3.0 Materials and Methods

3.1. Chemicals and reagents

All the experimental rats were obtained from SAHA Enterprise (Kolkata, India). Standard food ingredients were taken from Midnapore local market. Loba group had provided the Curcumin. Chitosan was taken from Himedia. Sodium arsenite, Methanol, ethanol, chloroform and hydrogen peroxide was brought from S D Fine Chem Ltd (SDFCL) India. NAD, NADP, NBT, agarose, Tris-base, Tween 20, sodium lactate, sodium acetate and potassium phosphate were obtained from SRL (Mumbai, Maharashtra, India). TPP, Glacial acetic acid, ethidium bromide and Potassium ferricyanide were purchased from Merck (Mumbai, Maharashtra, India). The Riboflavin was purchased from the chemical supplier Loba (Mumbai, Maharashtra, India). From the Ray Biotech, Georgia, USA, the IL-6 and TNF-α kit was purchased. Wuhan Fine Biological Technology Co., Ltd. (Wuhan, China) supplied folic acid (B_9), vitamin B_{12}, LH, FSH, Estradiol, Esr-1, NF-κB, and MT-1 ELISA kits. Ferric chloride, eosin and Hematoxylin were purchased from Qualigens (India). SGOT, SGPT, Urea and Creatinine kits were obtained from Tulip group (India). Cholesterol, triglyceride and HDL kits were buying from ARKRAY Healthcare Private Limited, (India).

3.2. Isolation and purification of Pectic Polysaccharide

Bitter gourd (*Momordica charantia*) fruits were sliced into tiny pieces. These were boiling for 11-13 hrs. The total extract was placed at low temperature 4˚C for overnight and after that filtered with a linen cloth. Collected parts were centrifuge at 8100 rpm for 35-40 minutes. The clear part was blended with the ethanol and kept it to precipitate pectic polysaccharide. Finally, precipitated polysaccharides were collected through the centrifugation and cleaned with the ethanol. The pectic polysaccharide was then freeze dried (Maji et al., 2012). The crude pectic polysaccharide was then purified. It was finally isolated through Sepharose-6B.

Structure of the pectic polysaccharide contains D-methyl galacturonate and D-galactose with a molar ratio of 4:1 (Panda et al., 2015).

3.3. Preparation of arsenic with Pectic Polysaccharide

The crude Pectic polysaccharide (0.2 gm) was added into 1.0-ppm arsenic solution. Then CCPS was mixed drop wise in the sodium arsenite (100 ml) solution using stirrer for 1 hr. Samples were filtrated through Whatman syringe filter (0.2 μm) and then collected. Finally, the arsenic-CCPS association is freeze-dried to use for our experimental purpose.

3.4. Spectrum analysis of CCPS and arsenic-CCPS association

3.4.1. UV spectroscopic analysis

The absorbance of CCPS and arsenic-CCPS association sample solution was examined over the range between 180-500 nm using the Evolution 201 UV-Vis-spectrophotometer (Thermo Fisher Scientific, Shanghai, China).

3.4.2. FTIR spectroscopic analysis

The wave numbers from 400 to 4000 cm^{-1} for each sample (CCPS, arsenic-CCPS conjugation and curcumin encapsulated chitosan nanoparticles) were also recorded in an FTIR spectrophotometer applying the KBr pellets (Perkin spectrum two FTIR system, model Spectrum two).

3.4.3. X-ray diffraction analysis

The XRD studies of the CCPS and arsenic-CCPS conjugation ware conducted by D8 ADVANCE system. Few amounts of dried powder of CCPS and arsenic-CCPS conjugation was kept on holder of the sample. This assessment was completed in the temperature range within 10° to 90°C.

3.4.4. ^{1}H NMR study

For NMR analysis CCPS and arsenic-CCPS association were lyophilized with D_2O. ^{1}H NMR spectra of CCPS and arsenic-CCPS association samples were determined using Bruker Avance DPX-300 spectrometer (Rheinstetten, Germany) which was operated at δ 4.60 ppm and 27˚C temperature.

3.4.5. Electron microscopy study

The morphology of the CCPS and arsenic-CCPS association was analyzed on a scanning and transmission electron microscope (SEM and TEM). One drop of each samples was allowed to deposit on a glass slide. It is evaporated in vacuum condition. The dried samples were dropped against a thin plated gold layer. Finally, the image of each sample was recorded at 5 kV accelerating voltage using a ZEISS EVO 18.

Furthermore, the accurate morphology of the arsenic-CCPS association was determined using a TEM which was operated with a voltage at 200 kV (CM-200, Philips). A small droplet (0.1%) of CCPS-arsenic association was assigned on a copper coated grid which also covered with carbon. Before TEM examination the samples were finally, permitted for lyophilize for 2-3 hrs.

3.5. Methods of curcumin encapsulated chitosan nanoparticles (ECNPs)

3.5.1. Preparation of Chitosan nanoparticles

600 mg powdered chitosan was melted in the glacial acetic acid (0.1%) solution under the stirrer for several hours. By NaOH, pH of chitosan solution was then adjusted (Calvo et al., 1997) and finally, solution was sprayed into 100 ml of distilled water under a pressure.

3.5.2. Preparation of encapsulated curcumin chitosan nanoparticles

The curcumin powder (60 mg) was mixed in ethanol. The TPP solution was arranged using the distilled water (1 gm of TPP in 300 ml DW). The Tween-20 was then mixed with the chitosan solution at the same time with constant stirring. The curcumin solution is allowed to

mix for 30 minutes using the stirrer. The solution was stirred continuously and the prepared TPP solution was added in total solution by drop wise during stirring. Finally, the supernatant part was then permitted to centrifuge at 12000 rpm. The supernatant part was removed and the remaining part was then washed using the double distilled water. Thick part of the ECNP was collected and lyophilized. It was then stored in a chill place. The ECNPs was synthesized according to Yadav et al with slight changes (Yadav et al., 2012).

3.5.3. Nanoparticles characterization

The encapsulated curcumin in the chitosan nanoparticles were examined by the UV spectrophotometry, XRD and TEM analyses respectively. The encapsulated curcumin in the chitosan was determined using Evolution 201 (Thermo Fisher Scientific, Shanghai, China) UV spectrophotometer. The nanoparticles KBr pellet samples were performed using the Perkin spectrum of two FTIR systems.

3.5.4. Particle size measurement by TEM

The nanoparticles size and morphology were taken using the TEM analysis at 200 kV (CM-200, Philips). The encapsulated curcumin in chitosan were dispersed in water. A small droplet (0.1%) of aqueous solution of nanoparticles was then arranged on copper grid which is coated with carbon. Next it allowed to lyophilize for several hrs and finally determined under an electron microscope. The average and standard particle size and distribution were examined using the Image J software.

3.6. Animal selection and treatment

Different weighed of female Wister rats were applied for our experiment. All the rats were taken from animal house of SAHA Enterprise. These studies were conducted following the instruction of Animal Ethical Committee, India (Ethical no-IEC/7-5/C-5/16). All the experimental rats were kept in animal house in our university campus. These rats were place in netted polycarbonate cage. Supply the sufficient temperature within 30-34°C with 12-h-

light-dark cycle and sufficient percentage of humidity of all animals in the room for 10 days. Animals were allowed water and standard pellet diet. During the course of experiment animals were served with a standard rat chow diet as listed below:

- Carbohydrate: 51.0%
- Crude protein: 23.0%
- Crude fibre: 7.0%
- Crude fat: 3.0%
- Moisture: 13.0%
- Phosphorus: 1.0%
- Sodium: 1.0%
- Calcium: 1.0%

3.7. Vaginal smear investigation

The droppers were used to collect the Vaginal smears of female rats. Containing 0.9% of normal saline dropper was inserted into the vagina. For several times saline was flushed into the vagina. Collected fluid was then placed onto the glass slide and kept to dry for few minutes (Champlin et al., 1973). Dry slides were allow to stain (Leishman) and after that estrous cycle of female rats was determined under a light microscope (40 x magnifications).

3.8. Assay of serum total protein

The biuret method has been used here to assay the total protein of serum. A colored chelate forms in alkaline solution, cupric ions and compounds containing at least two -$CONH_2$, -CH_2NH_2-, $CSNH_2$ or similar groups. These are joined directly through a carbon or nitrogen atom, one cupric ion and nearly six peptide bonds in proteins form a chelate. CU2+ + Proteins have formed blue colour complex.

3.9. Assessment of General Toxicity: Liver Function test

Serum creatinine, urea, SGPT and SGOT were assayed from the different groups of all female rats by the standard protocol using the assay kits. These kits were supplied by Tulip Group, India or other reputed company.

3.9.1. Estimation of serum creatinine

Presence of alkaline medium an orange coloured complex was formed by the reaction of creatinine with the picric acid. The formation of this complex was assayed by reading the alter in absorbance around 505 nm in a selective interval of time that is also proportional to creatinine concentration. The optimization of reaction time as well as the concentration of sodium hydroxide and picric acid has been done to avoid the interference from ketoacids.

3.9.2. Estimation of serum SGPT

SGPT also catalyzes the transfer of amino group between L-Alanine and α ketogluterate to form glutamate and pyruvate respectively. In the presence of Lactate dehydrogenase these reacts with NADH and form NAD. The oxidation rate of NADH to NAD was measured as decrease in absorbance that is proportional to the activity of SGPT in serum.

3.9.3. Estimation of serum SGOT

SGOT catalyzes the transfer of amino group between L-aspartate and α ketogluterate to form Glutamate and Oxaloacetate and these reacts with NADH in the presence of Malate Dehydrogenase to form NAD. The rate of oxidation of NADH to NAD is calculated as reduce in absorbance that is proportional to the SGOT activity in the serum sample.

3.10. Assessment of General Toxicity: Lipid Function test

The plasma level of lipid profile such as cholesterol, triglyceride (TG), HDL and LDL were measured from the several groups of all female rats by the standard protocol using the assay kits. (These kits were supplied by ARKRAY Health care Private Limited India.

3.10.1. Estimation of Total Cholesterol (Oxidase method)

Esterase hydrolyses was hydrolyzed esterifies cholesterol into free cholesterol. Then the free cholesterol is further oxidizing into H_2O_2 and then reacts with the phenol and 4-aminoantipyrine, under the catalytic influence of peroxidase to form a red quinoneimine complex.

3.10.2. Estimation of Triglycerides

The triglyceride level was measured using the standard protocol of assay kit. In presence of Lipoprotein Lipase, Triglycerides were hydrolyzed to produce glycerol and free fatty acids. Reaction between glycerol and glycerol kinase was producing the formation of glycerol 3 poshpate, H_2O_2 and glycerophosphate oxidase. Finally, H_2O_2 was further react with the phenolic compound and formed 4- amino anti pyrine and quinoneimine dye complex.

3.10.3. Estimation of HDL–cholesterol

The serum was reacted with the contained Polyethylene Glycol in the precipitating reagent. All the VLDL and LDL were then precipitated. The HDL residue presence in the supernatant was then analysing as a sample for cholesterol using the cholesterol reagents. Finally the absorbance was measured at 600 nm.

3.10.4. Estimation of LDL-cholesterol

LDL-cholesterol was precisely measured using the relation as:

LDL-cholesterol = Total cholesterol - Triglycerides /5-HDL-cholesterol.

3.11. MDA and CD assay

Uterine, ovarian and liver (20% w/v) tissue slices were homogenized using chilled PBS (0.1 mol/L, pH 7.4). All the homogenates slices were allowed to centrifuge for few a minute at 15,000×g. The supernatant parts of above slices were taken for the MDA and CD assay. MDA was done following the given method as in Devasagayam et al., 2003.

The uterine, ovarian and liver slices were done by the calculating amount of CD. The supernatant parts of tissues were extracted using the chloroform-methanol mixture (2:1). Following the centrifugation extracted lipids residue was evaporated by a dryer. The rest of lipids were then re-dissolved in a little amount of cyclohexane and measured at 233 nm. Finally, formed lipohydroperoxide was evaluated around 234 nm (Kumar, 2012).

3.12. Estimation of NPSH

The uterine horns and ovarian tissues were homogenized using 0.1 M chilled buffer (pH 7.4) and placed in 4°C to centrifuge at 10,000g for 10 minutes. After centrifugation, the supernatant part was taken for the assessment of NPSH by a standard DTNB method (Mieyal et al., 2008). Precipitation of protein was collected using of sulfosalicylic acid. Clear supernatant part was then added in sodium phosphate buffer (0.1M) containing DTNB. Finally, absorbance of NPSH level was recorded at 412 nm.

3.13. Spectrophotometric assay of SOD, Catalase, Px and Gpx

10% (w/v) of tissue's (uterus, ovary and liver) concentration was kept in homogenizer containing 100 mmol/L chilled tris HCl buffer (pH 7.4). All the homogenized tissue slices were centrifuged for a few minutes at 10000g at 4°C. The intracellular SOD contents in homogenizing tissue slices were examined by a standard method of Pattichis et al., 1994. The reacted cocktail was arranged by adding 800 µL of TDB, 40 µL of 7.5 mmol/L, NADPH, 25 µL of EDTA-$MnCl_2$ in 100 µL of tissue supernatant. Afterwards the SOD content in mixture was assayed at 340 nm at the oxidation rate of NADPH. The intracellular catalase contents in homogenizing tissue slices were done a standard method by Hadwan, 2016. The presence of acetic acid in dichromate was changed into the per chromic acid. In the presence of H_2O_2 it transformed into chromic acetate during heating procedure. The acetate was examined at 570 nm. Prepare catalase reaction was splits in the presence of H_2O_2 for several times. The same reaction was altered during the mixture of dichromatic acetic acid for several times. One unit

of activity was observed as consumed H_2O_2 one mole per minute per milligram of protein. The uterine, ovarian tissues were homogenized with the help of 0.1 M PBS (pH 7.0). Following the centrifugation separeted supernatant part was used for the peroxidase assay. In presence of 12.3 mM H_2O_2 0.1 ml of tissue supernatant part and 20 mM of guiacol cocktail were finally read at 436 nm (Sadasivan and Manickam, 1966). The uterine, ovarian and liver GPx activities were also measured using the procedure given by Paglia and Valentine 1967. The GPx activity of tissues was considered as a nmol NAD(P)H oxidized/min/mg of protein.

3.14. Assessment of SOD, Catalase, Px and GPx by gel electrophoresis

The uterine horns, ovarian and liver tissues (20%; w/v) were first allowed to homogenizer with chilled 1.0M PBS (pH 7.4). All homogenized tissues were centrifuged at 10000g at low temperature 4°C for a few minutes. This SOD activity was used in 12% native gel. 50 μg of proteins from the rest part of tissues were allowed in 12% native gel (Weydert and Cullen, 2010). The gels were then kept in mixing of NBT (2.3 mmol/L) and riboflavin (28 μmol/L) for a few minutes. Finally, gels were incubated with TEMED (28 mmol/L) in dark place for 20 minutes. The reduction NBT was done using the principle of inhibition. Exposing of the light on gels showed achromatic SOD bands against a dark blue background. Tissues extracts having 50 μg proteins were allowed in 8% native gels to analyze the catalase, peroxidase and GPx activity. For the detection of catalase activity after electrophoresis, gels were kept in dark place in H_2O_2 solution (0.003%) (Lewis et al., 2005). After incubation the gels were allowed to stain in the mixing solution of 2% of ferric chloride and potassium ferricyanide. Finally the bands were appeared bluish-yellow in colour against a green background. For the finding of peroxidase activity, after electrophoresis the gels were then stained with the solution of 100mg benzidine powder and 4.5ml glacial acetic acid in the presence of H_2O_2 (30%) (Hasan and Aburahma, 2014). The GPx activity was evaluated by eliminating peroxide. This peroxide played an important role to change potassium ferricyanide into

ferricyanide. GPx eliminates the peroxide which help to reduce the ferric chloride (Liu et al., 2006). Finally the clear achromatic bands were appeared due to ferric chloride against the blue-green background. The density measurement of all bands was done using the image J software.

3.15. Spectrophotometry assay of serum LDH

Serum LDH levels were computed using the LDH kits according to the (Tulip Group, Goa, India) manufacturer's directions. In the presence of NADH and formed NAD, the LDH changes pyruvate to lactate. The reducing in the absorbance was indicated the rate of oxidation of NADH to NAD and was proportional to the LDH activity.

3.16. Determination of serum LDH

Serum LDH activity was observed using agarose (1.2%) gels in 50mM Tris-HCl buffer solution (pH 8.2) (Brandt et al., 1987). The gels were placed onto electrophoresis tank and 15-25 µl serum were filled in wells of gel. Finally, gels were electrophoresed at 150-170 Volt. It was then developed using the mixing solution of Tris (1.0M), Na-lactate phenazine-methosulphate tetrazolium-blue NAD and H_2O. The gels were placed at 37°C to develop colour reaction. Finally, the gels were washed using distilled water and found serum LDH was made exposure to light. The band density was measured using the image J software.

3.17. Study of DNA Fragmentation

The uterine horns were taken for DNA preparation. The uterine tissues were placed in homogenizer and it was treated with lysis buffer comprise 20 mM EDTA, 1% SDS, 10 mM NaCl, 0.5 mg/ml proteinase K and 50 mM Tris (pH 8.0). The cell lysate was placed at 4°C for 15 minutes and centrifuged for a few minutes (Garcia-Martinez et al., 1993). Supernatant part was treated with the solution of chloroform and phenol (1:1) and softly mixed. It was precipitated into two parts of ethanol and one tenth part of sodium acetate and then allowed to centrifuge. After centrifugation, part of the pellet was gently mixed in 30µl deionized water–

RNAase solution along with loading buffer (5µl). It was then kept at 37°C for few minutes. The ethidium bromide containing agarose gel was electrophoresed at 65 Volt. Finally DNA gel was visualized under the Bio-Rad documentation system.

3.18. Comet assay

The glass slides were pre-coated into the prepared agarose solution (1%). The suspension of liver and uterine cells was exposed to prepare 0.6% of low melting agarose in PBS solution and it was then incubated at 37°C (Singh et al., 1988). After solidification, glass slides were precoated into 1% agarose solution and coverslips was gently placed onto the slides. After the solidification of 1% removed agarose coverslips and slides were dipped into chilled lysis buffer (10mM Trizma base, 10% DMSO, 2.5mM NaCl, 85mM EDTA, 1% Triton X-100 and 1% SDS, pH 10) for 2 hrs at 4°C with a slight modification. The slides were then separated from lysis buffer and cleaned several times using PBS solution. The slides were then kept at room temperature for 35-40 minutes. All the slides were dipped to wash thrice in water for removing of excess salts. After that, all slides were allowed to keep in electrophoresis tank. The electrophoresis tank was filled with 1mM EDTA and 0.3 M NaOH containing buffer. Electrophoresis was done at 30 Volt for a few minutes. After electrophoresis, slides were then soaked in PBS for neutralization for three times. The slides were then allowed to stain with ethidium bromide solution (10mg/ml) and kept at dark condition for 5-10 minutes. Slides were soaked in water to remove the excess stain and finally, slides were examined under a (Eclipse LV100 POL, Nikon-Tokyo, Japan) fluorescence microscope by Vis Comet software (Impuls Bildanalyse-Amsterdam, Netherlands).

3.19. Ovarian Δ^5, 3β-HSD and 17β-HSD activities

Ovarian tissues were homogenized using chilled buffer containing potassium phosphate (5.0mM), EDTA (1.0mM) and 20% spectroscopic-grade glycerol and centrifuged at 10000g for 30-40 minutes. The part of collected supernatant tissue was used for the detection of Δ^5,

3β-HSD. Then addition of DHEA (30 μg) and NAD (0.5 μM) into the supernatant parts were then allowed to mix properly together for a few minutes (Talalay, 1962). Finally, the absorbance data was taken at 340 nm against the without NAD of reagent blank.

For the measurement activity of 17β-HSD, homogenates tissue was added with crystalline BSA (25 mg), NADP (1.1 μM), and testosterone (0.3 μM) and it was then mixed properly (Jarabak et al., 1962). Finally, the absorbance was found at 340 nm against a blank without NADP.

3.20. Determination of serum Vitamin B_{12}, Folic Acid and Vitamin-C: HPLC

C_{18} column of reverse phase HPLC was applied for the measurements of vitamins levels in serum. Vitamin B_{12} was collected from the 5.0 ml serum using the addition of 1.0% acetate buffer (pH 4.6) (Stefova et al., 1997). The extract of vitamin $B_{12}$was placed in boiling water bath for 25-30 minutes. A clear supernatant part was collected following the centrifugation.

Folic acid (B_9) was collected from the 0.2 ml of serum by addition of potassium tetraborate (50 mM/L) and 1.0% sodium ascorbate (pH 9.0) (Kalmbach et al., 2011). The extracted folic acid was allowed to vortex and then boiled for 25-30 minutes and placed in a dark background for overnight at 4 °C. Here, an internal standard eTHF was used. Above extracted samples were allowed to filter and analyzed in a HPLC. For the detection of vitamin-C in serum it was centrifuged at 2750g for 5-10 minutes (Robitaille and Hoffer, 2015). The collected clear part was properly mixed in presence of disodium EDTA (2.0 mmol) with MPA (10%) and allowed to place for 5 minutes in ice a chamber. Following the centrifugation, the collected ascorbic acid (protein free) was placed in the dry ice. Finally, it was kept at −80 °C. Vitamin-C level in serum was finally detected in reverse phase HPLC.

3.21. Assessments of Vit-B_{12}, B_9 and Hcy: ELISA

Levels of the Vit-B_{12}, B_9 and Hcy in serum were assayed using the ELISA kits. All these procedures were given by manufacturers of Wuhan Fine test-China. Based on the competitive ELISA technique these vitamins levels were performed.

The ELISA microtiter plates were also pre-coated with the vitamin of homocysteine, B_{12}, and folic acid. The tested samples and standards on the solid phase supporter can compete with each other for binding with biotinylated detection antibody. The microtiter plates are also allowed to wash to eliminate the excess unbound sample or standard. In each microtiter of all wells, HRP-Streptavidin (SABC) is also added. The incubation step is then permitted for a few minutes. In every microtiter of all wells, a TMB substrate solution is added. A sulphuric acid solution is next to added to stop the enzyme-substrate reaction. The color change reaction was determined at around 450 nm. Finally, the vitamins of homocysteine, B_{12}, and folic acid concentration data was taken by the calculating of the sample's OD to the standard curve.

3.22. Assessments of Serum Hormone analysis

The levels of estradiol and gonadotrophin hormones (LH and FSH) were assayed using the ELISA kits. All procedures were given by manufacturers of Wuhan Fine test-China. Based on competitive ELISA technique these vitamins levels were performed.

The ELISA microtiter plates were pre-coated with Estradiol, LH and FSH. The tested samples and standard also incorporated for the binding with biotinylated detection antibody. The excess unbound, conjugates and standards samples were washed and the plates are allowed to incubation. HRP-Streptavidin (SABC) and TMB substrate are also added in microtiter plates. The sulphuric acid is finally terminated to the enzyme-substrate reaction. The color changed reaction was finally recorded at 450 nm wavelength which is compared to the OD of the samples to the standard curve.

3.23. Assessments of Esr-1, NF-κB, TNF-α, IL-6 and MT-1

The levels Esr-1 and NF-κB of uterine, TNF-α and IL-6 of serum, and MT-1 of liver, were assayed using the ELISA. All the procedures were recommended given by manufacturers of Wuhan Fine test-China and RayBio.Based on the sandwich ELISA method these inflammatory cytokines and pro-inflammatory markers levels were performed. For the detection of the above parameters, the ELISA microtiter plates were already pre-coated with Esr-1, NF-κB, TNF-α, IL-6 and MT-1. The tested samples, standards, and also biotin-conjugated detection antibody included in each well. The microtiter plates are allowed to washed and incubated for a few minutes. HRP-Streptavidin is then added in wells. In each well, the TMB substrate is added and allowed to incubation. After catalyzing the TMB by HRP which is formed the blue colour reaction. The blue colour reaction is then transferred to the yellow reaction adding the acidic stop solution. The concentration of each sample was finally recorded at 450 nm.

3.24. Uterine-Ovarian histopathology

During sacrifice uterine and ovarian tissues were removed and preserved in formalin. The uterine and ovarian tissues were dehydrated with increasing concentrations of ethanol. After that, tissues were cleared using the xylene and finally fixed in paraffin. Then the 5μm thickness sections of tissues were allowed to stain using the procedure of hematoxylin-eosin (Harris). Using a microscope (Olympus, CX21i, magnification 400x) all the above stained sections were examined and finally evaluated the uterine and ovarian tissues architecture alteration.

3.25. Calculation and Statistical analysis

Organs weight ratio was calculated according to the formula:

Organo Somatic Index = Organ weight / body weight ×100

All experimental results were defined mean ± SE, n=6 in variable groups. Variation between the control with vehicle and arsenic challenged groups were estimated using one-way

ANOVA with Dunnett's post Hoc t-test. Statistically, $p<0.05$ were expressed as a minimum level of significance.