Research Article

Subacute Toxicity Evaluation of Rauvolfia Tetraphylla Methanolic Leaf Extract in Sprague Dawley Rat

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ABSTRACT

The objective of this study was to evaluate the toxic characteristics of *Rauvolfia tetraphylla* leaf extract in Sprague dawley rats after treatment for minimum 7 days by oral route. The study was conducted according to OECD guideline 407. Animals from low, mid and high dose groups received leaf extract at 250, 500 and 1000 mg/kg/day, respectively. Animals from control group received Vehicle alone. Animals were assessed for mortality, clinical signs, food consumption, body weights, hematology, clinical chemistry, ophthalmoscopy, organ weights, gross and histopathology. 1000 mg/kg dose resulted in reduction in food consumption, body weight, percent body weight change and increase in total bilirubin in male rats and reduction in absolute and relative thymus weight in female rats. From results, it is concluded that the *R. tetraphylla* methanolic leaf extract is well tolerated up to 500 mg/kg/day in the present study. Further, toxicity observed in rat in the present study is an indication of possible risk to human which needs to be further explored in longer duration toxicity studies.

Keywords: *Rauvolfia tetraphylla*; Subacute Toxicity; Leaf Extract, OECD.

INTRODUCTION

Rauvolfia tetraphylla L. (Apocynaceae) is a small much branched woody shrub. It is native to West-Indies though found throughout the plains of India especially in Bihar, Orissa, Madhya Pradesh, West Bengal, Andhra Pradesh, Tamil Nadu, Karnataka and Kerala.[1] The leaf extract of the plant is useful in the treatment of fever, hypertension, cholera, eye-disease as well as for the treatment of diarrhea and dysentery (Anonymous, 1969).

In-house pilot studies (unpublished literature) have confirmed that *R.tetraphylla* leaf extract have larvicidal and oviposition deterrent activity against the vector of lymphatic fileriasis, Culex quinquefasciatus. But for successful development of plant extract as a biopesticide it needs to be characterized for its safety.

Prior work on methanolic leaf extract of *R. tetraphylla* has confirmed that it is non-mutagenic in Ames test.[2] Additionally, the leaf extract was also characterized for its acute toxicity potential in female Sprague Dawley rats.[3]

The objective of this study is to further characterize toxicity potential of *R. tetraphylla* methanolic leaf extract by using subacute toxicity testing in Sprague dawley rats by oral route of administration.

MATERIALS AND METHODS

Plant collection and extraction

R. tetraphylla plant leaves were collected from University of Pune campus, Pune, India. The specimen and samples were identified and confirmed by Botanical Survey of India (BSI), Western Regional Office, Pune.

In brief, Leaves were washed with water; shed dried and powdered using a mechanical grinder. Powdered leaves were extracted with methanol solvent in Soxhlet apparatus. Extract was then concentrated under reduced pressure 22-26 mm of Hg at 45 °C using rotary evaporator, and the residue was stored at 4 to 8 °C till further used for the experiment.[4]

Animals and husbandry

Sprague Dawley rats, approximately 6–7 weeks of age were used for the study. Animals were fed standard laboratory animal diet (Altromin 1324P, Spezialfutter GmbH & Co. KG, Germany) and given drinking water filtered through water filtration system *ad libitum*. The animal rooms were maintained at 19-26 °C temperature and 30-70 % relative humidity with a 12 hour light/dark cycle. The animals were acclimatized to the laboratory conditions for at least five days prior to first day of dosing. Based on body weights, animals were randomly distributed in different dose groups (Group 1-4) prior to initiation of dosing. Animals selected for study were within ± 20 % of the mean body weight for each gender at randomization. The study was conducted according to Organization for Economic Cooperation and Development (OECD) guideline for testing of chemicals, No.407, "Repeated Dose 28-Days Oral Toxicity Study in Rodents".[5] Institutional Animal Ethics Committee has approved procedures and use of laboratory animals for this experiment.

As per the OECD Guideline No. 407 the limit dose of 1000 mg/kg was selected as high dose and remaining two doses were selected to identify dose response and no observed adverse effect level.

Dose formulation and administration

Test item formulation was prepared daily. Weighed quantity of plant extract was triturated with small quantity of 0.5 % carboxymethylcellulose (CMC) with pestle and mortar. The final volume of formulation was made by adding required quantity of 0.5 % CMC. The formulation was stirred for 15 minutes using magnetic stirrer. Dose formulation was kept at room temperature and stirred using a magnetic stirrer prior to and throughout dosing. Dose formulation was administered through oral gavage. Animals were dosed once daily for consecutive 7 days at the dose volume of 10 mL/kg. Dose volumes were based on the most recent body weight.

Experimental design

Three groups (6 animals/gender/group) were exposed to the test item for 7 consecutive days. Control group (G1) was given vehicle alone. Daily all animals were observed for mortality and clinical signs. Body weight and food consumption were recorded weekly.

Ocular examination was performed on all animals once prior to the commencement of treatment and on treatment day-7. Towards study termination, blood was collected for analyzing the haematological and biochemical parameters. After completion of treatment period, animals were sacrificed and complete gross and histopathological studies were carried out on selected organs.

Data analysis

The mean and standard deviation were calculated using the Microsoft Excel Worksheet. Groups mean data (Mean, SD and n) were processed using Graph Pad Prism software (10855, Sorrento valley Road, # 203 San Diego CA 92121, USA). Continuous data was analyzed with ANOVA and Dunnett's multiple comparison test.

RESULTS AND DISCUSSION

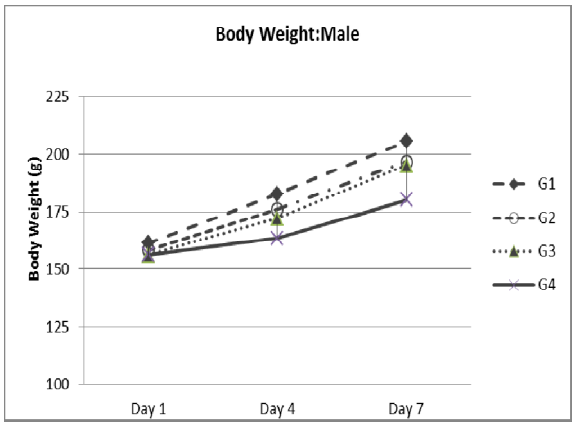

Figure 1: Effect of *R. tetraphylla* leaf extract on body weight

The assessment of the systemic toxicity of methanolic leaf extract obtained from the leaves of *R.tetraphylla* was performed based on the results obtained in subacute (7 days) toxicity study in rat.

No treatment related mortality was noticed during the study. However, one male and one female from mid dose group found dead due to gavage error. Daily administration of leaf extract was well tolerated and no visible signs of toxicity were observed during the study period.

Group mean body weight of high dose male animals on treatment day 7 was significantly decreased by 12.4 % (Table-2, Figure-1). These animals also revealed significant reduction in group mean percent body weight during treatment day 1-4 and 1-7 by 66.9 % and 46.3 % respectively as compared to control (Table-3). Further, group mean food consumption of high dose male animals during this period was found to be reduced. The reduction in body weight and percent body weight of high dose male animals were considered as treatment related adverse effect.

Marginal but statistically significant decrease in group mean body weight was observed in mid dose male animals on treatment day 7 however its percent body weight change was unaffected. Further, significant decrease in group mean percent body weight change was observed in mid dose male animals during treatment day 1-7 which was considered transient and not related to treatment.

Significant reduction (~25 %) in food consumption (Table-4, Figure-2) was noted in high dose male and female animals during treatment day 4-6 and 1-3 respectively, which is well correlated with the reduction in body weight and percent body weight during this period. Ophthalmological evaluation did not reveal any sign of ocular toxicity.

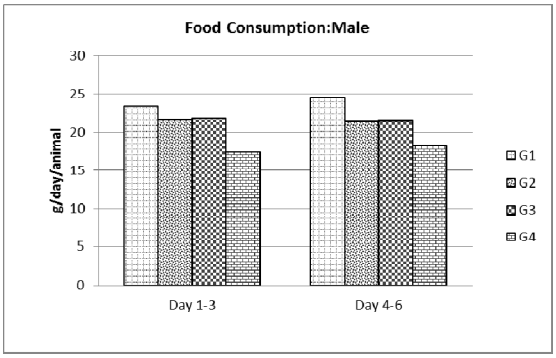

Figure 2: Effect of *R. tetraphylla* leaf extract on food consumption in male rat

Hematological parameters did not reveal any treatment related effect. However, statistically significant decrease in prothrombin time was observed in high dose female which is considered marginal and toxicologically non-significant. Additionally, significant decrease in eosinophil count was observed in all the treated female groups. Further, decrease in red blood cell (RBC) count (Mid dose female) and increase in activated partial thromboplastin time (APTT) (Low dose female) do not follow dose trend and were considered not related to treatment (Table-5 & 6).

Table 1: Group designation and dose levels

Group No.	Group	Dose (mg/kg b. wt.	Conc. (mg/ml)	No. of animal (M + F)	Animal No.	
					Male	Female
1	Control	0	0.0	6+6	1 - 6	25 - 30
2	Low	250	25	6+6	7 - 12	31 - 36
3	Mid	500	50	6+6	13 - 18	37 - 42
4	High	1000	100	6+6	19 - 24	43 - 48

Table 2: Summary of Body Weight (g) - Male & Female

Gender	Day	0 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg
Male	1	161.2 ± 8.4	158.2 ± 8.1	156.1 ± 7.5	156.3 ± 8.4
	4	182.7 ± 10.8	176.0 ± 12.1	172.2 ± 9.1	163.3 ± 13.5
	7	205.7 ± 12.0	196.5 ± 16.8	195.1 ± 12.2*	180.2 ± 16.9*
Female	1	137.5 ± 5.2	136.7 ± 8.3	138.6 ± 7.6	141.3 ± 7.3
	4	147.6 ± 5.8	146.8 ± 8.2	147.4 ± 6.1	142.6 ± 10.1
	7	154.6 ± 9.7	156.1 ± 10.3	160.2 ± 6.3	156.4 ± 14.5

***** - Statistical significance at P < 0.05); n=6; Values are expressed as Mean ± Standard Deviation

Table 3: Summary of Body Weight Change (%) - Male & Female

Gender	Day	0 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg
Male	1-4	13.3 ± 1.9	11.2 ± 3.6	10.3 ± 1.1	4.4 ± 5.4***
	1-7	27.6 ± 3.1	24.2 ± 7.5	25.5 ± 3.1	15.1 ± 6.0**
Female	1-4	7.4 ± 2.2	7.4 ± 3.2	6.5 ± 4.9	0.9 ± 3.1*
	1-7	12.5 ± 4.6	14.2 ± 4.1	15.7 ± 4.5	10.5 ± 5.8

***** - Statistical significance at P < 0.05, ****** - Statistical significance at P < 0.01, ******* - Statistical significance at P < 0.001; Values are expressed as Mean ± Standard Deviation; n=6

Table 4: Summary of Food Consumption (g/animal/day) - Male & Female

Gender	Day	0 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg
Male	1-3	23.35 ± 0.95	21.66 ± 1.09	21.88 ± 1.78	17.42 ± 5.38
	4-6	24.52 ± 1.58	21.52 ± 0.91	21.60 ± 2.54	18.33 ± 1.72**
Female	1-3	16.64 ± 1.44	15.76 ± 0.81	15.32 ± 1.88	12.00 ± 2.64*
	4-6	14.98 ± 2.09	15.38 ± 0.66	16.77 ± 0.56	15.85 ± 2.18

* - Statistical significance at P < 0.05, ** - Statistical significance at P < 0.01; Values are expressed as Mean ± Standard Deviation, n=3

Table 5: Summary of Hematology Values - Male

Hematological Parameters	0 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg
White Blood Cells (Thousand/µL)	12.06 ± 3.71	10.55 ± 2.51	10.76 ± 2.50	9.06 ± 1.04
Red Blood Cells (millions/µL)	7.02 ± 0.31	7.11 ± 0.18	7.00 ± 0.34	7.02 ± 0.24
Haemoglobin (g/dL)	15.10 ± 0.45	15.20 ± 0.33	15.06 ± 0.62	15.03 ± 0.55
Hematocrit (%)	40.22 ± 1.11	40.58 ± 1.40	40.12 ± 1.82	39.73 ± 1.34
MCV (femtoliter)	57.33 ± 2.56	57.02 ± 0.97	57.36 ± 1.68	56.67 ± 1.89
MCH (picogram)	21.55 ± 1.14	21.37 ± 0.39	21.52 ± 0.54	21.45 ± 0.72
MCHC (g/dL)	37.57 ± 0.43	37.48 ± 0.81	37.52 ± 0.26	37.83 ± 0.31
Platelets (thousands/µL)	1097.33 ± 110.74	1010.00 ± 376.50	1241.00 ± 71.58	1106.50 ± 86.79
Prothrombin Time (seconds)	15.95 ± 0.76	14.18 ± 2.47	15.44 ± 1.46	14.53 ± 0.69
APTT (seconds)	11.05 ± 1.30	13.42 ± 1.05	11.68 ± 1.09	9.57 ± 2.40
Neutrophils (%)	12.87 ± 2.04	14.38 ± 3.93	10.47 ± 3.12	10.40 ± 3.20
Lymphocytes (%)	81.82 ± 4.83	81.83 ± 3.11	84.24 ± 6.11	85.75 ± 3.44
Monocytes (%)	2.72 ± 3.11	1.77 ± 1.38	2.93 ± 3.32	1.60 ± 1.47

Eosinophils (%)	0.54 ± 0.42	0.05 ± 0.02	0.01 ± 0.01	0.03 ± 0.03
Basophils (%)	2.06±1.18	1.99 ± 1.08	2.34 ± 0.66	2.23 ± 0.91

MCV=Mean Corpuscular Volume, MCH=Mean Corpuscular Hemoglobin, MCHC=Mean Corpuscular Hemoglobin Concentration, APTT=Activated Partial Thromboplastin Time; n=6 except for 500 mg/kg dose where n=5; Values are expressed as Mean ± Standard Deviation

Table 6: Summary of Hematology Values - Female

Hematological Parameters	0 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg
White Blood Cells (Thousand/µL)	9.73 ± 1.84	9.79 ± 1.84	6.99 ±1.98	7.23 ± 2.23
Red Blood Cells (millions/µL)	7.09 ± 0.30	6.70 ± 0.25	6.56 ± 0.49*	6.97 ± 0.29
Haemoglobin (g/dL)	14.92 ± 0.83	14.87 ± 0.69	14.20 ± 1.04	14.90 ± 0.24
Hematocrit (%)	41.35 ± 1.71	40.64 ± 1.65	38.82 ± 2.65	40.38 ± 0.98
MCV (femtoliter)	58.30 ± 1.26	60.63 ± 1.41	59.26 ± 1.91	58.00 ± 2.00
MCH (picogram)	21.03 ± 0.94	22.15 ± 0.67	21.66 ± 0.86	21.40 ± 0.68
MCHC (g/dL)	36.10 ± 1.47	36.57 ± 0.37	36.52 ± 0.32	36.88 ± 0.48
Platelets (thousands/µL)	1259.83 ± 79.95	1161.50 ± 163.74	1211.60 ± 88.89	1165.83 ± 121.52
Prothrombin Time (seconds)	15.47 ± 0.75	13.82 ± 1.11	13.80 ± 1.64	13.52 ± 1.61*
APTT (seconds)	14.20 ± 1.72	14.48 ± 6.83*	13.38 ± 1.27	12.65 ± 1.52
Neutrophils (%)	11.65 ± 4.78	11.01 ± 1.74	13.41 ± 4.36	13.81 ± 6.25
Lymphocytes (%)	85.83 ± 6.20	86.18 ± 2.21	85.00 ± 3.68	81.52 ± 7.64
Monocytes (%)	1.35 ± 1.43	1.55 ± 1.10	0.82 ± 0.58	3.20 ± 1.68
Eosinophils (%)	0.23 ± 0.18	0.12 ± 0.09**	0.09 ± 0.07**	0.07 ± 0.08**
Basophils (%)	0.92 ± 0.22	1.15 ± 0.40	0.69 ± 0.39	1.42 ± 0.78

MCV=Mean Corpuscular Volume, MCH=Mean Corpuscular Hemoglobin, MCHC=Mean Corpuscular Hemoglobin Concentration, APTT=Activated Partial Thromboplastin Time; n=6 except for 500 mg/kg dose where n=5; * - Statistical significance at P < 0.05, ** - Statistical significance at P < 0.01; Values are expressed as Mean ± Standard Deviation

Table 7: Summary of Clinical Chemistry - Male

Biochemical parameters	0 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg
Albumin (g/dL)	1.15 ± 0.14	16.39 ± 37.04	1.21 ± 0.18	1.19 ± 0.14
Alanine aminotransferase (IU/L)	66.18 ± 8.21	76.48 ± 12.04	67.88 ± 6.46	69.52 ± 10.94
Aspartate aminotransferase (IU/L)	73.68 ± 11.94	65.17 ± 15.62	51.54 ± 9.42	65.45 ± 19.81
Alkaline phosphatase (IU/L)	430.75 ± 68.57	385.07 ± 83.09	396.88 ± 91.73	336.93 ± 52.54
Cholesterol (mg/dL)	123.60 ± 12.49	130.13 ± 19.52	108.26 ± 11.73	113.23 ± 12.55
Creatinine (mg/dL)	0.42 ± 0.06	0.38 ± 0.04	0.37 ± 0.06	0.32 ± 0.03**
γ-glutamyl transpeptidase (mg/dL)	5.24 ± 0.59	4.71 ± 0.56	4.80 ± 0.36	4.31 ± 0.79*
Glucose (mg/dL)	85.38 ± 7.27	88.67 ± 15.88	98.96 ± 12.48	81.37 ± 6.28
Total protein (g/dL)	6.01 ± 0.18	6.23 ± 0.27	6.05 ± 0.33	5.97 ± 0.14
Triglyceride (mg/dL)	98.00 ± 35.56	103.72 ± 18.18	114.0 ± 60.56	107.07 ± 27.54
Total bilirubin (mg/dL)	0.20 ± 0.03	0.20 ± 0.03	0.20 ± 0.01	0.26 ± 0.02**
Blood urea nitrogen (mg/dL)	11.93 ± 2.22	13.35 ± 1.87	13.68 ± 2.61	11.19 ± 2.46
Calcium (IU/L)	10.57 ± 0.21	10.57 ± 0.13	10.19 ± 0.13*	9.78 ± 0.26***
Phosphorus (mg/dL)	10.27 ± 0.50	10.20 ± 0.46	9.89 ± 0.77	9.33 ± 0.37*
Sodium (mmol/L)	143.83 ± 3.03	145.05 ± 3.82	142.02 ± 1.06	141.65 ± 0.94*
Potassium (mmol/L)	3.32 ± 0.25	3.56 ± 0.26	3.44 ± 0.16	3.79 ± 0.15
Chloride (mmol/L)	101.42 ± 2.35	102.58 ± 3.28	100.50 ± 0.80	101.57 ± 1.07

* - Statistical significance at P < 0.05, ** - Statistical significance at P < 0.01, *** - Statistical significance at P < 0.001, n=6 except for 500 mg/kg dose where n=5; Values are expressed as Mean ± Standard Deviation

Table 8: Summary of Clinical Chemistry - Female

Biochemical Parameters	0 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg
Albumin (g/dL)	1.44 ± 0.09	1.40 ± 0.04	1.34 ± 0.05*	1.35 ± 0.06
Alanine aminotransferase (IU/L)	52.78 ± 2.13	56.43 ± 4.63	53.00 ± 15.54	80.92 ± 45.86
Aspartate aminotransferase (IU/L)	75.72 ± 6.79	68.88 ± 7.57	64.88 ± 20.43	87.60 ± 16.17
Alkaline phosphatase (IU/L)	241.07 ± 52.07	221.47 ± 29.64	204.76 ± 52.02	210.28 ± 85.23
Cholesterol (mg/dL)	99.33 ± 9.94	100.20 ± 14.21	93.92 ± 10.37	90.95 ± 40.71
Creatinine (mg/dL)	0.33 ± 0.05	0.31 ± 0.03	0.35 ± 0.06	0.33 ± 0.03
γ-glutamyl transpeptidase (mg/dL)	4.97 ± 0.42	4.58 ± 0.63	5.09 ± 0.50	5.16 ± 0.71
Glucose (mg/dL)	99.48 ± 11.24	94.33 ± 13.82	99.42 ± 28.92	84.02 ± 15.59
Total protein (g/dL)	6.49 ± 0.30	6.47 ± 0.20	6.18 ± 0.42	6.59 ± 0.26
Triglyceride (mg/dL)	42.70 ± 21.46	49.08 ± 11.19	66.86 ± 31.35	63.60 ± 29.81
Total bilirubin (mg/dL)	0.10 ± 0.04	0.14 ± 0.04	0.11 ± 0.05	0.13 ± 0.03
Blood urea nitrogen (mg/dL)	15.64 ± 2.47	16.55 ± 3.20	17.15 ± 1.40	13.63 ± 2.91
Calcium (IU/L)	10.51 ± 0.33	10.51 ± 0.13	10.34 ± 0.46	10.00 ± 0.15*
Phosphorus (mg/dL)	8.35 ± 0.49	8.19 ± 0.50	9.19 ± 2.16	8.02 ± 0.34
Sodium (mmol/L)	144.13 ± 2.12	144.38 ± 3.36	146.26 ± 2.70	143.58 ± 2.44
Potassium (mmol/L)	3.17 ± 0.30	3.30 ± 0.17	3.95 ± 1.34	3.16 ± 0.38
Chloride (mmol/L)	103.27 ± 1.26	102.95 ± 3.31	104.34 ± 2.35	102.12 ± 1.63

n=6 except for 500 mg/kg dose where n=5; * - Statistical significance at P < 0.05; Values are expressed as Mean ± Standard Deviation

Treatment related increase (~30 %) in total bilirubin level was observed in high dose male animals. Marginal decrease in creatinine, GGT, phosphorus and sodium was observed in high dose male animals. Marginal decrease in levels of calcium was observed in mid dose male and high dose male and female animals. Further, decrease in albumin levels observed in mid dose female group was marginal and also do not follow dose related trend hence considered not related to treatment (Table-7 & 8).

Sacrificed animals showed normal appearance of visceral organs without any treatment related gross abnormality. Microscopic examination performed for high dose animals revealed spontaneous findings in various organs and were considered incidental in nature.

High dose female animals revealed ~30 % reduction of absolute and relative weight of thymus without any histopathological alterations. Reason for this weight reduction could not be ascertained. Increase or decrease in organ weight (either absolute or relative) has been observed as a sensitive indicator of organ toxicity by known toxicants.[6]

L Tédong have established toxicity of h*exane* extract of leaves from *Anacardium occidentale* in mice. Treated with this leaf extract in mice for 56 consecutive days revealed toxic effects such as reduction in weight gain, reduction in food intake, abnormal liver and kidney function tests and behavioral effects.[7]

Hausatu M. Babayi have studied sub-chronic (28-days) toxicity of aqueous whole extract of *Cassytha filiformis* in wistar albino rats and noticed reduction in body weight gain, absolute weight of kidneys and relative weights of heart and lungs.[8]

The present study clearly showed that the methanolic extract obtained from the leaves of *R. tetraphylla* when administered in rat by oral gavage for a period of seven days is well tolerated up to 500 mg/kg.[9] In conjunction with the further subacute and subchronic toxicity studies of this plant extract, it is likely to predict the safe use of this extract as biopesticide.

CONCLUSION

R. tetraphylla methanolic leaf extract when administered to rats by oral gavage at 1000 mg/kg for 7 consecutive days resulted in reduction in body weight, percent body weight change, food consumption and increase in total bilirubin in male Sprague dawley rats. Additionally reduction in absolute and relative weight of thymus was observed in female rats at high dose (1000 mg/kg).

Based on the above results, it is concluded that *R. tetraphylla* methanolic leaf extract is well tolerated up to 500 mg/kg/day in the present study.

Further, it is concluded that the toxicity observed in the present study is an indication that the extract may be toxic to human if consumed orally and hence there is need to further explore toxic characteristics of this extract using longer duration toxicity studies.

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