I. MATERIALS AND METHODS:

A. Animal selection:

Adult Wistar rats of either sex weighing 150-200g were used in the experiment. The animals had free access to laboratory chow diet and tap water. They were housed in galvanized iron cages in a thermostatically controlled room (28 ± 2^0 C) and maintained in 12 h natural dark/light cycle. The study has been approved by "Institutional Animal Ethics Committee", KLE University's College of Pharmacy, Belgaum.

B. Chemicals:

1.	Silymarin	Sigma-Aldrich Chemie, Germany
2	Quercetin	Sigma- Aldrich Chemical Pvt. Ltd. St Louis, MO, USA.
3	Chrysin	Sigma- Aldrich Chemical Pvt. Ltd. St Louis, MO, USA.
4.	p-nitrophenyl β-D-glucuronide	Sigma- Aldrich Chemical Pvt. Ltd. Bangalore.
5.	p-nitrophenol	Loba Chemie Pvt. Ltd., Mumbai
6.	Glycine	Himedia, Mumbai
7.	Carbon tetrachloride	Qualigens Fine Chemicals, Mumbai
8.	Triton X_{100}	Central Drug Gouse (P) Ltd., New Delhi
9.	Na_2EDTA	Ranbaxy Fine Chemicals Ltd., New Delhi
10.	Sucrose	Loba Chemie Pvt. Ltd., Mumbai
11.	Sodium Carbonate anhydrous	Nice Chemicals Pvt. Ltd., Cochin
12.	Phenobarbitone	Nicholas Piramal India Ltd., Gujarat
13.	p-nitrophenylphosphate	Sigma- Aldrich Chemical Pvt. Ltd. St Louis, MO, USA.
14.	Sodium hydroxide	Ranbaxy Fine Chemicals Ltd., New Delhi

Kits are used for estimation of: AST, ALT, ALP, SBR, TP, Adrenaline, Nor Adrenaline and Growth hormone.

All other reagents used were of analytical grade.

C. Instruments:

a. Tissue homogenizer - Remi motors Ltd., Mumbai (India).

b. Centrifuge instrument - Bangalore Genei Pvt., Ltd Bangalore.

c. Electronic balance - Adrir dutt instrument Pvt., Ltd.

d. UV visible spectrophotometer- Shimadzu (UV number 1201) Japan.

e. Biochemistry auto analyzer (Rapid star - 21 plus).

D. Animal treatment

Suspension of flavonoids were prepared by using 1% w/v Carboxy Methyl Cellulose (CMC) in water and the LD_{50} cutoff value was determined by OECD guidelines.[135] Acute toxicity studies revealed that Silymarin, Quercetin and Chrysin did not cause any mortality in the doses tested and found to be safe up to 5000mg/kg, therefore 1/10th and 1/20th of acute toxicity, i.e. 250 and 500mg/kg, p.o, b.w. were selected as daily dose in the experimental protocol.

The animals were randomly divided into 8 groups containing 18 animals in each. Distilled water containing Phenobarbitone at the concentration of 500 mg/L was the only source of drinking water for the rats 10 days prior to the first dose of CCl_4 vapour and throughout the treatment period. Phenobarbitone was co-administered with CCl_4 to hastens the development of cirrhosis.[136]

Twenty animals were exposed to CCl_4 vapors twice a week. After exposure to CCl_4 for 2 weeks, six rats were sacrificed 3 days after the last dose of CCl_4. Similarly six rats each were sacrificed after 6 weeks and 12 weeks of treatment with CCl_4 (3

days after the last dose of CCl_4). Similarly, in the control group and flavonoid treated groups, six rats each were sacrificed at the same time as CCl_4 treated animals.

Grouping of Animals:

S.No	Groups	Necrosis 2 Weeks	Fibrosis 6 Weeks	Cirrhosis 12 Weeks
1	Group 1 (Control)	Phenobarbitone (500mg/l	Phenobarbitone (500mg/l	Phenobarbitone (500mg/l
2	Group 2 ($_{CCl4}$ Treated)	CCl_4 Vapours + Phenobarbitone	CCl_4 Vapours + Phenobarbitone	CCl_4 Vapours + Phenobarbitone
3	Group 3 Silymarin	CCl_4 Vapours + Phenobarbitone+ 250mg/kg of Silymarin	CCl_4 Vapours + Phenobarbitone+ 250mg/kg of Silymarin	CCl_4 Vapours + Phenobarbitone+ 250mg/kg of Silymarin
4	Group 4 Chrysin Low dose	CCl_4 Vapours + Phenobarbitone+ 250mg/kg of Chrysin	CCl_4 Vapours + Phenobarbitone+ 250mg/kg of Chrysin	CCl_4 Vapours + Phenobarbitone+ 250mg/kg of Chrysin
5	Group 5 Chrysin High dose	CCl_4 Vapours + Phenobarbitone+ 500mg/kg of Chrysin	CCl_4 Vapours + Phenobarbitone+ 500mg/kg of Chrysin	CCl_4 Vapours + Phenobarbitone+ 500mg/kg of Chrysin
6	Group 6 Quercetin Low dose	CCl_4 Vapours + Phenobarbitone+ 250mg/kg of Quercetin	CCl_4 Vapours + Phenobarbitone+ 250mg/kg of Quercetin	CCl_4 Vapours + Phenobarbitone+ 250mg/kg of Quercetin
7	Group 7 Quercetin High dose	CCl_4 Vapours + Phenobarbitone+ 500mg/kg of Quercetin	CCl_4 Vapours + Phenobarbitone+ 500mg/kg of Quercetin	CCl_4 Vapours + Phenobarbitone+ 500mg/kg of Quercetin
8	Group 8 (Quercetin+ Chrysin)	CCl_4 Vapours + Phenobarbitone+ 250mg/kg Quercetin+ 250mg/kg Chrysin	CCl_4 Vapours + Phenobarbitone+ 250mg/kg Quercetin+ 250mg/kg Chrysin	CCl_4 Vapours + Phenobarbitone+ 250mg/kg Quercetin+ 250mg/kg Chrysin

II. SCREENING MODELS

Induction of cirrhosis:

Cirrhosis was induced in the rats by chronic exposure to CCl_4 Vapours according to the method of McLean *et al.* [137] A wooden box, with a glass front (72 x 45 x 46 cm, i.e., 150 L capacity) was fitted with an inlet tap and housed in a fume cupboard. Compressed air was passed via a flow meter, at 4 L/min, bubbling through a train of two wash bottles containing "Analar". CCl_4 was maintained at 20°C. Air left the box by leaking through the joints. CCl_4 was run in for 5 minutes and was then turned off and the rats left in the box for 5 more minutes (therefore, total period of exposure was 10 min). Treatment was carried out twice a week for 12 consecutive weeks.

Figure 12: CCl_4 Vapour Induction Chamber

Tap water containing Phenobarbitone at the concentration of 500 mg/L was only source of drinking water for the rats 10 days prior to the first dose of carbon tetrachloride (CCl_4) vapors and throughout treatment period.

Phenobarbitone was co-administered with carbon tetrachloride since it has been shown in earlier studies that Phenobarbitone hastens the development of cirrhosis.[36]

Biochemical analysis

The animals were fasted overnight and blood was drawn by heart puncture under light ether anaesthesia. The liver were excised, weighed and used for biochemical analysis and histological assessment. Sera were separated from the blood and used for the assay of N-acetyl glucosaminidase (NAG), β-glucuronidase (β- glc), acid phosphatase, ALT, AST, ALP, SRBN and total protein activity at 2, 6 and 12 weeks. Adrenaline, nor adrenaline and growth hormone levels were estimated at the end of 6 weeks of the study.

E. Histopathology

The animals were sacrificed at the end of 2, 6 and 12 weeks and liver sections were subjected for histopathological studies. Slices of liver tissue were fixed in 10% buffered formalin, processed and stained with Haematoxylin-Eosin, Foot's reticulin and Van Geison stains for histopathological observation at 20 – 80x magnification.

Preparation of the homogenate:

Liver homogenates were prepared as described by Kyaw *et al.*[138] Briefly, portion of the liver (approximately 250 mg) was homogenized in 10 ml of ice cold 0.25 M sucrose, 1mμ disodium EDTA in a potter-Elvehjem homogenizer. The homogenate was divided into two equal parts, marked 'maximal' and 'basal'. The activity in the presence of Triton X100 denotes "maximal" or "total" activity and the activity in the absence of Triton X100 denotes "basal" or "free" activity. They were incubated at 37^0 C for one hour with gentle shaking now and then. The homogenates

were centrifuged at 4^0C at 11,000 ×g for 30 minutes to remove unlysed particles. The supernatant was used for the assay of NAG, β glc and acid phosphatase.

Assay of β-glucuronidase[139]

β-glucuronidase activity was measured by the method of Kawai and Anno, using *p*-nitrophenol-β-glucuronide as the substrate. The enzyme solution (0.2 ml) was added to 0.5 ml of substrate and 0.3 ml of sodium acetate buffer in a test tube, shaken gently and incubated at 37°C for 1 hour. Glycine NaOH buffer (3.0 ml) was added for reaction termination, mixed, and read at 410 nm. The activity of β-glucuronidase was expressed as micromoles of *p*-nitrophenol liberated per hour per 100 mg protein.

Assay of acid phosphatase[140]

Acid phosphatase was assayed by the method of Rosenblit *et al*, using disodium phenyl phosphate as the substrate. The incubation mixture contained the following components in a final volume of 3.0:1.5 ml of citrate buffer, 1.0 ml of substrate, 0.3 ml of distilled water and the requisite amount of the enzyme source (0.2 ml plasma). The reaction mixture was incubated at 37°C for 15 min. The reaction was terminated by the addition of 1.0 ml of Folin's phenol reagent. If turbidity appears, the tubes were centrifuged. Controls without enzyme sources were also incubated and the enzyme source was added after the addition of Folin's phenol reagent; 1.0 ml of 15% sodium carbonate solution was added and incubated for a further 10 min at 37°C. The blue color developed was read at 640 nm using a Shimadzu-UV-1601 spectrophotometer against a blank. The standards were also treated similarly. The activity of the enzyme was expressed as micromoles of phenol liberated per hour per liter (plasma) or micromoles of phenol liberated per milligram protein (tissue).

Assay of N-Acetyl β-d-glucosaminidase[139]

N-Acetyl β-d-glucosaminidase (nag) was assayed in the liver supernatant and serum as described by Kawai and Anno using p-nitro phenyl-β-glucuoronide as substrate. The reaction mixture consisted of 0.3 ml of 0.15 molar citrate-phosphate buffer, pH 4.4, 0.2 ml of 1 milimol/ L substrate and made upto volume of 1 ml. The reaction was started by the addition of 0.1 ml of supernatant/serum and mixture was incubated at 37°C for 30 minutes. The reaction was stopped and added of 3 ml of 0.3M glycine-sodium hydroxide buffer, pH 10. The yellow colour developed was measured against the blank at 410 nm.

All the above mentioned assays p-nitrophenol was used as a standard.

Total / free activity[136]

It is the ratio of total/free lysosomal enzyme activity, indicates the stability of lysosomal membrane. Decreased value suggests an increase in vulnerability of lysosomal membrane which results in the leakage of lysosomal enzymes.

Assay of biochemical parameters[141]

ALT, AST, ALP, SRBN and total protein activity were colorimetrically estimated by International Federation of Clinical Chemistry (IFCC) methodology using commercial assay kits by ERBA diagnostics Mannheim GmbH (Germany).

5. Assay procedure for estimation of biochemical parameters:

a. Estimation of AST (Aspartate serum Transaminase):

This reagent kit was intended for *in-vitro* quantitative determination of (AST) activity in serum/plasma.

Method: International Federation of Clinical Chemistry (IFCC).

Principle:

L-Aspertate+2-Oxoglutarate ⟶ AST → Oxaloacetate + L- Glutamate.

Oxaloacetate + NADH → MDH→ Malate + NAD

Sample pyruvate + NADH →LDH → L- Lactate + NAD

Clinical Significance:

AST occurs in all human tissues and present in large amounts in liver, renal, cardiac and skeletal muscle tissue. AST Level increased in liver diseases, myocardial infraction, muscular dystrophy and cholecystitis, where as decreased in patients undergoing renal dialysis and those with B_6 deficiency.

Reagent composition:

Reagent-1: AST Reagent

2-Oxaloglutarate	12 mmol/L
L-Aspartate	200 mmol/L
MDH	≥ 545 U/L
LDH	≥ 909 U/L
NADH (Yeast)	≥ 0.18 mmol/L
Tris Buffer (pH = 7.8± 0.1 at 25^{o} C)	80 mmol/L
EDTA	5.0 mmol/L
Also contains fillers and stabilizers.	

Reagent reconstitution:

The amount of Aqua-4 supplied in the kit was added to the reagent-1indicated on the label.

Assay procedure:

The working reagent was allowed to attain 37^0C before performing the test. 1 ml of working reagent was mixed with 100µl of test solution and the absorbance was recorded at 340nm.

b. Estimation of ALT (Alanine serum transaminase):

This reagent kit was intended for *in-vitro* quantitative determination of ALT activity in serum/plasma.

Method: International Federation of Clinical Chemistry (IFCC).

Principle:

L–Alanine + 2- Oxoglutarate →ALT→ Pyruvate + L- Glutamate

Pyruvate +NADH →LDH → L- Lactate + NAD

Clinical Significance:

Even though glutamate pyruvate transaminase is widely distributed in various tissues of the body, it is a useful parameter in evaluating liver function. The elevated serum levels are found in case of hepatitis, obstructive jaundice, metastatic carcinoma, hepatic congestion and myocardial infarction or in kidney diseases.

Sample:

Fresh, unhaemolysed clear serum was used as sample.

Reagent composition:

Reagent 1: ALT reagent

L-Alanine	500 mmol/L
NADH (Yeast)	0.18 mmol/L
LDH	≥ 1820 U/L
LDH	≥ 909 U/L
2-Oxaloglutarate	12 mmol/L
Tris Buffer (pH = 7.8± 0.1 at 25^{o} C)	80 mmol/L

Also contains non-reactive fillers and stabilizers.

Reagent reconstitution:

The amount of Aqua-4 Supplied in the kit was added to the reagent-1indicated on the label.

Assay procedure:

The working reagent was allowed to attain 37^{0} C before performing the test. 1ml of working reagent was mixed with 100µl of test solution and the absorbance was recorded at 340nm.

c. Estimation of ALP:

ALP is an enzyme found in high concentrations in the liver, biliary tract epithelium and in the bones. Increased levels are associated mainly with liver and bone disease.

Methodology: Adaptation by Wilkinson *et al* of the Bessey. Lowry *et a*l. method.

Principle:

ALP at an alkaline pH hydrolyses p-Nitrophenyl phosphate to form p-Nitrophenol and Phosphate. The rate of formation of p-Nitrophenol is measured as an increase in absorbance, which is proportional to the ALP activity in the sample.

p- Nitrophenyl phosphate + water $\rightarrow$**ALP** $\xrightarrow{Mg^{++}}$**p- Nitrophenol + Phosphate**

Reagent composition:

Reagent 1: ALP reagent.

p-Nitrophenyl phosphate	16mmmol/L
Mg++	4mmmol/L
Tris carbonate buffer (pH 10.2 ± 0.2 at 25^{o} C)	-

Also contains non-reactive fillers and stabilizers

Reagent reconstitution:

The amount of Aqua-4 Supplied in the kit was added to the reagent-1indicated on the label.

Assay procedure:

The working reagent was allowed to attain 37^{0} C before performing the test. 1ml of working reagent was mixed with 20µl of test solution and the absorbance was recorded at 405 nm.

d. Estimation of Bilirubin:

This reagent kit was intended for *in-vitro* quantitative determination of direct and indirect bilirubin from serum/plasma.

Method: Diazo method of Peariman and Lee.

Principle:

Bilirubin reacts with diazotized sulphanilic acid in acidic medium to form pink colored azobilirubin with absorbance directly proportional to bilirubin concentration. Direct bilirubin, being water soluble directly reacts in acidic medium. However indirect or unconjugated bilirubin is solubilised using a surfactant and then it reacts similar to direct bilirubin.

Reagent composition

Reagent 1: Total bilirubin reagent

Surfactant	1.00 %
HCl	100 m mol/L
Sulphanic acid	5 m mol/L

Reagent 2: Sodium Nitrate reagent

Sodium nitrate - 144 m mo/L

Reagent preparation:

Test	**Volume of working reagent**	**Add**	
		Reagent 1	**Reagent 2**
Total bilirubin	10 ml	10 ml	0.2
	25 ml	25 ml	0.5
	50 ml	50 ml	1.0
	100 ml	100 ml	2.0

Assay procedure:

Pipette into test tubes marked	Blank	Standard	Test
Working reagent	500 µl	500 µl	500 µl
Distilled water	25 µl	-	-
Standard/ calibrator	-	25 µl	-
Test	-	-	25 µl

Mix well, incubate for 5 minutes at 37^0 C and read absorbance at 546 to 630 nm against reagent blank.

e. Estimation of Total Protein:

This reagent kit was intended for *in-vitro* quantitative determination of total protein from serum/plasma.

Clinical significance:

Total protein is useful for monitoring changes in protein levels caused by various disease states, including in chronic liver disease.

Method:

The peptide bonds of protein react with copper II ions in alkaline solution to form blue-violet complex (Biuret reaction). Each copper ion complexing with 5 or 6 peptide bonds. Tartarate was added as a stabilizer whilst iodide was used to prevent auto-reduction of the protein concentration and was measured at 546 nm (520-560).

Reagent composition:

Reagent 1: Total Protein reagent

Copper II sulphate	19 m mol/L
Potassium sodium tartarate	43 m mol/L
Potassium iodide	30 m mol/L
Sodium hydroxide	600 m mol/L

Total Protein standard

Protein standard	6.0 g/dl

Assay procedure:

Pipette into tubes marked	Blank	Standard	Test
Reagent	1000 µl	1000 µl	1000 µl
Distilled water	20 µl	-	-
standard	-	20 µl	-
Test	-	-	20 µl

Incubate for 10 min. at 37^0 C., read absorbance of the standard and each test at 546 nm against reagent blank.

Assay of growth hormone levels

Growth hormone levels were assayed in plasma sample by using rat growth hormone kit by ELISA method (SPI bio, France).

Assay of catecholamine levels

Adrenaline and nor adrenaline levels were assayed in plasma sample by using rat adrenaline and noradrenalin kits by ELISA method (LDN, GmbH & co.KG).

Assay procedure for Nor adrenaline:

Allow all reagents to reach room temperature and mix thoroughly by gentle inversion before use.

Pipette 10 µL of standards, controls and 300 µL of plasma samples into the respective wells of the extraction Plate and later samples were extracted and subjected to acetylation.

1. The acetylated samples were pipette out (25 µl) of the Enzyme solution into all wells of the nor adrenaline micro titer Strips.
2. Pipette 20 µL of the extracted standards, controls and samples into the appropriate wells.
3. Incubate for 30 min at RT (20-25°C) on a shaker (approx. 600 rpm).
4. Pipette 50 µL of the nor adrenaline Antiserum into all wells and cover plate with adhesive foil.
5. Incubate for 2 hours at RT (20-25°C) on a shaker (approx. 600 rpm).
6. Remove the foil. Discard or aspirate the content of the wells and wash each well 3 times thoroughly with 300 µL Wash buffer. Blot dry by tapping the inverted plate on absorbent material.
7. Pipette 100 µL of the enzyme conjugate into all wells.
8. Incubate for 30 min at RT (20-25°C) on a shaker (approx. 600 rpm).

9. Discard or aspirate the content of the wells and wash each well 3 times thoroughly with 300 µL Wash buffer. Blot dry by tapping the inverted plate on absorbent material.
10. Pipette 100 µL of the substrate into all wells and incubate for 25 ±5 min at RT (20-25°C) on a shaker (approx. 600 rpm). *Avoid exposure to direct sun light!*
11. Add 100 µL of the stop solution to each well and shake the micro titer plate to ensure a homogeneous distribution of the solution.
12. Read the absorbance of the solution in the wells within 10 minutes, using a microplate reader set to 450 nm and a reference wavelength between 620 nm and 650 nm.

Assay procedure for Adrenaline:

Allow all reagents to reach room temperature and mix thoroughly by gentle inversion before use.

Pipette 10 µL of standards, controls and 300 µL of plasma samples into the respective wells of the extraction plate and later samples were extracted and acetylated.

1. Pipette 25 µl of the Enzyme Solution (refer to 6.1*)* into all wells of the Adrenaline Micro titer Strips.
2. Pipette 100 µL of the extracted standards, controls and samples into the appropriate wells.
3. Incubate for 30 min at RT (20-25°C) on a shaker (approx. 600 rpm).
4. Pipette 50 µL of the respective Adrenaline Antiserum into all wells and cover plate with Adhesive Foil.

5. Incubate for 2 hours at RT (20-25°C) on a shaker (approx. 600 rpm).
6. Remove the foil. Discard or aspirate the content of the wells and wash each well 3 times thoroughly with 300 µL Wash Buffer. Blot dry by tapping the inverted plate on absorbent material.
7. Pipette 100 µL of the Enzyme Conjugate into all wells.
8. Incubate for 30 min at RT (20-25°C) on a shaker (approx. 600 rpm).
9. Discard or aspirate the content of the wells and wash each well 3 times thoroughly with 300 µL Wash buffer. Blot dry by tapping the inverted plate on absorbent material.
10. Pipette 100 µL of the Substrate into all wells and incubate for 25 ±5 min at RT (20-25°C) on a shaker (approx. 600 rpm). *Avoid exposure to direct sun light!*
11. Add 100 µL of the Stop Solution to each well and shake the micro titer plate to ensure a homogeneous distribution of the solution.
12. Read the absorbance of the solution in the wells within 10 minutes, using a microplate reader set to 450 nm and a reference wavelength between 620 nm and 650 nm.

Assay procedure for Growth Hormone

Plate Preparation:

1. Prepare the wash buffer as indicated in the reagent preparation section. Open the plate packet and select the sufficient strips for your assay and place the unused strips back in the packet (stored at 4°C). Rinse each well five times with the wash buffer (300 µl/well).

2. Just before distributing reagents and samples, remove the buffer from the wells by inverting the plate and shaking out the last drops.

3. A plate set-up is suggested on the following page. The contents of each well may be recorded on the sheet provided with the kit.

Pipetting the Reagents:

B : Blank

NSB : Non-Specific Binding

Bo : Maximum Binding

S1-S8 : Standards 1-8

Samples or Quality controls

1. EIA buffer: Dispense 100 µl to Non-Specific Binding (NSB) wells and 50 µl to Maximum Binding (Bo) wells.

2. Rat GH standard: Dispense 50 µl of each of the eight standards (S1 to S8) in duplicate to appropriate wells. Start with the lowest concentration standard (S8) and equilibrate the tip in the next higher standard before pipetting.

3. Quality control and samples: Dispense 50 µl in duplicate to appropriate wells. Highly concentrated samples may be diluted in EIA buffer.

4. Rat GH antiserum: Dispense 50 µl to each well except the Non-Specific Binding (NSB) wells.

Incubating the Plate:

5. Cover the plate with a plastic film and incubate for 20 hours at room temperature.

Distribution of Tracer

6. Rat GH AChE tracer: Dispense 50 µl to each well.

Incubating the plate:

7. Cover the plate with a plastic film and incubate for 20 hours at room temperature.

Developing and Reading the Plate:

8. Reconstitute the wash buffer and Ellman's Reagent as indicated in reagent preparation section. Empty the plate by turning over and shaking. Then, wash each well five times with the wash buffer (300 µl/well).

9. Dispense 200 µl of Ellman's Reagent to the 96 wells. Incubate in the dark (plate covered with an aluminium sheet) at room temperature. Optimal development is obtained using an orbital shaker. The plate should be read between 405 and 414 nm (yellow colour) when the Maximum Binding (Bo) wells reach an absorbance of 0.2-0.8 unit.

Data Analysis

10. Make sure that your Plate Reader has subtracted the absorbance readings of the blank well (absorbance of Ellman's reagent) from the absorbance readings of the rest of the plate.

11. Calculate the average absorbance for each NSB, Bo, standards and samples.

12. Calculate the B/Bo (%) for each standard and sample: (average absorbance of standards or sample -average absorbance of NSB) divided by (average absorbance of Bo - average absorbance of NSB) and multiplied by 100.

13. Using a semi-log graph paper, plot the B/Bo (%) for each standard point (y axis) versus the concentration(x axis). Draw a best-fit line through the points.
14. To determine the concentration of samples, find the B/Bo (%) value on the y axis. Read the corresponding value on the x axis which is the concentration of your unknown sample. Samples with a concentration greater than 40 ng/ml should be re-assayed after dilution in EIA buffer.
15. Most plate readers are supplied with curve-fitting software capable of graphing this type of data (logit/log or 4-parameter). If you have this type of software, we recommend using it. Refer to it for further information.

Statistical analysis:

The results were expressed as the mean ± SEM (n=6) for each group. Statistical differences were evaluated using One-way analysis of variance (ANOVA) followed by Dunnett's test. Results were considered to be statistically significant at $p < 0.01$.