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Safety Evaluation of *Picrorhiza kurroa* Rhizome Extract by Bacterial Reverse Mutation Test

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Abstract

Picrorhiza kurroa is a recognized herb in the Ayurvedic and Chinese system of traditional medicine. It is effective in treating a variety of diseases ranging from digestion problems to paralysis. Thus it is essential to rule out any kind of toxicity, mutagenicity or carcinogenicity in the plant to be used as a medicine. The study was conducted to evaluate ability of *Picrorhiza kurroa* to induce reverse mutations at the histidine locus in several strains of *Salmonella typhimurium* in the absence or presence of exogenous metabolic activation system (S9) containing microsomal enzymes. The bacterial reverse mutation assay has been shown to be a sensitive, rapid and accurate indicator of mutagenic activity of a wide range of chemical classes. All bacterial strains showed negative responses over the entire dose range. No significant dose related increase was observed in the number of revertants in the two independent experiments. Based on the results

of this study it is concluded that *Picrorhiza kurroa* rhizome extract is not mutagenic in the *Salmonella typhimurium* reverse mutation assay.

Keywords: *Picrorhiza kurroa, Salmonella typhimurium,* Metabolic Activation System, Mutagenicity, Reverse Mutations

Abbreviations: Histidine: *his;* Minimal glucose agar plates: MGA; Biological Oxygen Demand: BOD

1. Introduction

Picrorhiza kurroa Royle ex. Benth (Family Scrophularaceae) commonly called Kutki or Kutka is a recognized herb in the Ayurvedic and Chinese system of traditional medicine. It is a wonder herb with hepatoprotective, [1-4] anticholestatic, [5] antioxidant, [6] antidiabetic, [7] and immune-modulating properties. [8-10]

The leaves, root and rhizome of *Picrorhiza* are used to treat liver and upper respiratory tract disorders, chronic diarrhea, and scorpion sting. It is also effective in treating a variety of diseases ranging from digestion problems to malaria, jaundice, allergy, epilepsy, paralysis, rheumatoid arthritis and skin diseases [11-14]. *Picrorhiza* extract may be of therapeutic value in treating viral hepatitis as it has promising anti-hepatitis B surface antigen activity [15]. In rats infected with malaria, *Picrorhiza* restored depleted glutathione levels, thereby enhancing detoxification and antioxidation, and helping maintain a normal oxidation-reduction balance. [16]

Nowadays therapeutic importance of plants is well recognized in both Western and Eastern medicinal systems. However, some of them are potentially toxic, mutagenic or carcinogenic thus their toxicity profiling is essential for their use as medicine. We had earlier evaluated the ability of liposome-encapsulated *Picrorhiza kurroa* extract [17] to induce reverse mutations at the histidine locus in several strains of *Salmonella typhimurium* in the absence /presence of exogenous metabolic activation system. The liposome-encapsulated *Picrorhiza kurroa* extract was found to be non-mutagenic in the reverse mutation assay. The bacterial reverse mutation assay has been shown to be a sensitive, rapid and accurate indicator of mutagenic activity of a wide range of chemical classes.
In continuation of our work in this article we compared the mutagenic potential of *Picrorhiza kurroa* extract with liposome-encapsulated extract.

2. Material and Methods

2.1 Material & Extraction

Picrorhiza kurroa rhizomes were gifted by Patanjali Ayurved Ltd., Haridwar, India and stored in ambient conditions for further study. The sample specimen is

deposited at Patanjali Research Foundation, Haridwar, India. The other solvents and chemicals were purchase from Sigma-Aldrich, India. *Salmonella typhimurium* tester strains (TA1537, TA1535, TA98, TA100 and TA102) were received from Molecular Toxicology, Inc. USA. Other chemicals were purchased from Sigma-Aldrich, India.

The powdered *Picrorhiza kurroa* rhizomes were subjected to extraction using Methanol: Water (60:40). The extract was evaporated to dryness in a rotary flash evaporator at a temperature not exceeding 60°C, and then stored in air tight container.

2.2 Methods

The bacterial reverse mutation test (Ames test) was performed with *Salmonella typhimurium* strains TA1537, TA1535, TA98, TA100 and TA102 with/without metabolic activation as described. [18]

Dose Range finding Study

In the dose range finding study the *Picrorhiza kurroa* rhizome extract was tested at the dose levels of 78.13, 156.25, 312.5, 625, 1250, 2500 and 5000 µg/ml in the presence (10% S9 mix) and absence of metabolic activation system. The dose range finding study was performed at the above mentioned dose levels in TA98 and TA100 using plate incorporation method. The dose concentration for the main mutagenicity study was determined based on the results of solubility, precipitation and cytotoxicity test.

Precipitation Test

Precipitation test was carried out in all the phases of the study (Cytotoxicity and Mutagenicity test). During precipitation test the bacterial cultures were treated with various test concentrations of the extract in presence and absence of metabolic activation. At the end of treatment, mixture was poured on to minimal glucose agar plates (MGA) and observed for presence or absence of precipitates of test item on MGA plates.

Mutagenicity Assay

Based on data on number of revertant colonies observed during dose range finding study was also considered for main mutagenicity study. The assay was performed using TA1535, TA1537, and TA102 in the presence (10% S9 mix) and absence of S9 mix. Tester strains were exposed to the extract *via* the plate incorporation methodology at dose levels of 312.5, 625, 1250, 2500 and 5000 µg/ml with and without metabolic activation system.

Plating Procedures

The *Salmonella typhimurium* tester strains TA1537, TA1535, TA 98, TA 100 and TA102 were labeled with dose groups (A- E) and date of treatment respectively on minimal glucose agar plates at the time of treatment. The plates were labeled as "+S9" and "-S9" for the presence and absence of metabolic activation respectively.

All the treated plates were incubated for 48 to 72 hours at $37^{o}C \pm 2^{o}C$ in Biological Oxygen Demand (BOD) incubator in inverted position. At the end of incubation, the condition of the bacterial background lawn was evaluated for evidence of cytotoxicity and presence/absence of precipitation of test item. Evidence of cytotoxicity was scored relative to the vehicle control and recorded along with the revertant counts of that dose group.

3. Result and Discussion

Strains of *Salmonella typhimurium* used in this assay are histidine auxotrophs by virtue of conditionally lethal mutations in the histidine operon. When the histidine (*his*-) dependent cells are exposed to the *Picrorhiza kurroa* rhizome extract and grown under selective conditions (minimal media with trace amount of histidine and biotin) only those cells which revert to (*his*+) independence are able to form colonies (**Table 1**). The trace amount of histidine in the media allows all the plated bacteria to undergo a few cell divisions, which is essential for mutagenesis to be fully expressed. The *his*+ revertants are readily discernable as colonies against the limited background growth of the *his*- cells. By using several different tester strains, base pair substitution mutations and frame shift mutations can be detected. Spontaneous reversions occur with each of the strain, which will be considered as background level. Mutagenic compounds cause an increase in the number of revertant colonies relative to the background level.

Dose Range Finding Study

The *Picrorhiza kurroa* rhizome extract was tested at the dose levels of 78.13, 156.25, 312.5, 625, 1250, 2500 and 5000 µg/ml in the presence (10 % S9 mix) and absence of metabolic activation system in the tester strains TA98 and TA100 (**Table 2**).

Precipitation

Precipitation of the extract on the plate was not observed at the start or at the end of the incubation period (**Table 3**).

Toxicity

To determine the toxicity of test item on the reduction of the bacterial background lawn, the increase in the size of the micro colonies and the reduction of the revertant colonies were examined. The tester strains TA98 and TA100 at the concentration range of 78.13 – 5000 µg/plate treated with the extract in the absence and presence of metabolic activation revealed no background bacterial lawn inhibition when compared to vehicle control groups. No reduction of the bacterial background lawn and no decrease in the number of revertants were observed over the concentration range of 78.13 to 5000 µg/plate (**Table 4**).

Mutagenicity
In the dose range finding test, no increase in the number of revertants was observed upon treatment with *Picrorhiza kurroa* rhizome extract under all conditions tested.

Main Mutagenicity Assay
Based on the data on number of revertant colonies observed during dose range finding study was considered for main mutagenicity study. The main mutagenicity assay was performed with the strains TA1537, TA1535, and TA102 in the presence (10% S9 mix) and absence of metabolic activation (**Table 5**).

Precipitation
Precipitation of *Picrorhiza kurroa* rhizome extract on the plate was not observed at the start or at the end of the incubation period.

Toxicity
No reduction of the bacterial background lawn and no biologically relevant decrease in the number of revertants were observed in all strains at any concentration tested in the absence and presence of metabolic activation.

Mutagenicity
In the main mutagenicity assay, no increase in the number of revertants was observed upon treatment with *Picrorhiza kurroa* rhizome extract under all conditions tested (**Table 6**).
Similar results were obtained for liposome-encapsulated extract. [17]

4. Conclusion

All bacterial strains (TA1537, TA1535, TA98, TA100 and TA102) showed negative responses over the entire dose range, i.e. no significant dose related increase was observed in the number of revertants in the two independent experiments. The negative and strain-specific positive control values were within the normal acceptable ranges indicating that the test conditions were adequate and that the metabolic activation system functioned properly. The results obtained were similar those observed in the previous study with liposome-encapsulated extract. Based on the results of the study it can be concluded that *Picrorhiza kurroa* rhizome extract in either case is not mutagenic in the *Salmonella typhimurium* reverse mutation assay. However, detailed in vivo studies are required to confirm its toxicity.

Authors' Contributions
ABK conceived the study plan; **HKM** participated in the designing experimental protocol and performed the statistical analysis**. VKS** and **NS** helped to draft the manuscript**.**
All authors read and approved the final manuscript.

Conflict of Interest
The authors declare that they have no conflict of interest.

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Table 1

Selective MGA Plates	Tests Performed	Observation				
		TA1537	**TA1535**	**TA98**	**TA100**	**TA102**
With Histidine & Biotin	Histidine & biotin dependency	O	O	O	O	O
With Histidine, Biotin & Ampicillin	Presence of plasmid pKM101	X	X	O	O	O
With Histidine, Biotin, Ampicillin & Tetracycline	Presence of plasmid pKM101 & pAQ1	X	X	X	X	O
With Histidine, Biotin, Tetracycline	Presence of plasmid pAQ1	X	X	X	X	O
rfa Mutation	Crystal Violet (0.1%)	I	I	I	I	I

X = No Growth Observed, O = Growth Observed, I = Zone of Inhibition.

Table 2

Dose groups	Dose Levels (µg/plate)	Without Metabolic Activation System				With Metabolic Activation System			
		TA98		**TA100**		**TA98**		**TA100**	
		Mean ± SD	**RFV**	**Mean ± SD**	**RFV**	**Mean ± SD**	**RFV**	**Mean ± SD**	**RFV**
VC	0.00	19.03 ±1.53	1	185.00 ± 9.62	1	22.64 ± 3.11	1	148.18 ± 7.71	1
S1	5000.00	18.57 ±2.79	0.97	233.05 ± 4.43	1.25	22.13 ± 1.54	0.97	162.09 ± 10.10	1.09

Table 2 (Continued)

S2	2500.00	18.45 ±0.15	0.96	221.61 ± 7.00	1.19	17.65 ± 1.50	0.77	231.70 ± 5.41	1.56
S3	1250.00	23.63 ±3.77	1.24	222.65 ± 8.56	1.2	22.61 ± 4.00	0.99	173.06 ± 9.08	1.16
S4	625.00	22.43 ±0.68	1.08	194.31 ± 9.65	1.05	23.20 ± 5.37	1.02	168.61 ± 8.01	1.13
S5	312.50	19.53 ±0.70	1.02	200.00 ± 3.75	1.08	19.23 ± 3.16	0.84	173.34 ± 3.12	1.16
S6	156.25	21.67 ±2.22	1.13	216.90 ± 2.00	1.17	22.65 ± 5.64	1	154.57 ± 5.26	1.04
S7	78.13	21.77 ±4.41	1.14	254.63 ± 6.00	1.37	17.30 ± 2.50	0.64	173.38 ± 3.27	1.17
2 NF	25.00	1330 ± 8.68	69.8	-	-	-	-	-	-
SA	20.00	-	-	3644 ± 9.16	19.69	-	-	-	-
2 AA	20.00	-	-	-	-	1267.69 ±10.00	55.9 9	984.75 ± 6.28	6.64

RFV = Relative fold values as compared to VC, VC=Vehicle control, S1 to S7 = *Picrorhiza kurroa* rhizome extract. 2 NF= 2 Nitrofluorine, S.A= Sodium Azide, 2 AA= 2 Amino Anthracene.

Table 3

Dose Group	**Dose Levels (µg/plate)**	**With Metabolic Activation**		**Without Metabolic Activation**	
		Precipitation after Treatment	**Precipitation during Enumeration of colonies**	**Precipitation after Treatment**	**Precipitation during Enumeration of Colonies**
VC	0.00	No Ppt.	No Ppt.	No Ppt.	No Ppt.
S1	5000.00	No Ppt.	No Ppt.	No Ppt.	No Ppt.
S2	2500.00	No Ppt.	No Ppt.	No Ppt.	No Ppt.
S3	1250.00	No Ppt.	No Ppt.	No Ppt.	No Ppt.
S4	625.00	No Ppt.	No Ppt.	No Ppt.	No Ppt.
S5	312.50	No Ppt.	No Ppt.	No Ppt.	No Ppt.
S6	156.25	No Ppt.	No Ppt.	No Ppt.	No Ppt.
S7	78.13	No Ppt.	No Ppt.	No Ppt.	No Ppt.

VC=Vehicle Control, S1 to S7 = Test Item formulations in DMSO, No Ppt = No Precipitation observed

Table 4

Dose Group	**Dose Levels (µg/plate)**	**Background Bacterial Lawn Observation**			
		TA98		**TA100**	
		With S9 Mix	**Without S9 Mix**	**With S9 Mix**	**Without S9 Mix**
VC	0.00	Normal Lawn	Normal Lawn	Normal Lawn	Normal Lawn
S1	5000.00	Normal Lawn	Normal Lawn	Normal Lawn	Normal Lawn
S2	2500.00	Normal Lawn	Normal Lawn	Normal Lawn	Normal Lawn
S3	1250.00	Normal Lawn	Normal Lawn	Normal Lawn	Normal Lawn
S4	625.00	Normal Lawn	Normal Lawn	Normal Lawn	Normal Lawn
S5	312.50	Normal Lawn	Normal Lawn	Normal Lawn	Normal Lawn
S6	156.25	Normal Lawn	Normal Lawn	Normal Lawn	Normal Lawn
S7	78.13	Normal Lawn	Normal Lawn	Normal Lawn	Normal Lawn

VC=Vehicle control, S1 to S7 = Test Item formulations in DMSO

Table 5a

Dose Group	Dose Levels (µg/plate)	TA1537		TA1535		TA102	
		Mean ± SD	RFV	Mean ± SD	RFV	Mean ± SD	RFV
VC	0.00	3.60 ± 1.00	1.00	5.77 ± 3.12	1.00	216.62 ±10.04	1.00
S1	5000.00	3.30 ±1.50	0.91	11.38 ± 2.02	1.97	242.23 ± 6.12	1.11
S2	2500.00	5.86 ±1.00	1.62	8.60 ± 2.64	1.49	241.00 ± 5.80	1.11
S3	1250.00	4.61 ±1.46	1.28	10.02 ± 3.81	1.73	243.17 ± 2.54	1.12
S4	625.00	3.35 ±1.38	0.93	10.00 ± 1.08	1.73	252.64 ± 1.03	1.16
S5	312.50	3.43 ±0.50	0.95	10.44 ± 2.68	1.80	237.28 ± 7.16	1.09
SA	20.00	-	-	2750 ± 9.38	476.60	-	-
2NF	25.00	-	-	-	-	-	-
9AA	50.00	2238 ± 10.15	621.64	-	-	-	-
MMC	0.25	-	-	-	-	2352 ± 6.65	10.85

RFV = Relative fold values as compared to VC, VC=Vehicle control, S1 to S5 = *Picrorhiza kurroa* rhizome extract. 2 NF= 2 Nitro fluorine, S.A= Sodium Azide, 2 AA= 2 Amino Anthracene, 9AA = 9 Aminoacridine, MMC = Mitomycin C

Table 5b

Dose Group	**Dose Levels (µg/plate)**	**TA1537**		**TA1535**		**TA102**	
		Mean ± SD	**RFV**	**Mean ± SD**	**RFV**	**Mean ± SD**	**RFV**
VC	0.00	3.36 ± 0.70	1	10.36 ±4.56	1.00	240.07 ± 6.26	1.00
S1	5000.00	4.60 ± 1.05	1.36	7.02 ± 2.28	0.67	239.10 ± 2.04	0.99
S2	2500.00	1.83 ± 1.13	0.54	9.89 ±1.50	0.95	221.13 ± 1.21	0.92
S3	1250.00	3.88 ± 1.70	1.15	8.67 ±4.23	0.83	215.66 ± 2.01	0.89
S4	625.00	3.46 ± 0.54	1.02	8.65 ±1.59	0.83	211.60 ± 1.00	0.88
S5	312.50	3.12 ± 1.68	0.92	9.00 ±2.00	0.86	238.68 ± 3.29	0.99
2AA	20.00	670 ± 7.70	199.4	346.77 ± 2.90	33.47	1130 ± 8.33	4.66

RFV = Relative fold values as compared to VC, VC=Vehicle control, S1 to S5 = *Picrorhiza kurroa* rhizome extract. 2 NF= 2 Nitrofluorine, 2 AA= 2 Amino Anthracene

Table 6

Tester Strain	**Experimental Phase**	**Dilution factor of Bacterial Culture**	**Number of CFUs Observed/plate**		**Mean**	**Approximate number of viable cells per ml of culture**
			1	2		
TA1537	Mutagenicity Assay	10^{-6}	21	19	20	2.0×10^{9}
TA1535		10^{-6}	8	12	10	1.0×10^{9}
TA 98		10^{-6}	12	8	10	1.0×10^{9}
TA 100		10^{-6}	8	11	9.5	0.95×10^{9}
TA102		10^{-6}	8	12	10	1.0×10^{9}

Number of viable cells /ml = Number of colonies x dilution factor x10

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