Chapter 3

Materials & Methods
Fig. 5 Natural habitat of *Capparis aphylla* and site for samples collection.
Fig. 6. *Capparis aphylla* showing well developed stem, inflorescence and fruit (inset).
MATERIALS AND METHODS

3.1. Plant material: *Capparis aphylla* plant wildly growing in low irrigated area was collected from village Madina of district Rohtak, Haryana India (fig.6). The stem wood of the plant was chopped into small pieces and air dried over night for extraction purposes.

3.2. Hot percolation extraction:
Air dried stem (with bark and without bark) pieces was ground by hammer mill. The stem powder obtained was used for extraction with water and remainder plant material was subsequently extracted with appropriate solvent (1:10w/v) (patent applied) by hot percolation for 6 h each by using soxhlet distillation. The different solvent extracts obtained were then filtered with filter paper (Whatman Int.Ltd, Maidstone, U.K) and concentrated to dryness in oven. The concentrate was then layered on aluminium foil and freeze dried. The yield range varied, which was obtained (up to 1.5%) with different solvent system. Extract obtained was stored in sterile glass containers at – 4C° until used for study.

Similarly the conventional extraction (CE) of the air dried stem was done by keeping in big earthen vessel and heating for 6 h at high temperature, produced dark brown color extract. The CE was dried as described above and redissolved in deionised water to constitute different concentrations for use.

3.3. Animals:
After getting approval from the Institutional Animal Ethical Committee (ACBT/2009/1043-51), Albino Wister rats, weighing about 150 to 200 g were obtained from Deptment of pharmacy M.D.U.Rohtak after students demographic experiments selected, used for the experiment. They were housed in polypropylene cages measuring 12"x10"x8" (fig.7) under controlled temperature conditions (25 ± 2 C°) with 12:12 h light and dark cycle. Animals were fed on balanced diet of soaked maize, wheat and chicken beans supplemented with multivitamins and water ad
Animals were regularly checked throughout the investigation for any infection and if found infected, the animals were isolated and treated.

3.4. Experimental design:
The experimental models were administered various extracts for a period of 7 days and by single dose for oral glucose test. The extract was fed at an effective dose (patent applied). The control and experimental groups consisted of 4-5 animals each.

The study consisted of following groups:

Group 1: Normal Control + vehicle (0.5% CMC (carboxy methyl cellulose).
Group 2: Diabetic control + vehicle (0.5 % CMC).
Group 3: Diabetic + solvent extract treatment.
Group 5: Diabetic + glibenclamide ( 600µg/kg ).
Group 5: Diabetic + active compound at specific dose (patent applied).
Group 6: Diabetic with \textit{P. aeruginosa} + stem solvent extract treatment.
Group 7: Diabetic with \textit{P. aeruginosa} + active compound treatment.
Group 8: Diabetic with \textit{P. aeruginosa} + glibenclamide ( 600µg/kg ) treatment.
Group 9: Normal control with \textit{P. aeruginosa} + vehicle.
Group 10: Diabetic control with \textit{P. aeruginosa} + vehicle.
Group 11: Diabetic with \textit{S. aureus} + stem solvent extract treatment.
Group 12: Diabetic with \textit{S. aureus} + glibenclamide ( 600µg/kg ) treatment.
Group 14: Normal control with \textit{S. aureus} + vehicle.
Group 15: Diabetic control with \textit{S. aureus} + vehicle.
Group 16: Diabetic control with \textit{P. aeruginosa} + stem solvent extract.
Group 17: Diabetic with \textit{E. coli} + stem solvent extract.
Group 18: Diabetic with \textit{E. coli} + glibenclamide ( 600µg/kg ) treatment.
Group 19: Diabetic with \textit{E. coli} + active compound treatment.
Group 20: Normal control with \textit{E. coli} + vehicle.
Group 21: Diabetic control with \textit{E. coli} + vehicle.
Group 23: Diabetic with \textit{C. albicans} + glibenclamide ( 600µg/kg ) treatment.
Fig. 7 Animal housed in polypropylene cage under control conditions.

Group 26: Diabetic control with *C. albicans* + vehicle treatment.

Twenty four hours after the last administration, the animals were anaesthetized with diethyl ether vapor and dissected. The tissue samples (liver, heart, kidney, and pancreas) were removed, washed with normal saline and kept at -20°C until assayed for biochemical parameters.

### 3.5. Induction of diabetes:

Diabetes was induced in rats by the intraperitoneal injection of streptozotocin (STZ) at a dose of 55 mg/Kg b.w. dissolved in distilled water. Seven days after the injection, the blood glucose levels were measured. Each animal with a blood glucose concentration level above 250 mg/dL was considered to be diabetic and used in the experiments. To prevent the hypoglycemia which occurred during the first 24 h following the STZ administration, 5% glucose solution was orally given to the diabetic rats. In all experiments, rats were fasted for 16 h prior to STZ injection (Aslan et al. 2007).

### 3.6. Determination of acute toxicity:

The acute toxicity test (LD$_{50}$) of the extract was determined according to the OCED test guidelines (Organization for Economic Co-operation and development). Experiment was carried out on normal healthy rats. The behaviors of the treated rats appeared normal. No toxic effect was reported up to 5 and 10 times of the dose used here of the conventional and solvent extract. There was no death in any of these groups.

### 3.7. Serum preparation:

At the end of experimental period, the albino Wister rats was fasted overnight (12 hours). Whole blood was obtained by cardiac puncture from sacrificed rats and collected into sample bottles. The blood was centrifuged at 3000 rpm for 10 minutes and clear serum was aspirated, stored frozen and then used for biochemical analysis.
3.8. Serum lipids profile, creatinine and urea estimation:
Serum total cholesterol, triglyceride, High density Lipoprotein (HDL), creatinine were measured by enzymatic colorimetric method using Mennheim diagnostic kits. The concentration of low density lipoprotein (LDL) cholesterol and Very low density lipoprotein (VLDL) was calculated by the formula of Friedwald et al. (1972).

3.9. Blood glucose estimations:
In order to obtain antihyperglycemic effect of the test samples, blood samples collected by puncture of tail tip method and blood glucose level was estimated using an electronic glucometer Accu Check (Bayers diagnostic pvt. Germany) by of glucose dehydrogenase method (Strecker 1955, Owiredu et al.2009)

3.9.1. Acute antidiabetic effect of test samples: oral glucose tolerance test.
A combined methodology of Kato and Miura (1993) is followed for the activity assessment of extracts and compounds in order to avoid wasting animals; there are some modifications incorporated in the time pattern for blood glucose level determination (Wohaieb and Godin 1987). After overnight fasting (16 h) the blood glucose level of rats were determined and then were given test compounds. The extracts doses were administered orally by using a gastric gavage needle. The rats were loaded orally with 2 g/Kg glucose and simultaneously with test samples. The blood glucose concentrations were determined at 30, 60, 120 and 180 min after the dosing.

3.9.2. Antidiabetic effect of test samples after 7 days of treatment:
The solvent extract and glibenclamide were administered for 7 days consecutively. Blood glucose levels were determined at 10:00 a.m. on 1st, 3rd, 5th and 8th days after the administration of test samples. The effect on body weight and water consumption under treatment was also monitored at the same days. On 8th day, all animals were sacrificed and then the kidney, liver and heart tissue of animals were removed for measurement of antioxidative enzymes activity.
3.9.3. Histological studies:
The whole pancreas from each animal was removed after sacrificing the animal and was collected in 10% formalin solution, and immediately processed by the paraffin technique. Sections of 5μm thickness were cut and stained by hematoxylin and eosin (H & E) for histological examination. Photomicrograph were taken under binocular light microscope at 100 x optical zoom by using camera of 10 mega pixal with 4x optical zoom (Nagappa et al. 2003).

3.10. Antioxidative status: In order to obtained antioxidative potential of test samples the malonaldihyde(MDA) level, glutathione (GSH) level, Superoxide dismutase(SOD), Catalase, glutathione peroxidase (GPx) and glutathione -s-transferase(GST) activities were estimated.

3.10.1. Lipid peroxidation:

Homogenate preparation: The liver, kidney and heart of each rat were immediately excised and chilled in ice-cold 0.9% NaCl. After washing with 0.9% NaCl, 1.0 g of wet tissue was weighted exactly and homogenized in 9ml of 0.25M sucrose using a Teflon homogenizer to obtain a 10% suspension. The cytosolic compound was obtained by a two-step centrifugation first at 1000×g for 10 min and then at 2000×g for 30 min at 4 °C.

Estimation: The method of Ohkawa et al. (1979) as modified by Jamall and Smith (1985) was used to determine lipid peroxidation in tissue samples. A volume of the homogenate (200μl) was transferred to a vial and was mixed with 0.2 ml of 8.1% (w/v) sodium dodecyl sulphate solution, 1.50 ml of a 0.8% (w/v) solution of TBA and the final volume was adjusted to 4.0 ml with distilled water. Each vial was tightly capped and heated in a boiling water bath for 60 min. The vials were then cooled under running water. Equal volumes of tissue blank or test sample and 10% TCA were transferred into a centrifuge tube and centrifuged at 1000×g for 10 min. The absorbance of the supernatant compound was measured at 532 nm (Elico Spectrometer). 1,1,3,3-Tetraethoxypropan was used as standard for calibration of the curve.
3.10.2. Reduced glutathione (GSH) content:
Liver (200 mg), heart (400 mg), and kidney (400 mg) were homogenized in 8.0 ml of 0.02M EDTA in an ice bath. The homogenates were kept in the ice bath until used. Estimation of reduced glutathione content was done by using the method of Sedlak and Lindsay (1968).

An aliquots of 5.0 ml of the homogenates were mixed in 15.0 ml test tubes with 4.0 ml distilled water and 1.0 ml of 50% trichloroacetic acid (TCA). The tubes were centrifuged for 15 min at approximately 3000×g. The supernatant 2.0 ml was mixed with 4.0 ml Tris buffer (.4 M, pH 8.9), 0.1 ml Ellman’s reagent (19.8 mg of 5,5'-dithiobisnitro benzoic acid (DTNB)) added, and the mixture shaked. The absorbance was read within 5 min of the addition of DTNB at 412 nm against a reagent blank with no homogenate. Results were expressed as μ mol GSH/g tissue by using the extinction coefficient (ε₂₄ = 135.4μM⁻¹ cm⁻¹).

3.10.3. Estimation of Superoxide Dismutase (SOD) activity:
The rat liver, heart and kidney (200 mg) were homogenized (1:10 w/v) in 0.1M sodium phosphate buffer (pH 7.4) and centrifuged at 5000 rpm for 10 minutes at 4°C. The supernatant was used for estimation. Superoxide Dismutase activity was assayed by the method described by Kakkar et al. (1984) using nitroblue tetrazolium as the indicator reagent. The reagents contain sodium pyrophosphate buffer 1.2 ml (.052 M, pH 8.3), 0.1 ml phenazine methosulphate (186 μM), 0.3 ml nitro blue tetrazolium (300 μM) and 0.2 ml NADH (780 μM) and 0.1 ml of processed tissue sample. The mixture was then incubated for 90 min at 30°C then 4 ml of n-butanol and one ml of acetic acid were added. The mixture was shaken vigorously followed with centrifugation at 4000 rpm for 10 min, the organic layer was withdrawn and same used for absorbance measurement at 560 nm using a spectrophotometer (Elico Pvt.Ltd).

3.10.4. Estimation of Catalase (CAT) activity:

Preparation of reagents: Solution 1. Phosphate buffer (0.15 M, pH 7.0).
Solution 2. H₂O₂-phosphate buffer (0.15 M, pH 7.0) diluted by 0.16 ml hydrogen peroxide (30% w/v) to 100 ml with buffer (solution 1). The optical density of this solution was maintained about 0.5 at λ240 nm and prepared freshly.
Estimation:
Catalase activity was assayed by method of Luck (1971) with some modifications. Homogenized rat liver, heart and kidney tissue (600 mg) in 10 ml buffer solution phosphate buffer (solution 1, diluted 1:10) at 4° C and centrifuged at 5000 rpm for 5 minutes. Supernatant was used for assay and 100µl of sample was added to 3 ml of H$_2$O$_2$ buffer (solution2) and optical density was taken immediately at λ 240 nm. The decrease in optical density was observed for 3 minutes at interval of 30 s and activity of enzyme was expressed in µmole of H$_2$O$_2$ consumed/minute/g tissue by using the formula given by Luck et al (1971).

3.10.5. Determination of glutathione peroxidase (GPx):
Rat tissues (1g) were homogenized using glass homogenizer in 0.4M Tris hydrochloric acid buffer (10ml) containing pH 7.0 and 0.2ml of tissue homogenate was used for experiment. GPx activity was measured by the method described by Rotruck et al. (1973).

The reaction mixture contained 0.2 ml of 0.4 M Tris-HCl buffer pH 7.0, 0.1 ml of 10 mM sodium azide, 0.2 ml of tissue homogenate (homogenized in 0.4 M, Tris-HCl buffer, pH 7.0), 0.2 ml glutathione, 0.1 ml of 0.2 mM hydrogen peroxide. The contents were incubated at 37°C for 10 min. The reaction was arrested by 0.4 ml of 10% TCA, and centrifuged. Supernatant was assayed for glutathione content by using 50µl Ellman’s reagent (19.8 mg of 5,5'-dithiobisnitro benzoic acid (DTNB) in 100 ml of 0.1% sodium nitrate). Absorbance measured at 412 nm at interval of 30 seconds up to 2 minutes. Unit of enzyme activity expressed in µmole of GSSG formed/minute at 30° and pH 7 by using extinction coefficient of 9.6mM$^{-1}$ cm$^{-1}$.

3.10.6. Glutathione-S-transferase (GST):
Rat tissues (1g) were homogenized using glass homogenizer in 0.2M phosphate buffer (10ml) containing pH 8.0. The homogenate was centrifuged at 2000 rpm for 10 minutes and clear supernatant was used for estimation. The GST activity was determined by the method of Habig et al. (1974). The reaction mixture (3 ml) contained 1.0 ml of 0.3 mM phosphate buffer (pH 6.5), 0.1 ml of 30 mM 1-chloro-2, 4-dinitrobenzene (CDNB) and 1.7 ml of double distilled water. After pre incubating
the reaction mixture at 37°C for 5 min, the reaction was started by the addition of 0.1 ml of tissue homogenate and 0.1 ml of glutathione as substrate. The absorbance was followed for 5 min at 340 nm. Reaction mixture without the enzyme was used as blank. The activity of GST is expressed as µmoles of GSH-CDNB conjugate formed/min/mg protein using an extinction coefficient of 9.6 mM⁻¹ cm⁻¹.

3.10.7. Determination of protein content:

The protein content of the experimental samples was measured by the method of Lowry et al. (1951) using crystalline BSA as standard.

3.11. Antimicrobial potency:

The antimicrobial potential of test samples was observed both in vitro and in vivo methods for four human pathogenic strains of bacteria and fungi under standard conditions.

Three strains, of opportunistic bacteria Escherichia coli (MTCC no.729), Pseudomonas aeruginosa (MTCC no.3542) and Staphylococcus aureus (MTCC no.3160) obtained from Microbial type culture collection and gene bank (MTCC) Chandigarh India on kind request. Antifungicity of stem extract was assayed with Candida albicans from human culture isolates collected on request from stock culture of department of Microbiology Pt B.D.S.M.University, Rohtak, Haryana.

3.11.1. In vitro study:

The screening of most effective antibacterial and antifungal compound was performed by disc diffusion method and minimum inhibitory concentration of extract or isolated compound calculated by broth dilution method.

3.11.2. Preparation of Inoculum:

Stock cultures of S. aureus E.coli and P.aeruginosa were maintained by storing samples of an 18-h culture in Mueller Hinton broth at -20°C. Inoculum for each experiment was prepared by sub culturing a sample from the stock culture in Mueller Hinton broth (Hi media Pvt. Ltd) and incubating at 37°C for 18 h. The bacterial suspension was diluted 1:10 in Mueller Hinton broth and 25 µl of it was added to
culture media and mixed thoroughly. Stock culture of *C. albicans* maintained on Sabouraud dextrose agar slants at 37°C and inoculums was prepared by scraping colonies from slant and dissolving in Mueller Hinton broth. The final inoculum was of ~5 x 10^5 CFU/ml.

3.11.3. Disc diffusion assay:
Base plates were prepared by pouring 20 ml Mueller-Hinton agar (MHA) into sterile petri dishes (9 cm) and allowed to set. Filter paper discs (Wattman no.1, size 6 mm) were impregnated with the extract and the different compounds. The discs were air-dried and placed on the top layer of the agar plates. Each extract was tested in triplicate with a Ciprofloxacin, Amoxicillin, Kanamycin and Fluconazole as reference or positive control. Water saturated disc (air-dried) were used as negative controls. The activity was recorded by measuring the clear zone of growth inhibition on agar surface around the discs (Mothana and Lindequist 2005).

3.11.4. Determination of Minimum Inhibitory Concentration (MIC):
MIC was determined by Broth dilution method (Kouokam et al., 2002, Larrondo et al., 1995, Levine, 1987). Two-fold dilutions (six) of test substances (extract, fractions, isolated compounds) were carried out starting from the higher to lower concentration. Each inoculum was prepared in its respective medium and density was adjusted to 0.5 Mc farland standards (10^8 CFU/ml). Each tube was incubated at 37°C for 24 h. The lowest concentration of the tube which did not show any visible growth after macroscopic evaluation was considered as the MIC. The microbial growth was taken by taking the optical density at 600 nm at different incubation times of 10 min, and 2 to 36 h. To determine the MIC fresh culture from the broth was inoculated into Mueller-Hinton agar plates, incubated for 24 h and were observed for bacterial growth to confirm inhibition in growth. The standard drug amoxicillin, ciprofloxacin, kanamycin and fluconazole (Hi media Pvt.Ltd.) were used as controls in the tests conducted.

3.11.5. *In Vivo* antimicrobial studies:
In order to obtain the viability and antimicrobial potential of *C. aphylla* extract and modified compound under reference in severe diabetic condition, rats were pre
inoculated with pathogenic strains. The enumeration of microbes was performed after treatment by counting numbers of colonies per kidney tissue.

3.11.6. Determination of the ID$_{50}$. 
Diabetic and age matched control rats were divided into groups, and each group was given inoculation of various sizes intravenously of either $C. \text{albicans}$, $S. \text{aureus}$, $E.\text{coli}$ and $P.\text{aeruginosa}$. Rats were killed 5 days after inoculation. Rats were considered to be infected if the kidney allows to grew >102 CFU/g of pathogens. The 50% infective dose (ID$_{50}$) of microorganisms was estimated following Bancroft (1960) under treatment of all test samples of stem extract.

3.11.7. Inoculation of Microbes:
Diabetic, non diabetic control and treated rats were inoculated intravenously via the lateral tail vein at 1st day of treatment with single dose of $1 \times 10^5 \ C. \text{albicans}$, $5 \times 10^7 \ S. \text{aureus}$ or $P.\text{aeruginosa}$ and $7 \times 10^5 \ E.\text{coli}$ calculated by density adjusted to 0.5 McFarland turbidity standard (Raffel et al.1981).

3.11.8. Enumeration of microorganisms:
The number of organisms per gram of kidney was determined in the same manner, after homogenizing the kidney in 9 ml of Mueller Hinton broth with Teflon tissue grinders (Hi media Pvt.Ltd.). The number of organisms in broth was determined by plating 0.1 ml of each specimen on Mueller Hinton Agar media (Hi media Pvt.Ltd) by making serial 90-fold dilutions in Mueller Hinton broth. The total number of viable organisms in tissue or in broth was calculated from colony counts after incubation for 24 h at 37°C (Raffel et al. 1981).

3.12 Determination of total phenols and total flavanoids:
In order to check the level of contamination of phenols and flavanoids in test samples following methods used.
For phenols the solvent and conventional extracts (100 μl) as well as proposed test molecule mixed with 0.2 ml Folin-Ciocalteu reagent. About 2.0 ml of $H_2O$ and 1.0 ml of 15% $Na_2CO_3$ solution added to that and the mixture O.D was measured at 765 nm after keeping for 2 h at room temperature. The mean of three readings was used
and the total phenolic content was expressed as µg of gallic acid equivalents/1 g extract. The coefficient of determination was $r^2 = 0.9958$ (Gao et al., 2000).

Flavonoids in the solvent and conventional extract and test sample prepared from *C.aphylla* were estimated as quercetin equivalent. The standard extracts (0.5 ml) were mixed with 1.5 ml 95% ethanol (v/v), 0.1 ml 10% aluminum chloride (w/v), 0.1 ml of 1 mol/L sodium acetate and 2.8 ml water. The volume of 10% aluminum chloride was substituted by the same volume of distilled water in blank. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm. The mean of three readings was used and the total flavonoids content was expressed in µg of quercetin equivalents/1 g extract. The coefficient of determination was $r^2 = 0.9961$ (Kosalec et al., 2004).

### 3.13 Isolation and purification of active compound:

For isolation and purification the active compound, potent antidiabetic, antimicrobial and hypolipidemic from crude solvent extract of stem without bark was prepared by using thin layer chromatography and high performance liquid chromatography having various physical and chemical treatments during different steps of preparation.

The purity and isolation of the compound was undertaken using thin layer chromatographic system developed in Chloroform and methanol solvent system. A particular amount of sample after specified cold and light treatment (patent applied) was used for spotting on the activated silica G (5:1 w/v). A fixed temperature (patent applied) was maintained for chromatography to obtained desired active spot on T.L.C. A variety of solvent systems were tested and tried until ‘optimal’ systems (for the separation of the different constituents) were obtained for each of the extracts. The thin-layer plates were visualized with the aid of iodine vapors. Four different spots (including spot at origin) were obtained when the chromatogram was placed inside an Iodine chamber and subsequently a particular temperature treatment. All the compounds were separated and collected using preparative thin layer chromatography.
All spots designated as A (baseline), B, C and D estimated quantitatively showed as 8.3%, 3.33 %, 34.16% and 19.75 % yield respectively from crude extract (fig.8). Therefore, for further analysis all spots were scraped with sharp blade and redisolved into solvent. The selection of active spot was made on the basis of the antimicrobial screening by disc diffusion method. The band C was considered as the effective antimicrobial compound on the basis of hypoglycemic as well as antimicrobial test and subjected for further purification by thin layer chromatography after separation and reloaded (patent applied) onto Aluminum backed, 0.2 mm silica gel plates (Merck 0.20 mm or Machery-Nagel) by drawing a stripe across the plate with a ruler. A standard concentration was used for spotting. The plates were then developed in the chromatography tank with an appropriate solvent system (fig.9). The particular conditions (patent applied) were provided for color development marker of active molecule prepared. The clearly seen single spot was marked and scraped off by cutting a 1 cm strip from each side of the plate (fig.10). The compounds were removed from the silica named as test compound/ molecule/ bioactive compound.

3.13.1. Column Chromatography:
The C fraction was further subjected for purification by column chromatography separation. Column chromatography was performed with silica gel 60-100 (Merck 0.040 -0.063 mm) on column with a 2 cm diameter and with a desired length. The extract or fractions were dissolved in a small amount of solvent and loaded onto the column. The mobile phases (eluents) were comprised of chloroform and methanol at a particular ratio (patent applied). The collection of fraction was made of 4 ml at the flow rate of 1 ml/min and 50 fractions were collected of 4.0ml each at specified temperature.

3.13.2. High performance liquid chromatography:
The HPLC analysis was performed in different conditions in order to estimate the purity of compound isolated and to facilitate the structure of major active compound. Samples (20μL) were analyzed using a HPLC system coupled with a pump and controller (Waters 600), an auto sampler (Waters 717 plus) and a UV detector (Waters 996). The system was operated with Empower 2 software (Water, USA...
Fig. 8 Thin layer chromatography development under desired controlled conditions.
Fig. 9 T.L.C chromatography showing separation and concentrating test compound.
Fig. 10 Thin layer chromatography for isolation of test compound.
Fig. 11 H.P.L.C. chromatogram of test compound with retention time.
A reversed phase Bondapak C-18 column (300mm×3.9mm i.d. and particle size 10mm) was used. The Waters pump was operated at 1mL/min using the methanol as eluent and spectra obtained at 210 nm. The 40 compounds collected (4ml each) by column chromatography were pooled to obtained 10 fractions (E to N) on the basis of their H.P.LC spectrum. The compound I (21-26) and J (30-34) showed single peak at retention time 2 minutes (fig.11) were selected for further analysis by NMR and Mass spectroscopy on their results in Glucose tolerance test.

3.13.3. Nuclear Magnetic Resonance (NMR):
Nuclear magnetic resonance spectroscopy of the isolated compounds was performed on a Bruker Avance II 400 NMR spectrometer equipped with a proton probe 400 MHz for proton. The samples were dissolved in deuterated methanol and the spectra recorded at 30°C. Line broadening processing of 1 Hz was used. All spectra were recorded at 25 °C and the chemical shifts were recorded in ppm referenced to TMS or the solvent shift at the SAIF department Panjab University Chandigarh.

3.13.4. Mass Spectrometry
Electron ionization mass spectrometry of the compounds was performed by TOF MS ES+ mass spectrometer at the SAIF department Panjab University Chandigarh.

3.14. Statistical analysis:
The values are expressed as mean ±SD. The results were analyzed statistically using ANOVA to find out the level of significance. The minimum level of significance was fixed at p<0.05.