen, heart and brain were excised, blotted and weighed.

4.9.2. Subacute (15 and 45 days), subchronic (90 days) and chronic (135 and 180 days) toxicity study

One hundred twenty (120) Swiss albino mice were divided in to five sets (S 1–5) and each set was sub-divided in to four groups (G 1–4). Therefore, each group contained six mice. All the animals were administered orally as described earlier. Group 1 (G1) of all the sets were considered as control where 0.4 ml of distilled water was given. Group 2 (G2), Group 3 (G3) and Group 4 (G4) of all the sets were administered with 0.4 ml of BDE/CDE at the dose of 80, 160 and 320 mg/kg b.w., respectively. In this way, all the groups of S1 were treated daily for 15 days (sub-acute treatment 1), S2 daily for 45 days (sub-acute treatment 2), S3 daily for 90 days (subchronic), S4 daily for 135 days (chronic treatment 1) and S5 daily for 180 days (chronic treatment 2). The body weight of mice was recorded on day '0' and every following week. On 16^{th}, 46^{th}, 91^{st}, 136^{th} and 181^{st} day, mice from each set were sacrificed under proper anesthesia (chloroform and ether in 2:1 ratio), heart was punctured and blood was collected separately for serum separation as well as collected in EDTA vials for hematological examination. Vital organs were excised, blotted and weighed. Liver, kidney, testis and ovary were processed for histopathological examinations. Biochemical studies of serum were carried out for liver function (aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), acid phosphatase (ACP), γ-glutamyl transferase (GGT),

total bilirubin, and kidney function (urea, creatinine), using commercially available standard biochemical assay kits (Crest Biosystems, Goa, India).

4.10. STATISTICAL ANALYSIS

All data are given as the mean ± SD of six measurements. Statistical analysis was performed using KyPlot version 2.0 beta 15 (32 bit). The IC_{50} values were calculated by the formula Y= 100 x A1 / (X + A1), where A1 = IC_{50}, Y = response (Y = 100% when X = 0), X = inhibitory concentration. Differences between two groups (for example, plant extract and standard in DPPH radical scavenging assay) were determined by paired t test, whereas, differences among more than two groups were determined by one-way ANOVA followed by Dunnett's T test. $p < 0.05$ was considered significant.