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Antimutagenic activity of root extract of *Picrorhiza kurroa* using Ames test in both dose dependant cytotoxic assay and mutagenicity study

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ABSTRACT

The study was performed to evaluate mutagenic induction potential of *Picrorhiza Kurroa* root extract. The extract was tested in the *Salmonella typhimurium* reverse mutation assay with five histidine-requiring strains of *Salmonella typhimurium* (TA1537, TA1535, TA98, TA100 and TA102). The test was performed in two independent experiments (viz., Dose Selection Study/Cytotoxicity Study and Mutagenicity Study) in the presence (10% S9 v/v) and absence of metabolic activation system in triplicates. In the dose range finding study, the 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 v/v) and absence of metabolic activation system. The results of solubility, precipitation of test item and cytotoxicity study/dose range finding study, root extract of *Picrorhiza kurroa* was tested in the mutagenicity assay at the following dose levels. Mutagenicity Study with metabolic activation: 312.5, 625, 1250, 2500 and 5000 µg/ml. The root extract of *Picrorhiza kurroa* did not induce a significant dose-related increase in the number of revertant (his+) colonies in each of the five tester strains of *Salmonella typhimurium* (TA1537, TA1535, TA98, TA100 and TA102) in the presence (10% S9 v/v) and absence of metabolic activation system. Based on the results of this study it is concluded that root extract of *Picrorhiza kurroa* is not mutagenic in the *Salmonella typhimurium* reverse mutation assay (Ames test).Thus the study confirms the Antimutagenic effect of root extract of *Picrorhiza kurroa*.

Keywords: Antimutagenic, cytotoxic, Ames test, liposomal encapsulated, *Picrorhiza kurroa*.

1. Introduction

Picrorhiza kurroa is been used as traditional medicine across the world to treat many ailments like liver disorders, viral fevers, arthritis and also as immune modulator [1-2]. Many works has been published on its efficacy but very few concentrated on its safety and toxicology studies. In order find its mutagenic effects in the present study was done on the root extracts of *Picrorhiza kurroa*. The bacterial reverse mutation assay detects point mutations, both frameshifts and/or base pair substitutions. Strains of *Salmonella typhimurium* used in this assay are histidine auxotrophs by virtue of conditionally lethal mutations in the histidine operon [3, 4, 5]. When these histidine (his-) dependent cells are exposed to the test item 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. 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 frameshift mutations [6] 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. The bacterial reverse mutation assay has been shown to be a sensitive, rapid and accurate indicator of mutagenic activity of many materials including a wide range of chemical classes [7].

2. Materials

2.1 Vehicle Controls

Dimethyl sulphoxide (DMSO) purchased from RFCL Limited (BatchNo.R263K09) Solubility test for *Picrorhiza kurroa* root extract was performed and DMSO was found to be suitable

vehicle for the same. As per the solubility dimethyl sulphoxide was used as vehicle control.

2.2 Positive Controls

Following positive controls were used for with and without metabolic activation system.

Without Metabolic Activation Sodium Azide dissolved in distilled water with concentration 20 µg/plate on tester strains TA100 and TA1535, 2-Nitrofluorene in DMSO with concentration 25 µg/plate on tester strain TA98, 9-Aminoacridine dissolved in distilled water with concentration 50 µg/plate on tester strain TA1537 and Mitomycin C dissolved in distilled water with concentration 0.25 µg/plate on tester strain TA102 were prepared.

With Metabolic Activation 2-Aminoanthracene dissolved in DMSO at concentration 20 µg/plate on all tester strains were prepared. S9 mix [8, 9] was checked for sterility by plating 0.5 ml on NA plate.

2.3 Tester Strains

As per the recommendations of OECD and ICH (S2A and S2B) guidelines, *Salmonella typhimurium* TA1537 and TA1535 TA98, TA100 and TA102 were collected from Molecular Toxicology, Inc.157, Industrial Park, Dr. Boone, NC 28607. Specific genotypes of these strains are shown in table 1.

Table 1: Tester Stran Genotypes

Tester Strains	His Mutation	Additional Mutations		Plasmid
		Repair	LPS	-
TA98	*his*D3052	*uvr*B	*rfa*	pKM101
TA100	*his*G46	*uvr*B	*rfa*	pKM101
TA1535	*his*G46	*uvr*B	*rfa*	-
TA1537	*his*C3076	*uvr*B	*rfa*	-
TA102	*his*G428	-	*rfa*	pKM101 & pAQ1

In addition to a mutation in the histidine operon, the tester strains contain additional mutations that enhance their sensitivity to some mutagenic compounds. Mutation of the UvrB gene results in a deficient DNA excision repair system that greatly enhances the sensitivity of these strains to some mutagens. Since UvrB deletion extends through the biotin, *Salmonella typhimurium* tester strains containing the deletion also require biotin for growth.

Salmonella typhimurium tester strains also contain the rfa wall mutation, which results in the loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide (LPS) barrier that forms the surface of the bacterial cell wall. Resulting cell wall deficiency increases permeability to certain classes of chemicals such as those containing large ring systems. (i.e. benzo(a)pyrene) otherwise excluded by a normal intact cell wall.

Tester strains TA98, TA100 and TA102 also contain the pKM101 plasmid, which further increases the sensitivity of these strains to some mutagens. The suggested mechanism by which this plasmid increases sensitivity to mutagens is by modification of an existing bacterial DNA repair polymerase complex involved with the mismatch- repair process.

3. Methods

3.1 Preparation of Overnight Cultures

The bacterial cultures were inoculated from cryo vials of respective tester strains by scraping a small inoculum to the flask containing nutrient broth. Inoculated flasks were placed in a shaker/incubator (shaking 120±10 rpm; incubation, 37±2 ^{o}C) and kept for 12 to16 hrs. To ensure cultures are harvested in late log phase, cell density of inoculation (bacterial culture at the end of incubation) were determined by spectrophotometric monitoring. In addition to this bacterial cell viability was determined by colony forming units (CFUs) counts for each of the tester strains during the conduct of main mutagenicity study.

Each of the tester strains were checked for its genotypic characteristics viz., rfa wall mutation, presence of pKM101 and pAQ1 plasmid and characteristic spontaneous revertants range on the day of use in the mutagenicity assay.

3.2 Tester Strain Media and Reagents

Following media and reagents were used in the assay.

3.3 Culturing Broth

Oxoid Nutrient Broth # 2 (2.5% w/v) was used to grow overnight cultures of the tester strains.

3.4 Minimal Glucose Agar Plates

Vogel-Bonner minimal medium E supplemented with 1.5% w/v bacto agar and 0.5% (w/v) glucose was used as bottom agar/minimal glucose agar (MGA).

3.5 Top Agar for Selection of Revertants

Top (overlay) agar was prepared with 0.7% (w/v) bacto agar and 0.5% NaCl and supplemented with 10 ml of 0.5 mM histidine/biotin solution per 100 ml top agar for selection of histidine revertants. When S9 is required, 2.0 ml of supplemented top agar was used for the overlay. However when S9 is not required, 0.5 ml of 0.2 mM phosphate buffer was added to supplemented top agar (0.5 ml of phosphate buffer per 2 ml of supplemented top agar), and 2.5 ml of the diluted supplemented top agar was used for the overlay.

3.6 Metabolic Activation System

3.6.1 S9 Homogenate

As bacteria lack enzymes for metabolism, enzymes were added externally to mimic mammalian metabolism. For metabolic activation system, Aroclor 1254 induced male Sprague-Dawley rat liver post mitochondrial fraction was used for the entire assay. It was procured from Molecular Toxicology, Inc.157, Industrial Park, Dr. Boone, NC 28607

3.6.2 S9 Mix

S9 mix (Cofactors and liver homogenate) was prepared immediately prior to use in any experimental procedure. The post-mitochondrial fraction (liver homogenate) was usually used at concentrations at 10% v/v in the S9-mix.

3.6.3 Cofactor Mix

Cofactor mix was used for the preparation of S9 mix required for metabolic activation. The composition for the cofactor mix used is as follows.

Glucose-6-phosphate	:	0.16g
ß- NADP	:	0.35 g
$MgCl_2.6H_2O$	:	0.18 g
KCl	:	0.27 g
Na_2HPO_4	:	1.28 g
$NaH_2PO_4.H_2O$	:	0.28 g
Sterile water	:	100 ml

3.7 Preparation of root extract of *Picrorhiza kurroa*:

The roots of *Picrorhiza kurroa* were pulverised and extracted using non polar solvents and concentrated without solvent residues. The extract is then encapsulated using liposomal encapsulation by lecithin and used as test item. Root extract of *Picrorhiza kurroa* was dissolved in DMSO and the quantity of test preparation was done as shown in table 2.

Table 2: *Picrorhiza kurroa* root extract preparation for Mutagenicity test

Dose Selection Study		Main Mutagenicity			
		Without Metabolic Activation		With Metabolic Activation	
Dose Levels (µg/plate)	Con., of Test Item in DMSO (µg/ml)	Dose Levels (µg/plate)	Con., of Test Item in DMSO (µg/ml)	Dose Levels (µg/plate)	Con., of Test Item in DMSO (µg/ml)
5000.00	50000	5000.00	50000	5000.00	50000
2500.00	25000	2500.00	25000	2500.00	25000
1250.00	12500	1250.00	12500	1250.00	12500
625.00	6250	625.00	6250	625.00	6250
312.50	3125	312.50	3125	312.50	3125
156.25	1562.50	-	-	-	-
78.13	781.25	-	-	-	-

3.8 Dose Range finding Study

In the dose range finding study, root extract of *Picrorhiza kurroa* 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 v/v) 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. The growth inhibitory effect (cytotoxicity) of the test item on tester strain TA100 and TA98 is generally representative of that observed on other *Salmonella typhimurium* tester strains. Because of TA100's comparatively high number of spontaneous revertants per plate, gradations of cytotoxicity can be readily discerned from routine experimental variation. Cytotoxicity induced by a test item in the presence of microsomal enzymes may vary greatly from that observed in the absence of microsomal enzymes. Therefore, this would require that different test article dose ranges be tested in the mutagenicity assay based on the presence or absence of microsomal enzymes.

3.9 Evaluation of the Dose Range finding Study

Cytotoxicity was detected as a decrease in number of revertant colonies per plate and thinning or disappearance of the background lawn.

4. Precipitation Test

Precipitation test was carried out in all the phases of the study (i.e., Cytotoxicity test, Main Mutagenicity test). During precipitation check the bacterial cultures were treated with various test concentrations of root extract of *Picrorhiza kurroa* 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 the extract on MGA plates. Similarly the precipitation if any was also observed at the end of incubation of MGA plates during lawn observation.

5. 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% v/v S9 mix) and absence of S9 mix. Tester strains were exposed to the test item, root extract via the plate incorporation methodology at dose levels of 312.5, 625, 1250, 2500 and 5000 µg/ml with metabolic activation and without metabolic activation system.

6. Plating Procedures

The *Salmonella typhimurium* tester strains TA1537, TA1535, TA 98, TA 100 and TA102 were labeled as A, B, C, D and E with dose groups 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. Treatment groups denoted as "T" and concentrations subscribed serially from higher to lower concentrations. The replicate numbers were recorded as Rx (R1, R2 and R3). Negative control vehicle control and positive control groups were labeled as "VC" and "PC" respectively.

At the time of treatment each tube received the following constituents in order.

6.1 Without Metabolic Activation (-S9)

2 ml of top agar with trace quantity of histidine and biotin,
100 µl of bacterial culture
100 µl of test item formulation
500 µl of phosphate buffer

6.2 With Metabolic Activation (+S9)

2 ml of top agar with trace quantity of histidine and biotin
100 µl of bacterial culture
100 µl of test item formulation
500 µl of S9 Mix

The contents were mixed in cyclomixer and poured immediately onto MGA plate and allowed to solidify at room temperature. All the treated plates were incubated for 48 to 72 hours at 37±2 ^{0}C in Biological Oxygen Demand (BOD) incubator in inverted position.

At the end of incubation, the conditions 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.

7. Results

The root extract of picrorhiza kurroa was tested in the tester strains TA98 and TA100 with concentrations of 78.13, 156.25, 312.5, 625, 1250, 2500 and 5000 µg/plate in the absence and presence of S9 mix. The results are shown in Summary Table 1. To determine the toxicity of root extract of picrorhiza kurroa 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 results are shown in Table 3.

The tester strains TA98 and TA100 treated with root extract at the concentration range of 78.13–5000 µg/plate in the absence and presence of metabolic activation revealed a 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. In the dose range finding test, no increase in the number of revertants was observed upon treatment of root extract under all conditions tested.

Table 3: Mean Revertant Frequency: Dose Selection Study/Cytotoxicity Assay

Dose groups	Dose Levels (µg/plate)	Without Metabolic Activation System				With Metabolic Activation System			
		TA98		TA100		TA98		TA100	
		Mean ± SD	#	Mean ± SD	#	Mean ± SD	#	Mean ± SD	#
VC	0.00	21.00 ± 1.73	NA	187.00 ± 21.66	NA	24.67 ± 3.21	NA	150.33 ± 47.71	NA
T1	5000.00	20.67 ± 3.79	0.98	235.00 ± 34.77	1.26	24.33 ± 1.53	0.98	164.00 ± 10.15	1.09
T2	2500.00	20.33 ± 1.15	0.97	223.67 ± 47.08	1.20	19.67 ± 1.53	0.80	234.00 ± 25.51	1.56
T3	1250.00	25.33 ± 7.77	1.21	224.67 ± 58.56	1.20	24.67 ± 4.04	1.00	176.00 ± 19.08	1.17
T4	625.00	24.33 ± 0.58	1.16	196.33 ± 49.65	1.05	25.00 ± 5.57	1.01	171.67 ± 8.08	1.14
T5	312.50	21.33 ± 0.58	1.02	202.00 ± 73.75	1.08	21.33 ± 3.06	0.86	175.67 ± 53.69	1.17
T6	156.25	23.67 ± 2.52	1.13	218.00 ± 22.00	1.17	23.67 ± 5.69	0.96	159.67 ± 5.86	1.06
T7	78.13	25.67 ± 4.51	1.22	256.67 ± 36.00	1.37	19.33 ± 2.52	0.78	176.33 ± 3.21	1.17
2 NF	25	1328 ± 81.68	64.26	-	-	-	-	-	-
SA	20	-	-	3648 ± 79.16	15.52	-	-	-	-
2 AA	20	-	-	-	-	1268.67±70.78	52.14	981.67 ± 46.26	17.96

Note: # = Relative fold values as compared to VC, NA=Not applicable, VC=Vehicle control, SD= Standard deviation, T1 to T7 = Test item root extract formulation in DMSO. 2 NF= 2 Nitrofluorine, S.A= Sodium Azide, 2 AA= 2 Aminoanthracene.

7.1 Main mutagenicity assay

Based on the results of the dose range finding study the root extract of *Picrorhiza kurroa* was tested from concentration range of 312.5 to 5000 µg/plate in the absence and presence of metabolic activation system. The main mutagenicity assay was performed with the strains TA1537, TA1535, and TA102 in the presence (10% v/v S9 fraction) and absence of metabolic activation. The results are shown in Table 4.Based on the data on number of revertant colonies observed during dose range finding study was considered for main mutagenicity study.

There was no reduction of the bacterial background lawn and no biologically relevant decrease in the number of revertants at any of the concentration tested in all tester strains in the absence and presence of metabolic activation. In the main mutagenicity assay, no increase in the number of revertants was observed upon treatment with root extract of *Picrorhiza kurroa* under all conditions tested.

8. Discussion

The study was performed to evaluate mutagenic induction potential of *Picrorhiza Kurroa* root extract. The extract was tested in the *Salmonella typhimurium* reverse mutation assay with five histidine-requiring strains of *Salmonella typhimurium* (TA1537, TA1535, TA98, TA100 and TA102). The test was performed in two independent experiments (viz., Dose Selection Study/Cytotoxicity Study and Mutagenicity Study) in the presence (10 % S9 v/v) and absence of metabolic activation system in triplicates. In the dose range finding study, the 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 v/v) 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. Vehicle control and positive controls were also maintained concurrently along with treatment groups. Each concentration of test item including the controls was tested in triplicates. No toxicity in the form of background lawn inhibition and reduction in the number of revertant colonies was observed at a concentration range of 78.13 to 5000 µg/plate in the absence of metabolic activation and in the presence of metabolic activation (10% S9 v/v) respectively. At the start of treatment, no precipitation of test item, extract on the plates was observed at a concentration range of 78.13-5000 µg/plate.

Table 4: Mean Revertant Frequency: Main Mutagenicity Assay – Without Metabolic Activation System

Dose Levels (µg/plate)	TA1537		TA1535		TA102	
	Mean ± SD	#	Mean ± SD	#	Mean ± SD	#
(VC) 0.00	5.67 ± 1.15	NA	8.00 ± 3.00	NA	219.67 ± 21.94	NA
(T1) 5000	5.00 ± 1.00	0.88	14.33 ± 2.52	1.79	243.33 ± 36.12	1.11
(T2) 2500	7.00 ± 1.00	1.40	10.00 ± 2.65	1.25	243.67 ± 35.84	1.11
(T3) 1250	5.67 ± 1.53	1	11.00 ± 3.61	1.38	247.67 ± 2.552	1.13
(T4) 625	5.00 ± 1.00	0.88	11.67 ± 2.08	1.46	255.33 ± 21.03	1.16
(T5) 312.5	5.33 ± 0.58	0.94	12.00 ± 2.65	1.50	239.33 ± 17.16	1.09
(SA) 20	-	-	2747 ± 32.45	343.38	-	-
(2NF) 25.00	-	-	-	-	-	-
(9AA) 50.00	2434 ± 75.45	429.53	-	-	-	-
(MMC) 0.25	-	-	-	-	2245.33 ± 16.65	421

Note: # = Relative fold values as compared to VC, NA=Not applicable, VC=Vehicle control, SD= Standard deviation, T1 to T5 = Test Item root extract of *Picrorhiza kurroa* in DMSO, SA = Sodium Azide, 2NF = 2 Nitrofluorene, 9AA = 9 Aminoacridine, MMC = Mitomycin C.

Table: 4 (continued...) Mean Revertant Frequency: Main Mutagenicity Assay – With Metabolic Activation System

Dose Levels (µg/plate)	TA1537		TA1535		TA102	
	Mean ± SD	#	Mean ± SD	#	Mean ± SD	#
(VC) 0.00	5.33 ± 0.58	NA	12.33 ± 4.16	NA	241.67 ± 16.26	1.00
(T1) 5000	6.00 ± 1.00	1.125	9.33 ± 2.31	0.76	241.00 ± 42.04	0.93
(T2) 2500	3.33 ± 1.53	0.63	11.00 ± 1.73	0.89	224.33 ± 10.21	0.87
(T3) 1250	6.00 ± 1.73	1.13	10.00 ± 4.00	0.81	809.33 ± 12.01	0.89
(T4) 625	5.33 ± 0.58	1.00	10.67 ± 1.53	0.86	214.00 ± 11.00	1.00
(T5) 312.5	5.00 ± 1.00	0.94	11.33 ± 2.08	0.92	241.67 ± 33.29	1.00
(2AA) 20.00	671 ± 57.71	125.81	348.33 ± 22.90	28.25	1136 ± 18.33	213

Note: # = Relative fold values as compared to VC, NA=Not Applicable, VC=Vehicle Control, SD= Standard Deviation, T1 to T5 = Test Item, *Picrorhiza kurroa* root extract in DMSO, 2AA= 2 Aminoanthracene.

Based on the results of solubility, precipitation of test item and cytotoxicity study/dose range finding study, root extract of *Picrorhiza kurroa* was tested in the mutagenicity assay at the following dose levels. Mutagenicity Study with metabolic activation: 312.5, 625, 1250, 2500 and 5000 µg/ml. The root extract of *Picrorhiza kurroa* did not induce a significant dose-related increase in the number of revertant (his+) colonies in each of the five tester strains of *Salmonella typhimurium* (TA1537, TA1535, TA98, TA100 and TA102) in the presence (10% S9 v/v) and absence of metabolic activation system. The spontaneous revertant frequency of the negative and vehicle control groups were within normal range. The positive controls used in the study exhibited significant increase in revertant frequency respective to their strains, indicating that sensitivity of the strains towards specific mutagens and ensured that the test conditions were adequate and that the metabolic activation system functioned properly.

Based on the results of this study it is concluded that root extract of *Picrorhiza Kurroa* is not mutagenic in the *Salmonella typhimurium* reverse mutation assay (Ames test).

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