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Essential Safety Pharmacology and safety evaluation of bioactive fraction of Xylocarpus moluccensis: an antidyslipidaemic agent

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Abstract –

Background & objectives - Mangrove plants are used in folklore medicine and proven their medicated significance for humans. The present study showed the essential safety pharmacology (ESP) and toxicity profile of new patented (US 7959954 from CSIR-CDRI) bioactive fraction of mangrove Xylocarpus moluccensis for antidyslipidaemic activity (fraction code is CDR267F018). The effective dose (ED) of CDR267F018 is 50mg / kg in rodents.

Methods – Essential safety pharmacology (ESP) studies were performed to investigate the effect of CDR267F018 on central nervous system (CNS) and cardiovascular system (CVS). To explore the adverse effects of CDR267F018 on biochemistry, hematology and different organs of rodents and rhesus monkey, the toxicity studies were performed as per regulatory guidelines.

Results - Administration of CDR267F018 did not cause significant alteration CNS and CVS of animals as observed by ESP studies. No significant alteration in general behavior, body weight and urinalysis parameters were observed. Few biochemical parameters were altered significantly though the value remains with in range of normalcy. No significant change in organ weight was observed except the increase in liver weight of few animals of 10 day DRF study. CDR267F018 administration did not cause any significant histological changes in animals except few incidental findings of focal mononuclear cell collection (FMCC) in liver and bronchus associated lymphoid tissue (BALT) in lung and calcification in adrenal medulla.

Interpretation & conclusions - The maximum tolerated dose of CDR267F018 was 500 mg / kg and 250 mg / kg in rodents and primates respectively. Findings suggested that fraction is safe to be use as candidate drug for the treatment of dyslipidaemic disorder and should proceed for clinical trials.

Keywords: xylocarpus, antidyslipidaemia, essential safety pharmacology, toxicity studies

Introduction:

Marine flora and fauna have been extensively used in the treatment of many diseases. It also serves in their natural form as a template for synthetic modification or in combination of extracts of different plant parts[1]. Several molecules isolated from marine sources are at an advanced stage of clinical trials and some of them have already been marketed as drug[1,2]. Mangroves trees are up to medium height and shrubs that grow in saline coastal sediment habitats in the tropics and subtropics areas. Patanaik et al (2008)[3] have reported the utilization of mangrove forest in India. Xylocarpus genus is one of the mangrove plants in the mahogany family (Meliaceae). Xylocarpus is native to mangrove forests of the Western and Central Indo-Pacific[4]. It is commonly known as Pussur in the Hindi language. Xylocarpus trees range up to 20 meter in height with buttressed stem base. Bark is yellowish-white, peeling off as papery flakes. Leaves unijugate or bijugate, leaflets obovate, glabrous, entire, rounded at apex, tappering at base, flowers 5-7 mm. across, white with a reddish gland within, in axillary thyrses. Fruits are large as long as 30-40 cm. across, globose, septa fragal capsules, splitting tardily into 4 valves. Seeds 10-15 in number, pyramid shaped corky testa, flowering and fruiting throughout the year. In India the species is found in tidal forests along the coastal areas up to Maharashtra, and in the Andaman Islands. The plant X. molluccensis together with X. granatum has yielded 23 limonoids so far[5]. CSIR-CDRI prepared the bioactive fraction from fruits of Xylocarpus in 2011 for the treatment of dyslipidemia and diabetes and received the patent for the same (Patent number – US-7959954). The bioactive fraction of X. moluccensis was coded as CDR267F018.

Different parts of Xylocarpus moluccensis and Xylocarpus granatum trees like bark, leaves, roots etc are reported to have medicinal biological activities. Khairina et al (2011)[6] have reported the cell growth inhibition activity in leaf extract of X. Molluccensis and bark extract of X. granatum however, no anticancer activity was observed in root extract of both genus. Antioxidative activity of X. molleccensis is reported in methanolic fraction of leaves[7]. Antimicrobial effect has also been reported in the organic extract of X. granatum stem barks by Alam et al, (2006)[8]. Anti-diarrhoeal activity of the methanol extract of the barks of X. moluccensis in mice is also reported[9]. Neuropharmacological properties of X. moluccensis has been reported in mice by Sarker et al (2007)[10].

The bioactive fraction of X. moluccensis (coded as CDR267F018) has showed the antidyslipidemic and antidiabetic activity, as assessed in experimental models of dyslipidaemia and diabetes. In control and experimental models of dyslipidemia the level of triglyceride and sugar was assessed. X. moluccensis administration

offered significant lowering in the level of triglyceride and sugar (Patent number – US-7959954; for more details please see the patent available on line at website http://www.patentstorm.us/patents/7959954.html). Previous findings (published in patent) showed that bioactive fraction of X. moluccensis can be use in treatment of diabetes and dyslipidemia. Therefore the present study was performed to assess the effect of CDR267F018 on different biochemical, hematological and histology of rodents and primates. Depending on the findings of the present study the fraction can be proceed for further clinical trials and can be develop as a potent drug molecule for diabetes and dyslipidemia.

2. Materials and Methods:

Materials – Bovine serum albumin, dimethyl sulfoxide, ethylene ditetra acetic acid, Glucose, HEPES, Magnesium chloride, Disodium hydrogen phosphate, Monosodium dihydrogen phosphate, Potassium chloride, Sodium azide, Sodium Bicarbonate, Sodium carbonate, Sodium chloride, Sodium dihydrogen phosphate, Sodium hydroxide, Sodium potassium tartarate, Tris buffer, Folin & ciocalteu's reagent and ether were procured from Sisco Research Laboratory, India. All the biochemistry and hematology kits were purchased from Merck. Urine srips were procured from Bayer Company.

Study Design – The study was performed to evaluate both essential safety pharmacology and toxicological parameters after single and repeat administration of CDR276F018. The doses essential safety pharmacology (ESP) experiments were selected on the basis of inhibitory concentration of fraction i.e 22mg/kg. However for toxicity profiling the doses were selected on basis on effective dose of CDR267F018 and the animal on which the experiment need to perform. The parameters were selected as per regulatory guideline of schedule Y.

Methods –

A) The extract was prepared from the fruit of plant Xylocarpus moluccensis The fruits of the X. moluccensis were collected from South Andaman Coast of India in the month of January, 1999, March, 2003, March, 2004 with voucher specimen numbers 338,424,433 respectively and from Orissa Coast in June 2002 with voucher specimen number S96. The sample specimen has been preserved in the Herbarium of the Botany Division, Central Drug Research Institute, Lucknow, India. The bioactive fraction of CDR267F018 was provided by Medicinal Chemistry Division, CDRI.

In present study the swiss mice, Charles foster (CF) rats, spontaneous hypertensive rats, english albino guinea pigs, white albino rabbits and rhesus monkeys were used to evaluate different parameters. All animals were procured

from Animal house facility of CDRI after Institutional animal ethical committee (IAEC) approvals (56/06/R/Toxicol/IAEC; 103/06/Toxicol/IAEC for rats; CPCSEA number for rhesus monkey 25/184/2010-AWD). Animals were maintained under standard experimental conditions and provided with food pellets and water ad-libitum.

Aqueous suspension with gum acacia (1%) of CDR267F018 was administered to animals by oral route and control group received the vehicle (1% gum acacia) in all the studies.

B) Essential Safety Pharmacology studies:

To assess the effect of CDR267F018 on central nervous system (CNS), cardiovascular system (CVS) and respiration following studies were performed in mice, rats, guinea pigs and rabbit.

CNS studies: Study was conducted in swiss adult (10-12 weeks old) mice of either sex, weighing 18-22 g. The CDR267F018 was administered by oral route in doses of 90, 225, 450 and 900 mg/kg. Various activities like behavior, motor activity, sensory reflexes, neuromuscular coordination, maximal Electroshock Seizures (MES) and barbiturate induced hypnosis were evaluated to assess the effect of CDR267F018 as per regulatory guidelines.

Cardio-respiratory activity - Male albino rabbits (1.5-1.9 kg) were housed separately in the metallic cages. Test substance CDR-267-F018 was given in three different doses of 75, 150, 300 mg/kg, ig (intragastric routs) with anesthetia ketamine (50 mg/kg ip) and Xylazine (10 mg/kg ip). All the baseline (pre-drug administration) values (HR, MBP, amplitude and rate of respiration) were collated after a stabilization period of 30 minutes. Post-drug values were recorded for 90 min of observation period. Blood pressure was recorded by carotid artery on a Grass Polygraph (79G) using a pressure transducer (P23), while respiration was recorded by a Grass volumetric pressure transducer (PT5A). For systemic haemodynamic studies (effects on HR, MBP and respiration) 3 animals were used for 75 and 150 mg/kg, while one animal was used for 300 mg/kg. One rabbit was treated with highest dose (300 mg/kg, ig) observation (BP, HR, rate of respiration, and amplitude) were recorded for 200 min.

Procedure of telemetric recording for BP and HR: Effect of CDR-267-F018 was studied at a dose of 450 mg/kg p.o in conscious male spontaneous hypertensive rats weighing 250-275 gm. Blood pressure and heart rate were measured using biotelemetry system (DATAQUEST A.R.T., USA). A battery-operated transmitter (TL11M2-c50-PXT) was implanted through surgery. For surgery the animals were injected intraperitoneally with anesthesia ketamine (50 mg/kg ip) and Xylazine (10 mg/kg ip). During surgery, the tip of cannula, which measures blood

pressure and heart rate, was inserted into the left femoral artery. Post recovery the animals were subjected for drug administration (CDR267F018) and observations were done up to 5h after administration of CDR267F018.

Assessments of respiratory parameters in conscious rats - Animals were acclimatized in the chamber for a time period of 30 minutes prior to initiate the recording of the parameters. Test compound was administered by oral route (p.o) at 450mg/kg in rats and respiratory parameters were recorded in the form of numerical readings up to 4 h. Responses have been reported at an interval of 30 minutes up to four hours. Each reading was a mean of ten breaths. Each parameter as mentioned below was recorded at an interval of 30 minutes for a period of 240 minutes by using Whole Body Unrestrained Plethysmograph and data analysed by Buxco software. The respiratory parameters viz. frequency (breaths per minute), tidal volume (ml) and minute volume (ml/min.) were recorded.

Ex vivo recording action potential and QT interval: Ex vivo investigation for the effect of CDR267F018 was performed in papillary muscles of English albino guinea pigs (350-450 gm) of either sex. The animals were sacrificed under deep ether anesthesia and the heart quickly removed and placed in a dissecting chamber containing oxygenated PBS at a room temperature. Thin papillary muscles (n=3) of about 1-mm diameter were removed from the right or the left ventricle and placed in a 2-ml perspex electrophysiological chamber with a DC-controlled heating device to maintain the bath temperature (Hugo Sachs Electronics, March-Hugstetten, Germany) at 35° C. The preparation was super fused with oxygenated PBS maintained at 35^{0}C and driven electrically with rectangular pulse of twice the threshold potential and 1 to 2 milisecond duration delivered at 1Hz using teflon coated Ag-$AgCl_2$ bipolar electrodes that are connected to output of pulse generator (ST-02 Isolator ISO –100, Experimetria, Hungary). The preparation was allowed to equilibrate for 30 min before carrying out electrical recording. Papillary muscle showing arrhythmia or missed beats under control conditions were not used in the experiments. Recording of action Potentials (APs) was done by conventional intracellular recording technique using 3M KCl - filled glass microelectrodes (FMG -20 glass tubings, Dagan Corporation, U.S.A) of 10-20 MΩ tip impedance coupled to the input stage of a capacitance compensated preamplifier (Intracellular Amplifier Intr-01, Experimetria, Hungary). Vmax (maximum upstroke velocity) was also simultaneously measured with intracellular amplifier using SPEL ADVANCED INTRASYS software (Experimetria, Hungary). CDR267F018 dissolved in DMSO was applied cumulatively to circulating oxygenated perfusion solution to study dose and time dependence effect of its action on action potential parameters such as RP (resting membrane potential, mV), APA (action potential amplitude, mV), APD (action

potential duration at various level of repolarization, minute) and indirect assessment of QT interval was done via APD. Experiments only with stable impalements were included in this study. In order to examine the effect of vehicle (DMSO) on action potential parameters, experiments were done with DMSO alone and found that it had no significant effect on various parameters studied.

C) Systemic Toxicity Studies:

Single dose (single oral administration of CDR267F018 in rats), 10 days dose range finding (DRF, repeat dose for 10 day daily) and 28 day repeat dose studies (RDT, repeat dose administration for 28 days) were performed in CF rats (120-150gm bw) of both gender. In Rhesus Monkey (4-6 kg bw) of both genders the 28 day repeat dose toxicity (28 days repeat day administration of CDR267F018) was performed (as per guideline of schedule Y). Freshly prepared suspension of CDR267F018 in 1% gum acacia was orally administered in rats for study. Dosing was restricted between 10 am to 12 noon in empty stomach.

Dose schedule:

Single dose toxicity study in rats was performed to get the wide range of toxicity of CDR267F018. The selected doses were 500, 1000 and 2000 mg/kg depending on the effective dose of CDR267F018. The dose was selected as per recommendation of schedule Y. As the present compound is herbal / non-synthetic the maximum dose selected was 2000mg/kg. Groups with intermediate doses were also taken to get the complete toxicity profile of compound.

10 day dose range finding (DRF) study in rats was performed twice. For first study the doses were 250mg/kg, 500 mg/kg, 1000 mg/kg and 1500mg/kg body weight. In this study the mortality was observed therefore second DRF study was performed with doses 500 mg/kg and 750mg/kg body weight (based on findings of first DRF study).

For 28 day repeat dose toxicity (RDT) study the selected doses were 125 mg/kg, 250 mg/kg and 500 mg/kg (based on the findings of 10 day DRF study).

In primates the selected doses for 28 day RDT study were 62.5 mg/kg, 125 mg/kg and 250mg/kg depending on the studies conducted in rodent (FDA Guidelines, 2005).

Number of animals in studies – At initiation of the study CF rats (120-150 gm bw) were randomized and grouped. The number of rats per group of each gender was five per group each gender. The number of animals in RDT study in rhesus monkey was 3 animals / group / gender.

Parameters–

General Behavior, food-water intake and body weight: Recording of general behavior was done daily as per the guideline of schedule Y. Average

food and water intake in rats were recorded for 24 h (average of per group / gender). Body weights of individual animal were recorded at initial and final time point of study. At the end of the study also the body weights were recorded.

Haematology: Hemoglobin (Hb) levels, red blood cells count, total leukocytes count, number of platelets (PLT), hematocrit (HCT), mean corpuscular volume (MCV), mean corpusculare hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were assessed in peripheral blood of animals by using automated hematology analyzer (MS-9, Melet Schlosing, USA). In rats, the blood was collected by tail tip and different parameters were estimated at termination of study. In case of primates the hematology parameters were assessed at both initial and final time point of the study. Blood was collected from femoral vein.

Biochemistry: Biochemical parameters of blood like level of cholesterol, triglyceride, total protein, uric acid, creatinine, glucose, bilirubin, ALT, AST, levels of different ions like calcium and phosphorous were estimated in peripheral blood by automated biochemistry analyzer (Beckman Synchron CX-5, USA). In rats the parameters were estimated at termination of study and Blood was collected from heart directly during sacrifice. In case of primate study biochemistry parameters were assessed at both initial and final time point of the study and blood was collected from femoral vein.

Urine analysis: In rats and rhesus monkeys the urine samples were collected by metabolic cages. Urine parameters were estimated at initial and terminal time point of study by automated urine analyzer (Clintek 50, Bayer, USA).

Recording of organ weight and histological analysis: Animals were sacrificed to collect the different organs. Organs were washed separately and fixed in formalin minimum for 24 h and processed for dehydration and block preparation. Briefly the tissues were dehydrated in acetone (two changes of 30 m each). Transfer the tissue to 1:1 mixture of acetone and benzene for 30 minutes. Transfer the tissue to molten paraffin wax bath of histocenter 2 with wax at 65^0C and infiltrate with wax for 2 changes of 3 h. Blocks were prepared and labeled, fixed to the back side of block. Blocks were cooled sufficiently to form hard surface and moulds are removed. To cut the sections blocks were cooled and fixed in microtome aperture and 5 micron thick sections were cut down. Sections were processed for haematoxylin and eosin (HE) staining[11].

ECG Examination of rhesus monkey: To examine the effect of CDR267F018 on heart rate, regularity of heart beats, as well as the size and position of the chambers electrocardiogram (ECG) recordings were performed in primates (BPL,

India Sr. No. CT8G11835). Electrical activity of the heart over a period of time was detected by electrodes attached to the outer surface of the skin and recorded by a device external to the body. ECG tracing of the cardiac cycle (heart beat) consists of a P wave, a QRS complex and a T wave.

Ophthalmic Examination of rhesus monkey: The examination of eye was carried out by indirect ophthalmoscope (Make and model of ophthalmoscope- Heinz Accu Box II, Germany, CE). The parts of eye examined were conjunctiva, cornea, anterior chamber, lens, fundus- media, and blood vessels.

D) Genotoxicity Studies: AMES, micronucleus and chromosomal aberration assays were performed for genotoxic evaluation of CDR267F018 as per schedule Y guidelines. AMES assay was performed in four tester strains of Salmonella typhimurium TA-97a, TA-98, TA-100 and TA-102. Both spot assay and plate incorporation assay were done. Five doses were tested in spot assay (10µg, 33µg, 100µg, 333µg and 1000µg/plate) and three doses (100µg, 333µg and 1000µg/plate) were checked in plate incorporation assay. Each dose was checked in triplicate. For micronucleus assay swiss albino mice (20-25gm) were used (no. of animals /gr/gender-5).

Dose Schedule to animals for micronucleus and chromosomal aberration assay: CDR267F018 was dissolved in DMSO freshly and administered orally. Three doses 500 mg/kg, 1000 mg/kg and 1500 mg/kg body weight were selected on the basis of LD_{50} of the CDR267F018 in swiss mice. The administration of the CDR267F018 was done twice with a gap of 24 h. The autopsy was done after 6 h of the second treatment.

For chromosomal aberration assay the same doses were selected but the animals were sacrificed after 24 h of the treatment. Colchicine was injected 2 h prior to the autopsy to arrest metaphase.

Extraction of bone marrow and Preparation of smears for micronucleus assay: Animals were anesthetized with ether, dissected and femur bones were taken out. Muscles were removed from the bone and flushed out in 5ml of fetal calf serum with the help of a needle (22g) and a syringe as a fine suspension and cell mass if any was disrupted and incubated for 20 minutes. The cells in serum were centrifuged at 1000 x rpm for 10 minutes and the supernatant was removed by glass pasteur pipette carefully leaving a minute quantity. A small drop of the viscous suspension is put on the end of the clean slide and spread by pulling the material behind a cover glass held at an angle of 45° and coded and air dried. Three slides were made per animal. Slides were dipped in absolute methanol for five minute and processed for staining next day. The slides were stained in

freshly prepared May-Gruenwald stain for 2 minutes, in May- Grunwald : H_2O (1:1) for 5 minutes and in 10% Giemsa for 10 minutes. Finally slides were cleaned in xylene and mounted with cover glass in DPX.

Scoring: About 1000 polychromatic erythrocytes were scored for micronuclei under a microscope. The normochromatic erythrocytes were also scored along with the micronucleated ones. The number of polychromatic erythrocytes, micronucleated polychromatic erythrocytes, normochromatic erythrocytes, micronucleated normochromatic erythrocytes and the ratio of polychromatic erythrocytes and normochromatic erythrocytes for individual animal were recorded.

Preparation of metaphase plates, scoring of aberration: The bone morrow cells were collected in hypotonic solutions and centrifuged at 1000 x rpm for 10 minutes and the supernatant was discarded. 5 ml of Carnoy's fixative (3:1; methanol:acetic acid) was added drop by drop and the mass was broken. The sample was left for 2 h at 4^0C and centrifuged at 1000 x rpm. The supernatant was discarded and the pellet was again dissolved in few drops of fixatives to fix the cells. Then each sample was dropped onto the previously chilled glass slide from a distance so that the cells in the sample burst to show good metaphases. The slides were quick dried and allowed three days for maturation. The slides were stained in freshly prepared Giemsa for 10-20 minutes and cleaned with distilled water, air-dried and mounted with cover glass and DPX. About 100 metaphases were studied per animal under a light microscope. Each metaphase was observed for complete set of chromosomes and structural anomalies were counted in each individual.

Statistical Analysis: Statistical anaylsis was performed by using One-way ANOVA post-hoc Dunnette and Newmann Keuls Test. Data is represented as mean±SD. P less than 0.05 is considered as statistically significant.

3. Results –

(A) Essential Safety Pharmacology studies-

(I) Effects of CDR267F018 CDR 267 on CNS:

(a) Behavioral responses: No significant changes were observed on behavioral responses following the administration of CDR-267-F018 in doses of 90, 225, 450 or 900 mg/kg (p.o) up to 180 min.

(b) Motor activities: A mild decrease in spontaneous locomotor activity (SMA) was observed with 900 mg/kg dose at 60 min, which was transient in nature and normalized within 120 min. Ambulatory, vertical and total were also counted by Opto-Varimex at 1h after administration of CDR-267-F018. CDR-267-F018 in doses of 90, 225, 450 or 900 mg/kg, did not show significant effect on motor responses except mild transient decrease in SMA as compared to the control.

(c) Sensory Reflexes: Corneal, pinna and righting reflexes were intact (Score =1) in CDR-267-F018 treated mice. CDR-267-F018 showed significant increase in the nociceptive reaction time as compared to pre-treatment value for a short period i.e. upto 60 min (Table – 1 a). CDR-267-F018 in doses of 90, 225, 450 and 900 mg/kg po did not affect reflexes and showed transient anti-nociceptive activity.

(d) Neuromuscular coordination: To asses the muscle tonicity, all the mice in control as well as treated with both the doses of CDR-267-F018 showed normal hind limb rigidity. Rota rod test were performed for neuromuscular coordination. Control (vehicle) and CDR-267-F018 treated mice did not show any fall in rota-rod test i.e. stayed upto 2 min on the rod (5 rounds / min for 2 min) at all time-intervals – 0, 30, 60, 120 and 150 min. CDR-267-F018 in doses of 90, 225, 450 and 900 mg/kg, po did not show any significant effect on neuromuscular coordination.

(e) Maximal electroshock seizures (MES): Control and treated mice (-60 min) were subjected to electric shock (48 m Amp for 0.2 sec) through pinna electrode. All the mice showed tonic seizures – extension of hind limbs with loss of posture.

(f) Pentylenetetrazol induced seizure: Pentylenetetrazol (80 mg/kg, sc) was given to all the animals. Seizure pattern – alternate extension and flexion of limbs with loss of posture (clonic convulsions), was observed in each group. Pentylenetetrazol showed chronic convulsions in all the animals of control and treated groups. CDR-267-F018 in doses of 90, 225, 450 and 900 mg/kg, p.o did not show any significant anti-convulsant activity.

(g) Barbiturate sleeping time: Barbiturate induced hypnosis / sleeping time was calculated by the duration between loss and regain of righting reflex following administration of Pentobarbitone (30 mg/kg, ip). CDR-267-F018 in the doses 90, 225, 450 or 900 mg/kg, po was administered 30 min prior to pentobarbitone. Pentobarbitone induced sleep was not affected significantly by CDR-267-F018 (Table-1b). CDR-267-F018 in doses of 90, 225, 450 and 900 mg/kg, p.o showed no significant change in Barbiturate induced hypnosis.

(II) Effects of CDR267F018 on CVS: CDR-267 F018 had no significant effect on heart rate (HR), blood pressure (BP) and respiration in the anesthetized rabbits. No significant alteration was observed on any parameter recorded up to 6 hours after CDR-267-F018 in comparison to control rats (Fig.-1).

(III) Effect of CDR267F018 on respiratory parameters: CDR-267-F018 at 450mg/kg dose had no significant effect on the frequency of

respiration, tidal volume and minute volume in comparison to control rats (Table-1c).

(IV) Effect of CDR267F018 on action potential and QT interval: The effect of various doses (0.2, 0.4, 0.8, 1.6, 3.2 & 6.4 µg) of CDR-267F018 was seen on electrically driven action potentials of guinea pig papillary muscle. Various related parameters like resting potential (RP), action potential amplitude (APA), Vmax (the maximum rate of rise of action potentials) and QT interval was estimated in control, vehicle and CDR 267F018 administered animals. No significant effects on these parameters were observed after administration of CDR267F018 (Table- 1d).

(B) Toxicity Profile of CDR267F018:

(a) Mortality data of rats: In single dose toxicity study the animals were observed grossly for 14 days after single administration of CDR267F018. No significant mortality and adverse effects were observed in CDR267treated animals.

In 10 day DRF study, repeat dose administration of CDR267F018 for 10 day with doses of 500 mg/kg, 1000 mg/kg and 1500 mg/kg causes significantly mortality in rats. Approximate 70-80% mortality was observed in mid dose (1000 mg/kg) and high dose (1500 mg/kg) of CDR267F018 treated rats. Therefore the study was repeated with lower doses. The doses for second DRF study were 250mg/kg, 500 mg/kg and 750 mg/kg. Animals were administered for 10 day daily administration of CDR-267F018. In high dose (750mg/kg) group, 40% mortality was observed while in mid dose (500 mg/kg) and low dose (250 mg/kg) groups, no mortality was observed. Observations represented that the dose of 500 mg/kg is safe for further studies.

In 28 day RDT study in rats and rhesus monkey no mortality was observed after 28 day repeat dose administration of CDR267F018.

(b) General Behavior and food-water intake: No abnormality in general behavior was observed in animals during conduction of study. No alteration in food and water intake was observed in experimental animals and values are same as observed in control animals of respective study.

(c) Body weight: In animals of 10 day RDT study no significant alterations in body weight was observed after CDR267F018 administration (Fig.-2a). In animals of 28 day RDT study increase in body weights were observed in comparison to their initial weight (Fig.- 2b). No significant alterations were observed in body weights of both male and female rhesus monkey after 28 day repeat dose administration of CDR267F018 in comparison to control animals (Fig.-2c).

(d) Hematology: All the main clinically relevant hematological parameters viz. Hb, RBC, TLC, PLT, HCT, MCV, MCH and MCHC were estimated at the initial and final time point (terminal) in rats and rhesus monkey depending on type of study. Alteration in numbers of

platelets was observed in both male and female rats of 10 day RDT and 28 day RDT study. In rhesus monkey also 28 day repeat dose administration of CDR267F018 cause increase in platelet number though the count was with in normal range therefore considered as non-responsive of CDR267F018 administration.

Biochemistry: Biochemical parameters of blood like level of cholesterol, triglyceride, total protein, uric acid, creatinine, glucose, bilirubin, ALT, AST, levels of calcium and phosphorous ions were estimated. Significant ($p<0.01$) decrease in glucose level was observed in both male and female rats of 10 day RDT study. ALT level was also significantly altered in male rats of group III though values remained with in normal range. In female rats of group III significant ($p<0.05$) increase in calcium level was also observed although the values remained in normal range (Table-2a).

Cholesterol level was significantly ($p<0.01$) decrease in male rats of 28 day RDT. Total protein was significantly ($p<0.01$) increased in male animals of group II and female animals of group III. Creatinine level was significantly ($p<0.01$) decreased in female rats of group III. Decrease in calcium level ($p<0.05$) was observed in male rats of group II and female rats of group III. ALT and AST levels were also altered significantly though the levels varied within normal range (Table-2a).

In rhesus monkey the biochemical parameters were estimated in blood at initial and final time point of study. No significant alteration was observed except decrease ($p<0.05$) in TG level in female animal of group IV in comparison to the initial level (Table-2a). Nevertheless, the decreased value remained with in normal range.

(f) Urine analysis: In 10 day RDT study the samples were collected at termination of study. In 28 day RDT study samples were collected at both initial and final time point of study. No evidence of damage to kidney, liver or metabolic function was observed in routine parameters of urine analysis. However, in microscopic examination few epithelial cells per hpf were observed in animals of all group though the number varied within normal range (Table-2b). In rhesus monkey also no significant alterations were observed after CDR267F018 administration (Table – 2b).

(g) Organ weight: Animals were sacrificed after completion of dosing as per study. Different organs were isolated and weighed and relative organ weight was calculated. Significant increase in liver-weight was observed in male rats of group II and IV of 10 day RDT study (Table – 2c). However in 28 day RDT no significant alterations in organ weight were observed in both rats and rhesus monkey.

(h) Histological findings: Sections of all the collected organs were cut down and stained with

heamatoxylin and eosin to visualize any histological change under microscope. In 10 day RDT no abnormal morphology was observed though two animals showed the increased BALT and focal mononuclear cell collection in liver (Fig. -3). The total number of animals in study was 40 (20male+20female) thus the affected animals were only 5%. 28 day RDT study in rats have showed the incidence of increased BALT and focal mononuclear cell collection (FMCC). In rhesus monkey also increased BALT and calcification in adrenals was observed in rhesus monkey 28 day RDT (Fig.-3), which are considered as incidental finding.

(i) ECG and ophthalmoscopic findings - ECG examination of all the monkeys was normal. None of the monkeys showed any ocular abnormality. The CDR267F018 did not have any adverse ophthalmologic effect in the anterior or posterior segment of the eye at any dose level.

(C) Genotoxicity findings –

(a) Mutagenic effects - No mutagenic effect was found at any of the tested doses in all the four Tester strains of Salmonella in the both the studies after CDR267F018 treatment.

(b) Clastogenic and aneugenic effects - All the three doses (500mg, 1000mg and 1500mg/kg bw) of CDR267F018 were found to be non clastogenic or non aneugenic. The aberrant cells were increased only in animals of highest dose group (1500mg/kg bw). The numbers of aberrant cells in control samples were 3.7±0.9 while in experiment group (1500mg/kg bw) the number was 5.6±0.7. In positive control the number of aberrant cells was 9.6±1.5 (Table – 3).

4. Discussion:

Marine flora and fauna is currently receiving global attention as potential source for drugs now-a-days. Marine mangroves have shown the medicinal values from decades. Numerous mangrove plants are used in traditional medicine and the extracts of different parts like leaf, stem bark etc showed their efficacy against human diseases, animal and plant pathogens. Though only limited investigations have been carried out to identify the metabolites responsible for their bioactivities[12] thus need further investigations.

Our Institute (CSIR-CDRI) has prepared the bioactive fraction from Xylocarpus (code – CDR267F018) for the antidyslipidemic activity and received the patent for the same in 2011. Present study showed the safety pharmacology and toxicity profile of CDR-267F018 to confirm the potential of this new candidate drug for its future use as drug for treatment of dyslipidemic disorders.

Safety pharmacology studies were conducted to investigate the potential undesirable pharmacodynamic effects of a substance on

physiological functions in relation to exposure of patients within the therapeutic range and above. Evaluations were done to investigate the effects of the test agent on vital organ systems such as central nervous systems, cardiovascular respiratory and overall any adverse effects on physiology. CNS parameter motor activity, behavioral changes, neuromuscular coordination and motor reflex responses did not show any adverse after administration of CDR-267F018. CVS parameters like blood pressure, heart rate and respiration was estimated in rats and rabbits. No change in blood pressure and heart rate was observed in comparison to control animals. Observations showed that administration of CDR267F018 did not cause any adverse effects in general gross behavior, no alteration in coordination and motor responses at the dose of effective dose and above.

Further single dose and chronic (10 day repeat dose and 28 days repeat dose) toxicity studies in rats and rhesus monkey were conducted to evaluate the effect of CDR267F018 in animals by assessing different parameters like biochemistry, hematological, urinalysis and histological investigation in body organs. No significant alterations in body weight of animals were observed in comparison to control reflecting that CDR267F018 is not altering the normal metabolic functions. Hematological parameters indicate the alterations in numbers of platelets. The number of platelets was increased significantly in rodents and primates in dose dependent manner but the number was lying within normal range. Some of the biochemical parameters were altered in 10 day DRF study in animals administered with highest dose of CDR267F018. In animals of 10 day RDT study the glucose levels were decreased significantly with highest (1000 mg/kg) dose of CDR267F018. Due to significantly decreased level of glucose the mortality was observed. However at lower doses no significant decrease was observed. Results have indicated the antidiabetic property of compound with high dose. Though at high dose of CDR267F018 few biochemical parameters were significantly altered therefore for further 28 day RDT study, the dose of CDR267F018 was reduced. 28 day repeat administration of CDR267F018 in rats caused altered level of enzyme AST and ALT reflecting slight alterations in liver functions. However the levels were ranged between the normal ranges of parameters. Observations of 28 day study in rhesus monkey showed the increase in TG level though the levels were within normal range. Urine analysis parameters also showed no significant abnormality in kidney, liver and in metabolic function. Repeated administration of CDR267F018 did not show any increase in overall weight gain and nor any increase in weight of different organs. In few animals certain incidental histological findings were observed like FMCC in liver and BALT in lung. BALT is an accumulation of lymphoid cells with a typical localization of B lymphocytes preferentially in a follicle and T

lymphocytes, more peripherally around high endothelial venules in the wall of bronchi[13]. BALT is not present in all species and age groups and can be classified as a tertiary lymphoid organ. It is considered as a part of the integrated mucosal-associated lymphoid tissue system[13] and therefore does not considered as responsive of CDR267F018. Genotoxic studies have also revealed that CDR267F018 did not cause any mutagenic effects after administration in salmonella strains and rodents. The few incidental findings must be considered during the clinical trial of CDR267F018. Previous studies (as described in patent) showed the ED of CDR267F018 was 50 mg/kg for antidyslipidaemic activity. Toxicity studies have showed no mortality at dose of 500mg/kg thus further confirming the potential of compound to proceed for the use as drug.

In conclusion, results have suggested that the NAOEL dose of bioactive fraction of Xylocarpus is 500mg/kg in rats and 250mg/kg in rhesus monkey. Fraction is safe up to five times of effective dose in rats and up to ten times in case of rhesus monkey, indicated that CDR267F018 could be used as therapeutic agent and clinical trials can be initiated.

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Conflict of Interest: Authors have no conflict of interest.

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Figure Captions –

Fig. - 1 Effect of CDR 267 F018 on cardiovascular activity in rats

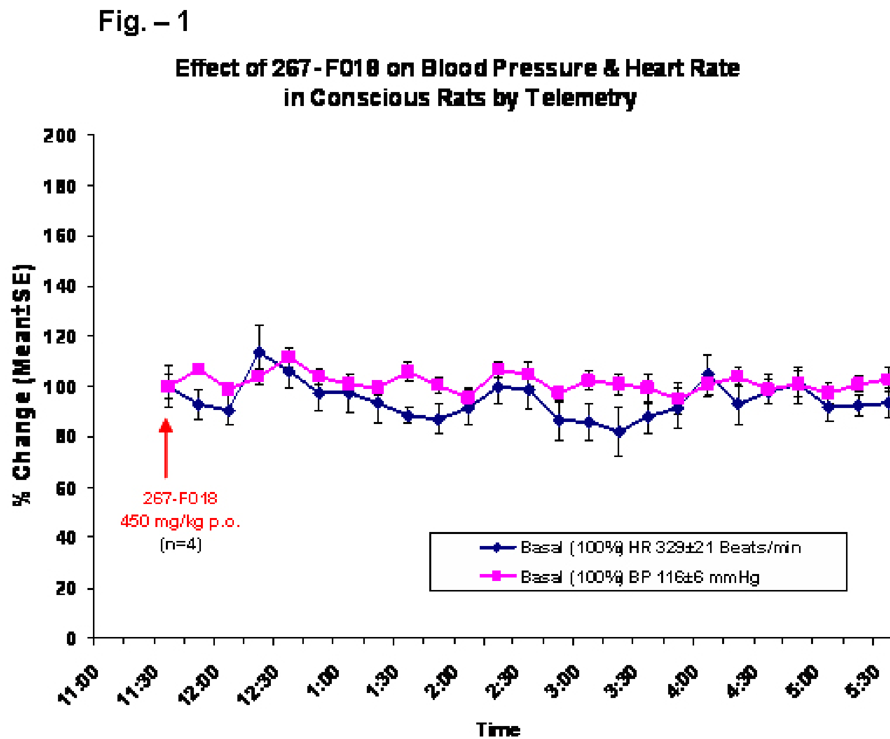
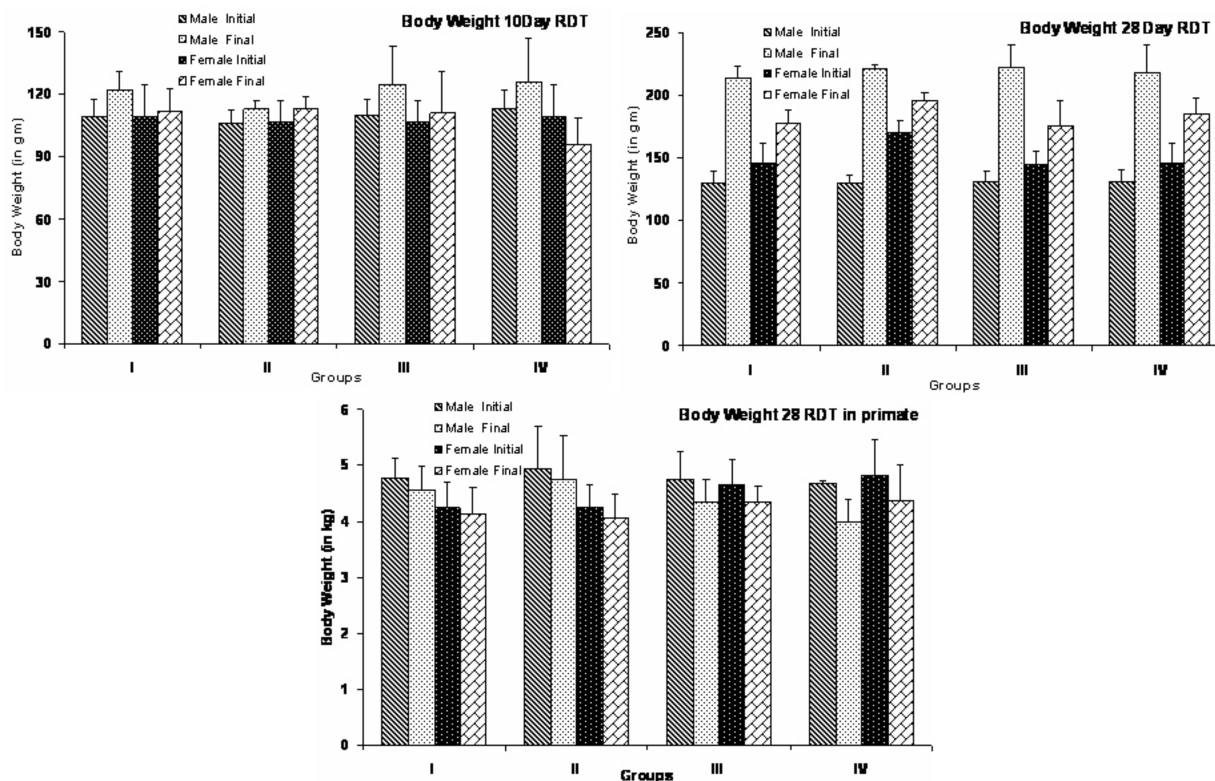


Fig.- 2 Graphs indicate the alteration in body weight of rats (rodents) and rhesus monkey (primates) before (initial) and after (final) 10 day and 28 day repeat dose (daily) administration of CDR267F-018.

Fig.-2



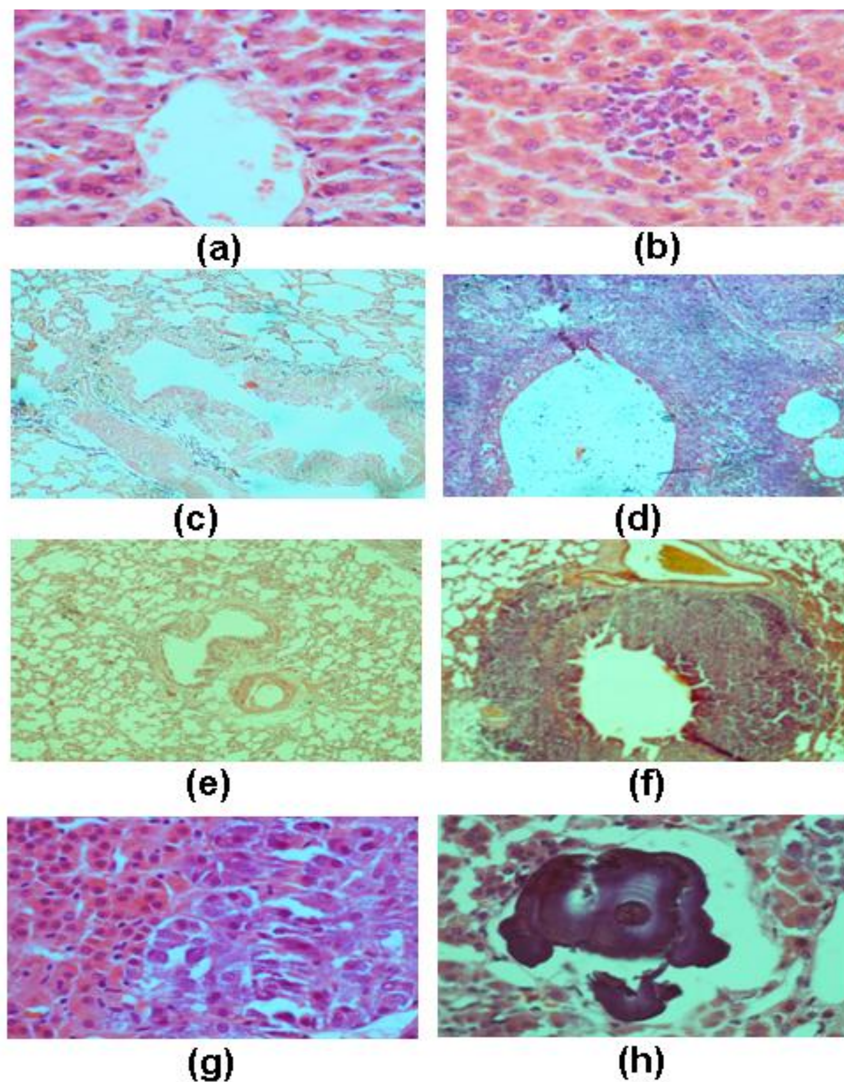


Fig.- 3 Images showing the histological findings in (a) rats liver normal 40x and (b) rat liver with focal mononuclear cell collection 40x (FMCC) 40x (c) rat lung normal 4x (d) rat lung with Bronchus associated lymphoid tissue (BALT) 4x (e) Rhesus Monkey lung normal 4x (f) Rhesus Monkey lung with BALT (g) Rhesus monkey adrenal medulla normal 40x (h) Rhesus monkey adrenal medulla with calcification 40x after administration of CDR267F018

Table-1 a & b

(a)

	Time interval (min) after drug administration					
	Pre-tt (0)	30	60	90	120	150
CONTROL	3.2±0.3	3.4±0.2	3.3±0.3	2.9±0.2	2.6±0.1	2.3±0.1
COMPOUND CDR-267-F018 (mg/kg, po.)						
90	3.1 ±0.2	4.3 ±0.3	3.7±0.3	3.4±0.2	2.8±0.2	2.7±0.1
225	3.5 ±0.1	3.6 ±0.2	3.3 ±0.2	3.0±0.1	2.9±0.1	2.6±0.1
450	3.3 ±0.1	3.5 ±0.2	3.2 ±0.2	3.1±0.1	2.7±0.1	2.6±0.2
900	3.4 ±0.2	3.9±0.2	3.4±0.1	3.3±0.1	3.0±0.2	2.9±0.1

Table- 1(a) Nociceptive Reaction Time (Sec) with and without CDR267F018 administration. Data is presented as Mean±SD

(b)

	SLEEP-DURATION (min) Mean ± SE
CONTROL (Vehicle)	76.2± 16.2
CDR-267-F018 (mg / kg, po)	
90	80.2 ± 9.6
225	60.9 ± 5.1
450	80.8 ± 8.5
900	77.2 ± 13.9

Table- 1(b) Effect of CDR-267-F018 on Barbiturate Induced Hypnosis at different doses

Table-1 c

Frequency (BPM)

Time Interwal		Basal	30 min	60 min	90 min	120 min	150 min	180 min	210 min	240 min
Vehicle	Average	92.517	115.416	102.568	115.138	101.746	110.190	95.543	124.002	105.847
	SE ±	6.149	11.076	10.875	17.393	11.410	12.642	8.149	14.652	12.024
Compd	Average	80.820	142.999	118.016	113.010	106.333	99.071	96.024	124.995	120.766
	SE ±	7.125	19.081	13.607	7.141	16.311	5.731	5.599	23.424	11.238

Tidal volume

Time Interwal		Basal	30 min	60 min	90 min	120 min	150 min	180 min	210 min	240 min
Vehicle	Average	1.498	1.564	1.493	1.445	1.456	1.381	1.416	1.461	1.689
	SE ±	0.050	0.157	0.095	0.104	0.113	0.083	0.105	0.063	0.135
Compd	Average	1.655	1.648	1.505	1.457	1.411	1.413	1.496	1.370	1.447
	SE ±	0.104	0.326	0.225	0.093	0.098	0.069	0.081	0.064	0.053

Minute volume

Time Interval		Basal	30 min	60 min	90 min	120 min	150 min	180 min	210 min	240 min
Vehicle	Average	126.109	162.827	139.649	153.921	133.405	134.014	121.639	145.395	109.061
	SE ±	9.653	14.089	11.324	25.555	7.805	11.360	8.127	10.979	8.780
Compd	Average	126.069	201.304	157.989	152.234	136.329	129.809	132.431	150.773	152.513
	SE ±	11.712	22.659	20.852	13.522	14.654	8.432	8.458	21.857	15.604

Table- 1(c) Effect of CDR-267-F018 on respiratory parameters viz. frequency, tidal volume and minute volume

Table – 1 (d)

Parameters	Control	Vehicle	Con.(µg /ml) of CDR-267-F018					
			(0.2)	(0.4)	(0.8)	(1.6)	(3.2)	(6.4)
RP	79.95 ±1.25	80.34 ±4.54	79.25 ±0.50	78.5 ±0.70	77.25 ±0.95	80.0 ±0.0	78.25 ±1.25	80.25 ±0.95
APA	117.0 ±0.0	119.64 ±3.86	120.75 ±0.50	115.0 ±2.30	118.0 ±0.0	117.0 ±2.0	117.75 ±0.5	117.5 ±0.57
APD 10%	99.66 ±9.71	98.86 ±8.42	96.5 ±1.73	98.75 ±8.95	96.25 ±6.13	98.0 ±3.36	100.0 ±3.36	101.5 ±4.72
APD 20%	173.33 ±4.72	176.52 ±6.6	176.25 ±2.06	173.0 ±1.15	176.25 ±5.31	177.5 ±2.08	183.25 ±8.80	182.0 ±8.98
APD 50%	293.5 ±7.93	293.6 ±4.2	297.25 ±0.95	295.75 ±2.87	307.5 ±4.12	310.5 ±1.29	311.25 ±4.34	310.5 ±2.64
APD 75%	338.0 ±2.30	339.6 ±4.2	342.75 ±1.70	342.75 ±2.06	341.75 ±1.25	350.0 ±1.63	342.75 ±0.5	345.75 ±2.06
APD 90%	355.25 ±2.5	358.20 ±4.22	358.0 ±0.0	357.0 ±1.15	362.0 ±0.81	364.5 ±0.57	360.25 ±0.5	360.5 ±1.0
Vmax(V/sec)	327.5 ±3.0	332.43 ±3.68	334.75 ±7.21	330.5 ±4.84	334.66 ±3.78	324.5 ±4.70	325 ±5.56	336.0 ±5.12

Table- 1(d) Effect of CDR-267- F018 on resting potential (RP), action potential amplitude (APA), Vmax and QT interval

Table -2 (a)

28 Day RDT Study in Rhesus Monkey								
	Male							
Groups -	I		II		III		IV	
Parameters	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Cholesterol	185.1 ± 15.1	152.6 ± 10.1	194.0± 23.46	177.6 25.9	200.9 ± 16.6	162.7 ± 30.2	186.5 ± 20.4	171.1± 20.7
Triglyceride	92 ± 13.53	47.7 ± 9.85	88.5 ± 19.54	92.7± 6.4	97± 28.2	98 ± 9.47	96.8 ± 4.61	87.97 ± 8.34*
Total Protein	8.27 ± 0.23	7.62 ± 0.54	8.57 ± 0.40	8.0 ± 0.78	8.4 ± 0.43	8.19 ± 0.65	8.39 ± 0.31	7.94 ± 0.43
Creatinine	1.02 ± 0.12	1.00 ± 0.10	1.03 ± 0.06	1.1 ± 0.04	1.1 ± 0.15	1.14 ± 0.05	1.04 ± 0.01	0.90 ± 0.07
Glucose	116.7 ± 30.3	77 ± 12.48	91.7 ± 12.77	78.4 ± 3.56	100 ± 19.7	83.3 ± 9.27	104.1 ± 6.06	67.7 ± 11.05
Total Bilirubin	0.16 ± 0.01	0.17 ± 0.02	0.20 ± 0.02	0.14 ± 0.04	0.14 ± 0.06	0.13 ± 0.03	0.14 ± 0.05	0.20 ± 0.01
Calcium	12.03 ± 0.29	11.08 ± 0.26	11.97 ± 0.65	10.25 ± 0.4	12.03 ± 0.58	10.11 ± 0.48	12.25 ± 0.22	11.46 ± 0.52
Phosphorus	5.36 ± 0.63	3.33 ± 0.29	4.83 ± 0.88	2.46 ± 0.30	4.92 ± 0.63	1.98 ± 0.23	5.39 ± 1.09	2.95 ± 0.38
AST	53.6 ± 2.55	57.7 ± 27.54	45.9 ± 6.31	63.7 ± 27.4	68.1 ± 25.01	55.6 ± 7.47	46.2 ± 4.61	55.2 ± 4.60
ALT	38.8 ± 10.13	27.5 ± 4.48	31.6 ± 5.92	27.6 ± 4.6	28.5 ± 6.5	30.5 ± 5.70	29.9 ± 8.95	28.8 ± 2.35
	Female							
Groups -	I		II		III		IV	
Parameters	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Cholesterol	173.4 ± 17.01	167.1 ± 15.2	166.9 ± 8.8	155 ± 12.03	168.7 ± 12.2	170.2 ± 8.9	179.8 ± 7.44	144.03 ± 34.5
Triglyceride	67.4 ± 20.7	45.23 ± 3.90	77.7 ± 15.1	102.2 ± 26.2	131.7 ± 24.2	189.1 ± 85.6	103 ± 16.60	194.3 ± 100.7
Total Protein	7.84 ± 0.43	7.59 ± 0.24	8.35 ± 0.25	7.88 ± 0.22	8.33 ± 0.46	8.92 ± 0.28	8.22 ± 0.12	7.71 ± 0.60
Creatinine	0.86 ± 0.07	0.93 ± 0.10	1.00 ± 0.18	1.08 ± 0.08	1.10 ± 0.17	1.15 ± 0.07	0.96 ± 0.09	0.93 ± 0.15
Gucose	92.43 ± 9.04	86.3 ± 13.6	138.4 ± 9.7	95.3 ± 5.9	88.6 ± 12.7	93.6 ± 18.1	93.8 ± 22.6	76.37 ± 7.98
Total Bilirubin	0.17 ± 0.01	0.17 ± 0.02	0.14 ± 0.05	0.13 ± 0.04	0.20 ± 0.02	0.13 ± 0.01	0.02 ± 0.09	0.16 ± 0.06
Calcium	11.34 ± 0.48	10.52 ± 0.23	11.8 ± 0.13	10.21 ± 0.37	11.7 ± 1.09	10.83 ± 0.36	11.41 ± 0.64	11.17 ± 0.80
Phosphorus	4.34 ± 0.20	2.72 ± 0.51	4.54 ± 0.44	2.22 ± 0.32	4.44 ± 0.43	2.36 ± 0.47	4.12 ± 0.59	2.50 ± 0.58
AST	46.07 ± 5.25	46.61 ± 5.36	56.07 ± 5.17	84.9 ± 17.5	65.2 ± 13.6	57.3 ± 1.5	58.5 ± 10.2	50.83 ± 4.11
ALT	25 ± 0.75	28.33 ± 7.54	29.3 ± 3.8	43.7 ± 22.5	30.2 ± 11.7	25.9 ± 3.3	24.9 ± 7.1	31.87 ± 12.7

(a) Biochemical parameters in rhesus monkey after 28 day of repeat administration of compound

Table-2 (b)

10 Day DRF Study in rats								
Groups -	I		II		III		IV	
Parameters	Male	Female	Male	Female	Male	Female	Male	Female
No. of Animals	10	10	10	10	10	10	10	10
Colour	Straw	Straw	Straw	Straw	Straw	Straw	Straw	Straw
Specific Gravity	1.025 ± 0.005	1.025 ± 0.005	1.027 ± 0.004	1.024 ± 0.004	1.029 ± 0.002	1.023 ± 0.003	1.028 ± 0.003	1.024 ± 0.006
Reaction (pH)	6.20 ± 0.27	6.00 ± 0.61	6.30 ± 0.27	6.20 ± 0.76	6.10 ± 0.20	6.10 ± 0.65	6.10 ± 0.65	5.83 ± 0.73
Protein (mg/dl)	VARIATION WITHIN NORMAL RANGE (Trace to30mg/dl)							
Glucose	NIL							
Ketone	NIL							
Bilirubin	NIL							
Occult Blood	NIL							
Urobilinogen	NIL							
Microscopic Examination	FEW EPITHELIAL CELLS SEEN PER HPF IN ALL THE GROUPS (NO ABNORMAL CELLULAR/CRISTALLINE CONSTITUENT)							

28 Day RDT Study in rats								
Parameters	Male							
Groups -	I		II		III		IV	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
No. of Animals	10	10	10	10	10	10	10	10
Colour	Straw	Straw	Straw	Straw	Straw	Straw	Straw	Straw
Specific Gravity	1.021 ± 0.005	1.020 ± 0.006	1.023 ± 0.003	1.025 ± 0.004	1.024 ± 0.005	1.025 ± 0.004	1.026 ± 0.005	1.024 ± 0.006
Reaction (pH)	6.35 ± 0.47	6.15 ± 0.75	6.05 ± 0.60	6.25 ± 0.54	6.20 ± 0.54	6.11 ± 0.49	6.25 ± 0.26	6.20 ± 0.79
Protein (mg/dl)	VARIATION WITHIN NORMAL RANGE (Trace to30mg/dl)							
Glucose	NIL							
Ketone	NIL							
Bilirubin	NIL							
Occult Blood	NIL							
Urobilinogen	NIL							
Microscopic Examination	FEW EPITHELIAL CELLS SEEN PER HPF IN ALL THE GROUPS (NO ABNORMAL CELLULAR/CRISTALLINE CONSTITUENT)							

	Female							
Groups -	I		II		III		IV	
Parameters	Initial	Final	Initial	Final	Initial	Final	Initial	Final
No. of Animals	10	10	10	10	10	10	10	10
Colour	Straw	Straw	Straw	Straw	Straw	Straw	Straw	Straw
Specific Gravity	1.023 ± 0.007	1.022 ± 0.007	1.022 ± 0.007	1.005 ± 0.005	1.023 ± 0.006	1.023 ± 0.007	1.024 ± 0.008	1.027 ± 0.004
Reaction (pH)	6.10 ± 0.46	6.05 ± 0.64	6.00 ± 0.58	6.10 ± 0.74	6.25 ± 0.26	6.30 ± 0.35	6.05 ± 0.44	6.25 ±0.59
Protein (mg/dl)	VARIATION WITHIN NORMAL RANGE (Trace to30mg/dl)							
Glucose	NIL							
Ketone	NIL							
Bilirubin	NIL							
Occult Blood	NIL							
Urobilinogen	NIL							
Microscopic Examination	FEW EPITHELIAL CELLS SEEN PER HPF IN ALL THE GROUPS (NO ABNORMAL CELLULAR/CRISTALLINE CONSTITUENT)							

Urine – parameters in rats after 10 and 28 day repeat dose (daily) administration of compound

28 Day RDT Study in Rhesus Monkey								
	Male							
Groups -	I		II		III		IV	
Parameters	Initial	Final	Initial	Final	Initial	Final	Initial	Final
No. of Animals	3	3	3	3	3	3	3	3
Colour	Straw	Straw	Straw	Straw	Straw	Straw	Straw	Straw
Specific Gravity	1.010 ± 0.013	1.005 ± 0.005	1.010 ± 0.009	1.003 ± 0.003	1.003 ± 0.003	1.008 ± 0.003	1.003 ± 0.008	1.008 ± 0.008
Reaction (pH)	8.00 ± 0.50	8.00 ± 0.50	8.17 ± 0.57	7.67 ± 0.58	7.16 ± 0.57	8.00 ± 0.50	7.83 ± 0.28	8.00 ± 1.00
Protein (mg/dl)	VARIATION WITHIN NORMAL RANGE (Trace to30mg/dl)							
Glucose	NIL							
Ketone	NIL							
Bilirubin	NIL							
Occult Blood	NIL							
Urobilinogen	NIL							
Microscopic Examination	FEW EPITHELIAL CELLS SEEN PER HPF IN ALL THE GROUPS (NO ABNORMAL CELLULAR/CRISTALLINE CONSTITUENT)							

Parameters	Female							
	I		II		III		IV	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
No. of Animals	3	3	3	3	3	3	3	3
Colour	Straw	Straw	Straw	Straw	Straw	Straw	Straw	Straw
Specific Gravity	1.012 ± 0.008	1.002 ± 0.005	1.015 ± 0.009	1.005 ± 0.005	1.008 ± 0.008	1.003 ± 0.006	1.008 ± 0.008	1.007 ± 0.008
Reaction (pH)	7.83 ± 0.76	8.17 ± 0.29	7.83 ± 0.76	8.00 ± 0.87	7.83 ± 0.28	8.33 ± 0.29	8.17 ± 0.57	8.17 ± 0.29
Protein (mg/dl)	VARIATION WITHIN NORMAL RANGE (Trace to30mg/dl)							
Glucose	NIL							
Ketone	NIL							
Bilirubin	NIL							
Occult Blood	NIL							
Urobilinogen	NIL							
Microscopic Examination	FEW EPITHELIAL CELLS SEEN PER HPF IN ALL THE GROUPS (NO ABNORMAL CELLULAR/CRISTALLINE CONSTITUENT)							

Urine – parameters in Rhesus monkey after 10 and 28 day repeat dose (daily) administration of compound

Table-2(a) & (b) Showed the alteration in biochemical parameters in rhesus monkey before (initial) and after (final) 28 day of repeat dose (daily) administration of CDR267F-018Table -2(b) Indicates the alteration in urine - parameters in rats after 10 and 28 day and in rhesus monkey after 28 day repeat dose (daily) administration of CDR267F-018. *=$p<0.05$ control vs. experimental grp.

Table-1 c

Frequency (BPM)

Time Interwal		Basal	30 min	60 min	90 min	120 min	150 min	180 min	210 min	240 min
Vehicle	Average	92.517	115.416	102.568	115.138	101.746	110.190	95.543	124.002	105.847
	SE ±	6.149	11.076	10.875	17.393	11.410	12.642	8.149	14.652	12.024
Compd	Average	80.820	142.999	118.016	113.010	106.333	99.071	96.024	124.995	120.766
	SE ±	7.125	19.081	13.607	7.141	16.311	5.731	5.599	23.424	11.238

Tidal volume

Time Interwal		Basal	30 min	60 min	90 min	120 min	150 min	180 min	210 min	240 min
Vehicle	Average	1.498	1.564	1.493	1.445	1.456	1.381	1.416	1.461	1.689
	SE ±	0.050	0.157	0.095	0.104	0.113	0.083	0.105	0.063	0.135
Compd	Average	1.655	1.648	1.505	1.457	1.411	1.413	1.496	1.370	1.447
	SE ±	0.104	0.326	0.225	0.093	0.098	0.069	0.081	0.064	0.053

Minute volume

Time Interwal		Basal	30 min	60 min	90 min	120 min	150 min	180 min	210 min	240 min
Vehicle	Average	126.109	162.827	139.649	153.921	133.405	134.014	121.639	145.395	109.061
	SE ±	9.653	14.089	11.324	25.555	7.805	11.360	8.127	10.979	8.780
Compd	Average	126.069	201.304	157.989	152.234	136.329	129.809	132.431	150.773	152.513
	SE ±	11.712	22.659	20.852	13.522	14.654	8.432	8.458	21.857	15.604

Table-2(c) Indicates the alteration in organ weight in rats after (final) 10 day repeat dose (daily) administration of CDR267F-018. **=p<0.01 control vs. experimental grp.

Table .-3

Groups	Cells with chromosomal aberrations	Cells with chromatidal aberrations	Cells with gaps	Aberrant cells	Aberrant cells and cells showing gaps
GroupI: Control, DMSO (10ml/kgbw)	1.40±0.70	2.30±0.67	1.00±0.47	3.70±0.95	4.70 ±1.16
GroupII: Low Dose, CDR267(F018) (500mg/kgbw)	1.40±0.97	2.10±0.88	1.00±0.82	3.50±1.18	4.50±1.27
GroupIII: Middle Dose CDR267(F018) (1000mg/kgbw)	1.20±0.63	2.30±0.48	0.90±0.74	3.50±0.97	4.40±1.26
GroupIV: High Dose CDR267(F018) (1500mg/kgbw)	2.20±0.63	3.40±0.84	1.10±0.88	5.60±0.70**	6.70±1.34
GroupV: Positive Control Cyclophosph-amide (25mg/kgbw)	4.60±0.70	5.00±1.25	2.00±0.47	9.60±1.65**	11.60±1.90

Effect of **CDR267(F018)** on Chromosomal aberration of bone marrow cells in 100 metaphases/animal

Groups	No. of polychromatic cells with Mn (mean ± SD)	No. of Normochromatic cells with Mn (mean ± SD)	Poly chromatic/ Normochromatic (mean ± SD)
Group I: Control (DMSO-10ml/kgbw)	3.50±1.58	0.90 ± 0.88	3.81±1.11
Group II: Low Dose CDR267(F018) (500mg/kg bw)	3.10±1.37	0.60 ± 0.84	3.70±0.97
Group III: Middle Dose CDR267(F018) (1000mg/kg bw)	3.20±1.32	0.80 ± 0.79	3.23±1.01
Group IV: High Dose CDR267(F018) (1500mg/kg bw)	3.20±1.32	0.90 ± 0.74	4.22±0.91
Group V: Positive control (cyclophophamide-25mg/kgbw)	18.40±4.06**	4.70 ± 1.16**	3.44±0.81

Effect of **CDR267(F018)** on bone marrow cells for producing micronucleated polychromatic erythrocytes in 1000 polychromatic erythrocytes /animal

Table-3 Effect of CDR267F018 on Chromosomal aberration of bone marrow cells and micronucleated polychromatic erythrocytes in 1000 polychromatic erythrocytes / mice. **=p<0.01 control vs. experimental grp.

Table-1

10 Day DRF Study in rats								
	Male				Female			
Parameters	I	II	III	IV	I	II	III	IV
Cholesterol	34.6 ±6.9	38.6 ± 10.5	44.40 ± 12.38	43.40 ± 10.11	39.20 ± 5.26	34.00 ± 2.92	38.40 ± 4.32	37.33 ± 2.52
Triglyceride	35.8 ± 7.92	30.8 ± 4.87	32.80 ± 15.22	31.00 ± 9.95	43.6 ± 7.09	38.4 ± 7.96	47.4 ± 13.02	56.00 ± 19.0
Total Protein	6.88 ± 0.54	6.88 ± 0.41	7.54 ± 0.60	7.20 ± 0.30	6.68 ± 0.16	6.88 ± 0.31	7.02 ± 0.35	6.37 ± 0.50
Creatinine	0.66 ± 0.05	0.64 ± 0.05	0.60 ± 0.1	0.62 ± 0.04	0.64 ± 0.05	0.64 ± 0.05	0.66 ± 0.08	0.57 ± 0.06
Glucose	73.6 ± 10.92	63.2 ± 7.43	64.00 ± 8.89	31.20 ± 11.5**	69.80 ±7.50	61.60 ± 6.91	66.00 ± 6.84	28.00 ± 13.1**
Total Bilirubin	0.36 ± 0.11	0.40 ± 0.1	0.24± 0.05	0.26 ± 0.11	0.50 ± 0.12	0.32 ± 0.13	0.46 ± 0.10	0.47 ± 0.06
Calcium	9.96 ± 0.60	9.56 ± 0.42	9.90 ± 0.34	10.16 ± 0.67	8.48 ± 0.33	8.46 ± 0.74	9.68 ± 0.62*	9.27 ± 0.31
Phosphorus	12.8 ± 0.84	12.2 ± 2.05	12 ± 0.71	12.80 ± 1.64	11.80 ± 1.48	13.00 ± 1.00	13.20 ± 1.17	12.67 ± 1.15
AST	170.2±6.3	177±25.1	143.6 ± 9.49	160.8 ± 19.51	60.20 ± 6.83	44.60 ± 7.70	64.20 ± 12	64.67 ± 14.74
ALT	63.8 ± 15.88	56 ±15.68	40.80 ±3.03*	56.40 ± 6.80	192.6 ± 27.62	153.4 ± 9.13	200.6 ± 46	184.0 ± 38.16
28 Day RDT Study in rats								
	Male				Female			
Parameters	I	II	III	IV	I	II	III	IV
Cholesterol	36.9 ±9.52	26.1 ± 7.45**	35.3 ± 5.72	31.40 ± 6.75	39.6 ± 3.75	43.6 ± 9.13	43.4 ± 10.6	42.3 ± 10.01
Triglyceride	25.8 ± 5.98	24.6 ± 8.60	22.7 ± 9.56	27.50 ± 8.45	32.5 ± 7.89	29.6 ± 8.57	28.7 ± 5.74	28.2 ± 8.46
Total Protein	7.57 ± 0.42	6.3 ± 0.84**	7.09 ± 0.43	7.70 ± 0.86	7.72 ± 0.55	8.06 ± 0.89	6.81 ± 0.50**	6.76 ± 1.03*
Creatinine	0.63 ± 0.07	0.56 ± 0.07	0.58 ± 0.07	0.59 ± 0.07	0.63 ± 0.07	0.64 ± 0.10	0.52 ± 0.04**	0.58 ± 0.04
Glucose	84.6 ± 10.99	81.9 ± 10.17	77.2 ± 18.67	80.7 ± 15.97	70.6 ± 9.56	70.8 ± 19.2	72.7 ± 10.04	71.5 ± 15.55
Total Bilirubin	0.25 ± 0.05	0.27 ± 0.11	0.36 ± 0.12	0.33 ± 0.11	0.38 ± 0.11	0.39 ± 0.15	0.35± 0.17	0.39 ± 0.11
Calcium	10.7 ± 0.56	9.19 ± 0.89**	10.18 ± 0.53	10.14 ± 1.18	10.75 ± 0.79	10.6 ± 0.85	9.69 ± 0.67*	9.55 ± 1.04
Phosphorus	11.20 ±1.32	12 ± 1.15	11.33 ± 1.50	11.80 ± 1.62	12.2 ± 1.03	11.5 ± 1.29	11.3 ± 1.49	11.6 ± 1.26**
AST	183.9 ± 48.5	147.2 ± 47.3	141.8± 28.69	130.6± 29.44**	153.3 ± 22.4	13.9 ± 37.06	98.9 ± 15.4**	90.6 ± 16.7**
ALT	65.3 ± 14.59	53.1 ± 10.6*	47.78 ± 5.74*	46.5 ± 10.68*	57.3 ± 6.73	45.6 ± 12.85	47.8 ± 16.7	39.2 ± 9.45**

Suppl Table -1 Illustrate the alteration in biochemical parameters in rats after 10 day and 28 day of repeat dose (daily) administration of CDR267F-018. *=p<0.05 control vs. experimental grp., **=p<0.01 control vs. experimental grp.