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ACUTE AND 28 DAYS REPEATED ORAL TOXICITY STUDIES OF A SIDDHA DRUG PIRANDAI UPPU ON WISTAR ALBINO RAT

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ABSTRACT: *Pirandai uppu* (PU) a herbo mineral formulation has been employed as a traditional siddha remedy for diarrhea since long time. Diarrheal diseases remain one of a leading cause of morbidity and mortality in the world, particularly in developing countries. As a mandate, steps were taken to evaluate safety profile of PU in animal model under OECD guidelines. Acute toxicity studies done on female wistar albino rat under OECD guidelines 423 and 28 days repeated oral toxicity studies done on both sex of wistar albino rat under OECD guidelines 407. Acute oral toxicity study of PU revealed no mortality at the dosage of 2000 mg/kg body weight and the median lethal dosage of PU is estimated above 2000 mg/kg body weight. Repeated oral toxicity study of PU does not exhibit mortality at the high dosage of 200 mg/kg body weight given up to the period of 56 days including 28 days of drug administered. At the end of 28 days no specific changes are observed in hematological, hepatic, renal and other biochemical parameters. No gross morphological and histological changes are observed in the organs. The above studies recommend that PU is a safest drug under intended human adult dosage (500 mg) as illustrated in the literature.

INTRODUCTION: The interventional drug *Pirandai Uppu* (PU) is a compound formulation of siddha medicine containing juice of *Pirandai (Cissus quadrangularis) and Kariuppu (Sodium Chloride)* has been quoted in *Noikalukku Siddha Parikaram Part-I* [1], but the claim of the efficacy and safety is not yet to be tested. Cissus quadrangularis, a perennial climber widely used in traditional medicinal systems of India has been reported to posses bone fracture healing, antibacterial, antifungal, antioxidant, anthelmintic, antihemorrhoidal and analgesic activities [2] . *Sodium chloride* has stomachic, anthelmintic [5].

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It has been found to contain a rich source of carotenoids, triterpenoids, ascorbic acid [3] and useful for treatment of bloody diarrhoea [4]. In clinical aspect, PU is remedy for diarrhea[1]. Diarrhea is the passage of 3 or more loose or liquid stools per day, or more frequently than is normal for the individual. Diarrheal diseases remain one of a leading cause of morbidity and mortality in the world, particularly in developing countries [6].

An estimated about two billion cases of diarrheal disease worldwide every year [7]. It is usually a symptom of gastrointestinal infection, which can be caused by a variety of bacterial, viral, parasitic organisms and other digestive disorders [8]. Diarrhea may be accompanied by cramping, abdominal pain, nausea, loss of bowel control [9]. However, a toxicity study of *pirandai uppu* has not been studied. The preclinical toxicity studies are essential for determining a safe dose for human trials [10].

The present study has been planned to explore the real toxicity study of herbo-mineral siddha formulation of *Pirandai Uppu* in Wistar albino rats.

MATERIALS & METHODS:

Preparation of the *Pirandai Uppu*:

Ingredients: Pirandai charu *(cissus qudrangularis* juice*),* Kariuppu (sodium chloride) [1].

Procedure: 650 ml of Well growned *Cissus quadrangularis* stem juice (Pirandai charu) is taken and mixed with 300gm of sodium chloride (Kariuppu). It boiled till dry in mud pot (agal), and keep it into calcination process [1].

Animals: Rat of either sex weighing more than 100 g were obtained from the animal house of King Institute of Preventive Medicine. The animals were used with the approval of the Institute animal ethics committee (IAEC) of Sairam Advanced Centre for Research, Chennai approval no. (1545/PO/a11/CPCSEA/1-3/2013). They were fed with a balanced standard pellet diet, maintained under standard laboratory condition providing 20 - 24^0 C temperature, standard light cycle (12 h light, 12 h dark) and water *ad libitum*.

All the animals were randomly selected and maintained under laboratory conditions for an acclimatization period of 5 days before performing the experiments.

Acute Toxicity Study – OECD 423 guidelines [11-12]:

Acute oral toxicity test for the *Pirandai Uppu* was carried out as per OECD Guidelines 423. Female wistar albino rat were fasted over night prior to dosing. Following the period of fasting, the animals were weighed and then the test substance was administered. After the substance has been administered, food was withheld for a further 3-4 hours. The principle of laboratory animal care was followed.

Observations were made and recorded systematically and continuously observed as per the guideline after substance administration. The visual observations included skin changes, mobility, aggressively, sensitivity to sound and pain, as well as respiratory movements. They were deprived of food, but not water 16–18 h prior to the administration of the test suspension. Finally, the number of survivors was noted after 24 h and these animals were then maintained for a further 14 days and observations made daily. The toxicological effect was assessed on the basis of mortality.

Animals were observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days, to observe any death or changes in general behavior and other physiological activities. It should be determined by the toxic reactions, time of onset and length of recovery period, and may thus be extended when considered necessary. The times at which signs of toxicity appear and disappear are important, especially if there is a tendency for toxic signs to be delayed.

Observations include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behavior pattern.

28 days repeated oral toxicity study [13]:

This study was carried out as per OECD Guidelines 407.In a 28 days repeated oral toxicity study, forty rats of either sex were divided into four groups. Each group consists of 10 rats (5+5).Group I served as normal control and administered with 2 ml of distilled water (p.o) while group II, III and IV were administered daily with the *Pirandai Uppu* for 28 days at a dose of 20, 100, 200 mg/kg respectively (p.o).

The weight of each rat was recorded on day 0 and weekly intervals throughout the course of the study, food and water consumption per rat was calculated.

On 29th day, the animals were fasted for approximately 18 h, then slightly anesthetized with ether and blood samples were collected from the retro-orbital plexus into two tubes: one with EDTA for immediate analysis of haematological parameters, the other without any anticoagulant and was centrifuged at 4000 rpm at 4 °C for 10 minutes to obtain the serum. Serum was stored at 20 °C until analyzed for biochemical parameters.

RESULTS:

TABLE 1: DOSE FINDING EXPERIMENT AND ITS BEHAVIORAL SIGNS OF TOXICITY

Dose mg/kg	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
2000	+	-	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-

1. Alertness 2. Aggressiveness 3. Pile erection 4. Grooming 5. Gripping 6. Touch Response 7. Decreased Motor Activity 8. Tremors 9. Convulsions 10. Muscle Spasm 11. Catatonia 12. Muscle relaxant 13. Hypnosis 14. Analgesia 15.Lacrimation 16. Exophthalmos 17. Diarrhoea 18. Writhing 19. Respiration 20. Mortality

TABLE 2: BODY WEIGHT (G) CHANGES OF ALBINO RATS EXPOSED TO PIRANDAI UPPU FOR 28 DAYS

Dose (mg/kg/day)	Days				
	1	7	14	21	28
Control	102.47±4.22	104.25±4.11	108.36±3.18	107.10±4.10*	113.21±5.85*
20	120.31±4.14	120.20±5.28	120.44±6.37	123.30±6.08	124.55±8.04*
100	118.00±6.17	119.34±8.16	120±10.15	122.46±8.50	125.21±10.24
200	116.24±10.10	116.77±10.11	118.64±10.67	120.08±4.16*	122.16±10.46*

Values are mean of 6 animals ± S.D. (Dunnett's test). *P<0.05; **P<0.01. N=6.

TABLE 3: FOOD (g/day) INTAKE OF ALBINO RATS EXPOSED TO PIRANDAI UPPU FOR 28 DAYS

Dose (mg/kg/day)	Days(g/rats)				
	1	7	14	21	28
Control	38.46±3.10	36.17±2.78	39.45±2.10	36.11±2.80	39.19±2.12
20	37.45±2.44	37.07±2.18	38.44±2.30	38.21±2.72	38.32±3.00
100	40.60±3.40	39.40±2.42	39.10±3.44	40.77±3.04	41.46±3.16
200	41.2±2.07	42.15±2.12	41.88±2.45	42.16±3.08	42.56±2.05

Values are mean of 6 animals ± S.D. (Dunnett's test). *P<0.05; **P<0.01. N=6.

TABLE 4: WATER (ml/day) INTAKE OF ALBINO RATS EXPOSED TO PIRANDAI UPPU FOR 28 DAYS

Dose (mg/kg/day)	Days(ml/rat)				
	1	7	14	21	28
Control	46.68±2.58	44.15±3.66	45.10±2.15	40.14±2.40	41.0±3.02
20	50.34±3.17	49.45±3.28	51.11±3.60	49.02±3.81	51.00±2.80
100	49.76±2.04	47.30±3.08	47.43±3.11	48.10±2.18	47.12±2.32
200	48.10±2.16	46.30±2.37	47.14±2.09	47.11±2.19	48.10±2.54

Values are mean of 6 animals ± S.D. (Dunnett's test). *P<0.05; **P<0.01. N=6.

Hematological and blood biochemical analyses:

At the end of the study, the animals were fasted for approximately 18 h, then slightly anesthetized with ether on the 29th day. Blood samples for hematological and blood analyses were taken from retro-orbital plexus. Heparinized blood samples were taken for determining complete blood count **Table 5** (white blood cell count, differential white blood cell count, platelet count, red blood cell count, hematocrit, and hemoglobin) by semi-automated hematology analyzer.

TABLE 5: EFFECT OF PIRANDAI UPPU ON HAEMATOLOGICAL PARAMETERS AFTER 28 DAYS

Parameter	Control	20 mg/kg	100 mg/kg	200 mg/kg
RBC (mm^3)	7.51±0.16	6.87±0.31	6.52±0.22	6.25±0.11
HB (%)	15.30±0.41	15.17±0.38	15.38±0.60	15.92±0.52
Leukocyte ($x10^6$/mL)	10138±126.53	10137±286.10	10209±246.75	10290±264.1
Platelets/ul	1367±39.67	1268±70.17	1291±97.57	1299±23.45
MCV (gl)	59.67±4.12	56.37±3.12	57.85±2.28	55.01±2.51
DLC (%)				
N	4.79±0.82	5.25±1.50	4.24±0.63	5.22±2.22
L	92.12±3.56	90.42±3.61	91.12±3.12	91.14±2.88
M	2.10±0.48	2.60±0.42	2.34±0.40	2.44±0.30
E	1.12±0.22	1.24±0.30	2.10±0.28	1.98±0.24
B	0	0	0	0
ESR(mm)	1±00	1±00	1±00	1±00
PCV	48.20±1.88	46.32±2.56	46.14±3.65	47.30±2.11
MCH (pg)	18.38±0.45	17.41±0.44	18.46±0.32	18.78±0.42
MCHC (g/dl)	30.67±0.98	31.04±0.42	31.18±1.30	30.69±0.55

Values are mean of 6 animals ± S.D. (Dunnett's test). *P<0.05; **P<0.01. N=6.

The serum from non-heparinized blood was carefully collected for blood chemistry and enzyme analysis, glucose, Creatinine, total protein, albumin, total and direct bilirubins, serum glutamate-oxaloacetate trans-aminase (SGOT), serum glutamate pyruvate transaminase (SGPT), and alkaline phosphatase (ALP) were automatically determined using auto analyzer **Table 6, 7, 8 and 9.**

TABLE 6: EFFECT OF PIRANDAI UPPU ON HEPATIC PARAMETERS

Dose (mg/kg)	Control	20 mg/kg	100 mg/kg	200 mg/kg
Total Bilirubin (mg/dL)	0.206±0.05	0.207±0.04	0.206±0.06	0.208±0.05
Bilirubin direct (mg/dL)	0.1±0.07	0.1±0.05	0.1±0.09	0.1±0.04
Bilirubin Indirect (mg/dL)	0.1±00	0.1±00	0.1±00	0.1±00
SGOT (U/L)	168.44±6.21	164.78±5.54	160.42±5.82*	158.14±5.40*
SGPT(U/L)	46.4±2.24	45.4±2.18	44.00±2.14	46.62±4.11
Total Protein(g/dl)	10.12±1.30	9.16±0.30	8.42±0.27	9.11±0.46
Albumin(g/dl)	3.11±0.25	3.11±0.20	3.17±0.21	3.48±0.11
Globulin(g/dl)	6.12±0.18	5.24±0.15	6.77±0.17	6.48±0.20
A/G Ratio(g/dl)	0.56±0.05	0.55±0.12	0.66±0.11	0.64±0.10
GGT(U/L)	7.3±0	7.0±0	7.1±0	7.2±0

Values are mean of 6 animals ± S.D. (Dunnett's test). *P<0.05; **P<0.01. *vs. control group* N=6.

TABLE 7: EFFECT OF PIRANDAI UPPU ON RENAL PARAMETERS

Dose (mg/kg)	Control	20 mg/kg	100 mg/kg	200 mg/kg
Urea(mg/dL)	58.19±1.56	62.45±2.56	64.±2.31	68.21±2.45
Creatinine (mg/dL)	0.82±0.06	0.84±0.15	0.80±0.25	0.82±0.24
Uric acid (mg/dL)	1.5±0.20	1.6±0.24	1.6±0.21	1.56±0.27
Na m.mol	138.12±7.30	137.4±6.88	138.12±6.32	141.18±5.12
K m.mol	21.50±2.84	19.51±2.08	20.10±2.18	20.23±2.10
Cl m.mol	99.24±3.18	100.10±5.26	98.28±4.64	102.20±6.98*

Values are mean of 6 animals ± S.D. (Dunnett's test). *P<0.05; **P<0.01. *vs. control group* N=6.

TABLE 8: EFFECT OF PIRANDAI UPPU ON LIPID PROFILE

Dose (mg/kg)	Control	20 mg/kg	100 mg/kg	200 mg/kg
Total cholestrol(mg/dL)	41.48±2.78	40.12±2.59	44.20±3.23	46.14±2.98
HDL(mg/dL)	12.29±1.65	12.28±1.46	12.35±1.30	13.10±2.00
LDL(mg/dL)	32.18±2.88	44.11±3.18	36.14±2.47	34.14±1.88
VLDL(mg/dl)	16.29±2.46	16.12±2.10	16.58±1.66	14.20±1.10
Triglycerides (mg/dl)	82.14±3.38	81.22±2.58	81.11±2.26	84.21±2.99
TC/HDL ratio (g/dl)	3.32±0.21	3.28±0.26	3.46±0.27	3.50±0.28
Blood glucose(mg/dl)	112.16±8.62	113.0±3.33	111.37±4.12	112.4±2.58

Values are mean of 6 animals ± S.D. (Dunnett's test). *P<0.05; **P<0.01. *vs. control group* N=6.

TABLE 9: EFFECT OF PIRANDAI UPPU ON URINE PARAMETERS

Parameters	Control	20 mg/kg	100 mg/kg	200 mg/kg
Colour	Yellow	Yellow	Yellow	Yellow
Transparency	Clear	turbid	cloudy	Slightly turbid
Specific gravity	1.010	1.010	1.010	1.010
PH	>7.2	>8.0	>8.0	>9.0
Protein	Nil	2+	2+	1+
Glucose	Nil	Nil	Nil	Nil
Bilirubin	-ve	-ve	-ve	-ve
Ketones	-ve	+ve	+ve	+ve
Blood	Absent	Absent	Absent	Absent
Urobilinogen	Normal	Abnormal	Abnormal	Abnormal
Pus cells	0-cells/HPF	1-cell/HPF	2-cells/HPF	1-cell/HPF
RBCs	Nil	Nil	0-1cells/HPF	Nil
Epithelial cells	Nil	1-cell/HPF	Nil	1-cell/HPF
Crystals	Nil	Nil	Nil	Nil
Casts	Nil	Nil	Nil	Nil
Others	Bacteria seen	Bacteria seen	Bacteria seen	Bacteria seen

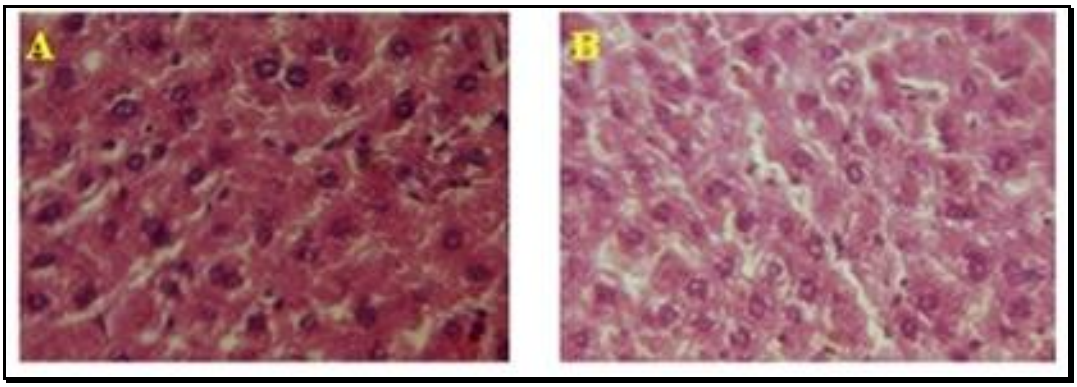
Values are mean of 6 animals ± S.D. (Dunnett's test). *P<0.05; **P<0.01. *vs. control group* N=6.

Necropsy: All the animals were sacrificed on day 29. Necropsy of all animals was carried out and the weights of the organs including liver, kidneys, adrenals, spleen, brain, heart, uterus and testes/ovaries were recorded **Table 10**. Histopathological investigation of the vital organs was done. The organ pieces (3-5µm thick) of the highest dose level of 400 mg/kg were preserved and were fixed in 10% formalin for 24 h and washed in running water for 24 h. Embedding was done by passing the cleared samples through three cups containing molten paraffin at 50°C and then in a cubical block of paraffin made by the "L" moulds. It was followed by microtome and the slides were stained with Haematoxylin-eosin and were examined microscopically **Panel-1, 2, 3 and 4.**

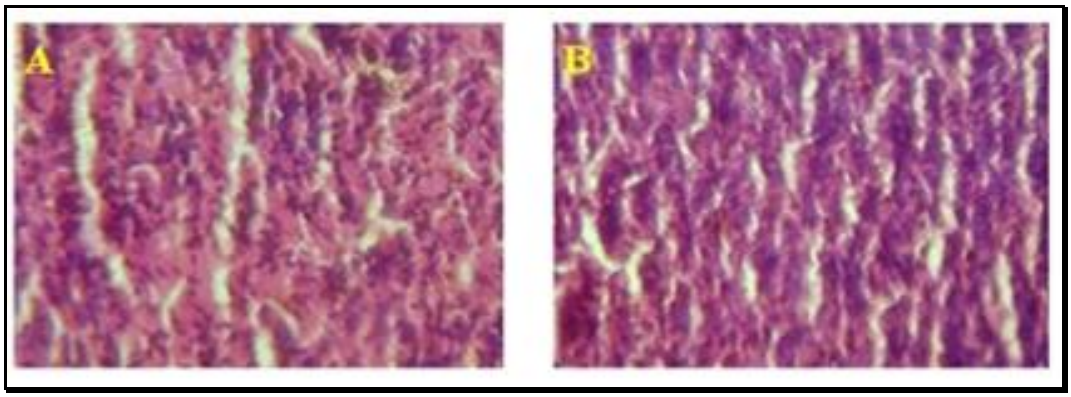
TABLE 10: EFFECT OF PIRANDAI UPPU ON ORGAN WEIGHT

Dose (mg/kg)	Control	20 mg/kg	100 mg/kg	200 mg/kg
Liver (g)	5.16±0.14	4.72±0.21	4.69±0.20	4.70±0.22
Heart (g)	0.68±0.05	0.62±0.02	0.63±0.05	0.64±0.02
Lung (g)	1.54±0.25	1.16±0.21	1.17±0.22	1.16±0.10
Spleen (g)	0.67±0.07	0.65±0.05	0.67±0.05	0.64±0.04
Ovary (g)	1.92±0.14	1.87±0.12	1.65±0.18	1.62±0.15
Testes (g)	1.42±0.12	1.41±0.12	1.42±0.12	1.44±0.19
Brain (g)	1.44±0.18	1.42±0.17	1.45±0.16	1.46±0.18
Kidney (g)	0.71±0.05	0.71±0.04	0.72±0.05	0.73±0.04
Stomach (g)	1.41±0.10	1.42±0.20	1.40±0.17	1.43±0.12

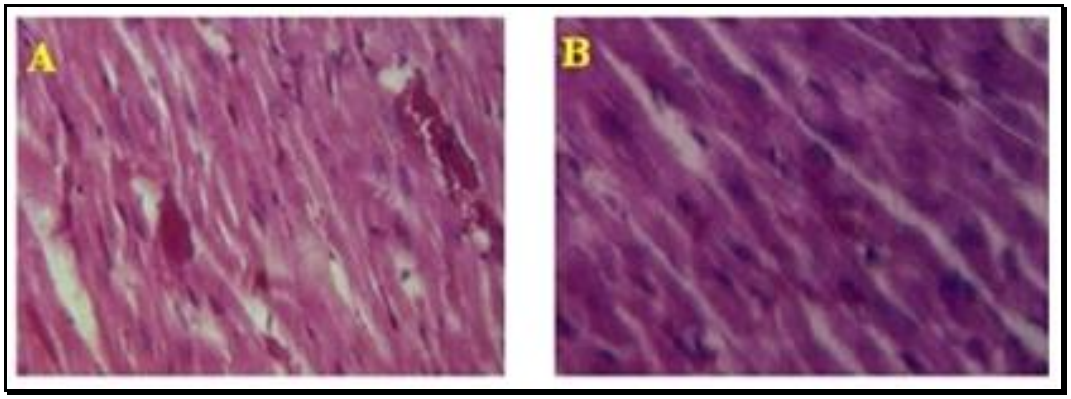
Values are mean of 6 animals ± S.D. (Dunnett's test). *P<0.05; **P<0.01 *vs control* N=6**.**



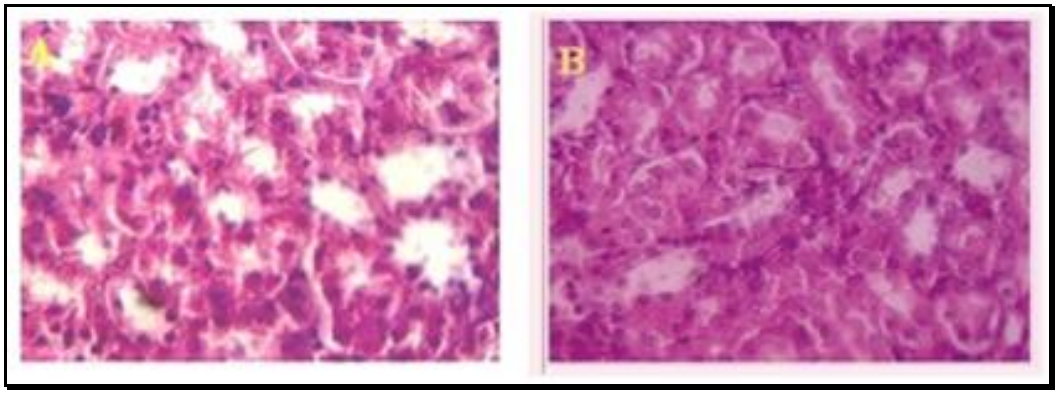
PANEL 1: LIGHT PHOTOMICROGRAPHY OF LIVER OF A CONTROL RAT
FIGURE A – Control
FIGURE B – Treated on high dose, no abnormality is seen in hepatocytes, sinusoids.



PANEL 2: LIGHT PHOTOMICROGRAPHY OF SPLEEN OF A CONTROL RAT
FIGURE A – Control
FIGURE B – Treated on high dose, no abnormality is seen in trabeculae, capsule.



PANEL 3: LIGHT PHOTOMICROGRAPHY OF HEART OF A CONTROL RAT
FIGURE A – Control
FIGURE B – Treated on high dose, no abnormality is seen in nuclei of myocytes, myocardium



PANEL 4: LIGHT PHOTOMICROGRAPHY OF KIDNEY OF A CONTROL RAT
FIGURE A – Control
FIGURE B – Treated on high dose, no abnormality is seen in glomeruli, Bowman's capsule, capillaries.

Statistical analysis: Findings such as clinical signs of intoxication, body weight changes, food consumption, hematology and blood chemistry were subjected to One-way Anova followed by dunnet't' test using a computer software programme -INSTAT-V3 version.

DISCUSSIONS: The results of acute toxicity study of *Pirandai Uppu* revealed no mortality, abnormal signs and behavioral changes in rats at the dose of 2000 mg/kg body weight administered orally. **Table 1**. All animals from control and all the treated dose groups survived throughout the dosing period of 28 days. The results for body weight determination of animals from control and different dose groups show comparable body weight gain throughout the dosing period of 28 days **Table 2**. During dosing period, the quantity of food and water consumed by animals from different dose groups was found to be comparable and normal with that by control animals **Table 3 and 4.**

The results of hematological investigations such as Erythrocytes, Total Leucocytes and Platelets count **Table 5** conducted on day 29, revealed no significant changes in the values when compared with those of respective controls. This gave clear justification that *Pirandai Uppu* did not influence bone marrow and spleen. Among the differential count of WBC, only the Eosinophil's count was slightly increased at the *Pirandai Uppu* dosage of 20 mg/kg and 100 mg/kg. This might be occurred due to stress.

Results of Biochemical investigations conducted on days 29 and recorded: Urea, SGOT, SGPT, and Bilirubin were within the limits. LDL level was elevated in animals of 20 mg/kg dose group ($P<0.05$) and at the dosage of 200mg/kg, total cholesterol level was slightly increased but these were within the normal limits **Table 6, 7** and **8**. The other cardio vascular risk markers were also within normal, ensured that *Pirandai Uppu* did not influence the Cardio vascular system.

Urine analysis data **(Table 9)** of control group and treated group of animals determined in week 4 did not reveal major abnormalities rather than transparency, pH and deposits. Group Mean Relative Organ Weights (% of body weight) are recorded **(Table 10).** Comparison of organ weights of treated animals with respective control animals on day 29 was found to be comparable with respective control group. Gross pathological examination of animals in control as well as the treated groups did not reveal any abnormalities. Histopathology: The vital organs such as liver, heart, Spleen and kidneys were removed from the test groups at the end of the study they did not reveal any abnormal macroscopic changes. Microscopically, these organs of the test groups revealed normal histological appearance when compared with the control group **(Panel 1-4).**

CONCLUSIONS: The authors concluded based on the above findings, the median lethal dose of *Pirandai uppu* estimated as above 2000 mg/kg body weight, and results of 28 days repeated oral toxicity study revealed there is no significant changes found at 200 mg/kg body weight. It can be recommend that PU is a safest drug under intended human adult dosage (500 mg) as illustrated in the literature for diarrhea.

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