

BACTERIAL REVERSE MUTATION TEST WITH NARDOSTACHYS JATAMANSI

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ABSTRACT

Objective: To perform Bacterial Reverse Mutation Test[1] with jatamansi (Nardostachysjatamansi plant rhizome powder) using *Salmonella typhimurium* tester strains TA 1535, TA 1537, TA 98, TA 100, and TA 102 both in the presence and absence of metabolic activation system for finding the mutagenicity as per the OECD guideline No. 471[2].

Method: Plate Incorporation Method.

Results: Substantial increase in revertant colony number was not observed in all the *Salmonella typhimurium* tester strains both in the presence and absence of metabolic activation system up to the dose 5.0 mg/plate, where negative control results are within the range and positive controls shown significant increase in the revertant colony number.

Conclusion: Jatamansi did not induced any gene mutation in the genome of *Salmonella typhimurium* tester strains, hence Jatamansi is non mutagenic and non cytotoxic in this test.

Keywords: Mutation, *Salmonella typhimurium*, Metabolic Activation, Jatamansi, Cytotoxicity, Mutagenicity.

INTRODUCTION

Point mutations are the cause of many human genetic diseases and there is substantial evidence that point mutations in oncogenes and tumor suppressor genes of somatic cells are involved in tumor formation in humans and experimental animals. The bacterial reverse mutation test is rapid, inexpensive and relatively easy to perform. Many of the test strains have several features that make them more sensitive for the detection of mutations, including responsive DNA sequences at the reversion sites, increased cell permeability to large molecules and elimination of DNA repair systems or enhancement of error-prone DNA repair processes. The specificity of the test strains[3] can provide some useful information on the types of mutations that are induced by genotoxic agents. The bacterial reverse mutation test uses amino-acid requiring strains of *Salmonella typhimurium* to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs. The principle of this bacterial reverse mutation test is that it detects mutations which revert mutations present in the test strains and restore the functional capability of the bacteria to synthesize an essential amino acid. The revertant bacteria are detected by their ability to grow in the absence of the amino acid required by the parent test strain. A very large data base of results for a wide variety of structures is available for bacterial reverse mutation tests and well-established methodologies have been developed for testing chemicals with different physico-chemical properties, including volatile compounds[4][5].

Spikenard (*Nardostachysjatamansi*), also called nard, nardin and muskroot is a flowering plant of the Valerian family that grows in the Himalayas of Nepal, China, and India. Spikenard rhizomes (underground stems) powder is commonly called as jatamansi. The rhizomes of the plant are used in the Ayurvedic system of medicine as a bitter tonic, stimulant, antispasmodic, and to treat hysteria, convulsions, and epilepsy. The root has been medically used to treat insomnia and mental disorders. The rhizome powder also helps in regularize digestion, respiratory tract in the body. Jatamansi is useful for urine-related problems and maintaining the circulatory system. The herb proves useful in hepatitis and treats enlargement of the liver. It is used as an adjunct in the treatment of sexual debility and impotence. Jatamansi relieves symptoms like vertigo and seizures in fever. Since it exerts a cleansing effect on the uterus, it is used in menstrual ailments like dysmenorrheal and inflammation of the uterus. The herb stops fermentation and gas in the stomach. Spikenard rhizomes can be crushed and distilled into an intensely aromatic amber-colored essential oil, used as a perfume, an incense,

a sedative, and an herbal medicine said to fight birth difficulties, and other minor ailments. The Spikenard was known in ancient times and was part of the ayurvedic herbal tradition of India. The plant has a rich history of medicinal use and has been valued for centuries in Ayurvedic (Indian) and Unani (ancient Greco-Arab) systems of medicine.

The rhizomes and roots of the plant have medicinal value[6][7][8] and, therefore, have been the focus of chemical studies[9]. They contain a variety of sesquiterpenes and coumarins. Other terpenoids include spirojatamol, nardostachysin, jatamols A and B and calarenol. Coumarins include jatamansin.

Toxicology data on jatamansi is not well documented.

The Bacterial Reverse Mutation Test was conducted to evaluate the mutagenic potential of natural compound[10]jatamansi in *salmonella typhimurium* test strains by following plate incorporation method both in the presence and absence of metabolic activation system.

MATERIALS AND METHODS

Chemicals and Media

Magnesium sulphate($MgSO_4 \cdot 7H_2O$), Citric acid monohydrate, Potassium phosphate dibasic (K_2HPO_4), Sodium ammonium phosphate, ($NaNH_4PO_4 \cdot 4H_2O$), Dextrose, L- Histidine, D- biotin, Sodium chloride, Sodium dihydrogen phosphate (NaH_2PO_4), Disodium hydrogen phosphate (Na_2HPO_4), D- Glucose -6-phosphate, β NADP, Magnesium chloride, Dimethyl sulphoxide, Potassium chloride, 9-aminoacridine (90-45-9), Sodium azide (26628-22-8), 2-nitrofluorene (607-57-8), Sodium azide (26628-22-8), Mitomycin-C (50-07-7), 2-aminoanthracene (613-13-8), Benzo (a) Pyrene (50-32-8) were obtained from Sigma. Ampicillin, Tetracycline, Nutrient agar from Himedia. Agar (BD falcon), Nutrient broth (oxoid), Crystal violet (merck), Rat liver S9(moltox).

Statistics: statplus 2009 software was used for statistical analysis.

Culture: The *Salmonella Typhimurium* bacterial strains TA 1535, TA 1537, TA 98, TA 100 and TA 102 were obtained from Molecular Toxicology, INC, Boone, NC 28607, USA.

The histidine dependent strains have several features that make them sensitive for the detection of mutations.

The tester strains are derived from *Salmonella typhimurium*[11,12,13] strain through mutations in the histidine locus. Additionally due to

the "deep rough" (*rfa*) mutation they possess a faulty lipopolysaccharide envelope, which enables substances to penetrate the cell wall more easily. A further mutation (deletion of the *uvrB* gene) causes an inactivation of the excision repair system. The latter alteration also includes a deletion in the biotin genes. In the strains TA 98, TA 100, and TA 102 the R-factor plasmid pKM101 carries *umuDC* analogous genes that are involved in error-prone repair and the ampicillin resistance marker. The strain TA 102 does not contain the *uvrB*-mutation. Additionally TA 102 contains the multicopy plasmid pAQ1, which carries the HisG428 mutation and a tetracycline resistance gene. TA 102 contains the ochre mutation in the His G gene. Refer **Table 1** for Mutations in tester strains [14][15].

The cultures were stored as frozen permanent stock in cryo vials with Oxoid nutrient broth no.2 and 9% DMSO at -80°C. Over night cultures were prepared by transferring a small quantity of frozen culture to a flask containing Oxoid nutrient broth no.2. Inoculated bacterial culture flask(s) were incubated in a shaking bath at 100 strokes for 14 hours at 37 ± 2°C. 1-2 x 10⁹ cfu (colony forming units)/ml of cells were used for the study. 100 µL (1-2 x 10⁸ cfu) of culture was used for each plate.

Genotyping

Genotyping of the *Salmonella typhimurium* strains was performed prior to study initiation to ensure the genetic integrity.

Salmonella typhimurium [16]

Histidine dependence: Added 0.1 ml of bacterial culture, to 2 ml of top agar and poured on MGA (Minimal Glucose Agar) plate supplemented with an excess biotin. Incubated the plates for 48 hours at 37 ± 2°C.

Biotin dependence: Added 0.1 ml of bacterial culture, to 2 ml of top agar and poured on MGA plate supplemented with excess histidine.

Histidine and biotin dependence: Added 0.1 ml of bacterial culture, to 2 ml of top agar supplemented with histidine and biotin, poured on MGA plate.

***rfa* mutation:** Added 0.1 ml of bacterial culture, to 2 ml of top agar and poured on Nutrient Agar plate. Placed a sterilized filter paper disc soaked in crystal violet solution (1 mg/ml) in the center of the plate.

***Uvr B* mutation:** Added 0.1 ml of bacterial culture, to 2 ml of top agar and poured on MGA plate supplemented with excess histidine and biotin. Expose to UV lamp for 4-8 seconds.

Presence of plasmid pKM101 (Ampicillin resistance): Added 0.1 ml of bacterial culture, to 2 ml of top agar and poured on the MGA plate containing Ampicillin (800 µg).

Presence of plasmid pAQ1 (Tetracycline resistance): Added 0.1 ml of bacterial culture, to 2 ml of top agar and poured on the MGA plate containing Tetracycline (67 µg).

All the plates are incubated for 48 hours at 37 ± 2°C in bacteriological incubator.

Metabolic Activation System [17]

The tester strains do not possess enzyme systems which are present in mammals to metabolize pro-mutagens to active electrophilic metabolites capable of reacting with DNA. Sometimes, these pro-mutagens interact with a mammalian enzyme system, and yield mutagenic metabolites. Hence it is necessary to add external metabolic activation system that is post mitochondrial fraction (S9) [18].

S9 fraction was obtained from Molecular Toxicology, INC, Boone, NC 28607, USA. Stored at -80°C. S9 fraction was mixed with cofactor solution (D-Glucose -6-phosphate: 0.80g, β-NADP: 1.75g, Magnesium chloride: 0.90g, Potassium chloride: 1.35g, Sodium phosphate, dibasic: 6.40g, Sodium phosphate, monobasic: 1.40g were dissolved in 450 ml distilled water and filter sterilized) 10% v/v in the S9 mix. 10% v/v S9 mix is used for the study. During the

test, the S9 mix will be prepared fresh and stored in ice. 500 µL S9 mix was used for plating of each plate.

Nardostachys jatamansi

Dried *Nardostachys jatamansi* rhizome was purchased from ayurvedic medicine supplier and was confirmed by Dr. Sailaja Vani MD (Ayurveda), Senior Medical Officer, ESIC (Employees State Insurance Corporation, Govt of A.P.) Model Hospital, Hyderabad, Andhra Pradesh, India. *Nardostachys jatamansi* rhizome was powdered and dissolved in DMSO (Dimethyl sulphoxide), filtered and used. DMSO is the suitable solvent for this assay and history of no adverse effect on tester strains and S9 fraction.

Solubility [19] and Precipitation Test

50 mg jatamansi was completely soluble in 1.0 ml of DMSO, from this (50 mg/ml) 100 µL was mixed with top agar and added to MGA plate, no precipitation was observed. Based upon these results 5 mg/plate was selected as highest dose.

Cytotoxicity Test

The toxicity of the jatamansi was evaluated by performing Cytotoxicity test with strain TA 100. Eight concentrations, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039 mg/plate were tested for toxicity in triplicate both in the absence and presence of metabolic activation system. The maximum concentration is 5 mg/plate. 100 µL of DMSO/plate used as negative control. Plate incorporation method was followed.

Plate incorporation method [20][21]: 100 µL Test solution at each dose level (treatment plates) or 100 µL of DMSO (negative control plates), 500 µL S9 mix (for test in the presence of metabolic activation) or 500 µL, 0.1 M Sodium phosphate buffer (for test in the absence of metabolic activation), 100 µL Bacteria suspension (1-2 x 10⁹ cfu/ml), 2000 µL top agar were added to each tube. The mixture was poured on minimal glucose agar (MGA) plates. After solidification the plates were incubated in inverted position for a period of 48 hours at 37 ± 2°C.

Number of revertant colonies per plate was counted by using colony counter and bacterial background lawn was observed with microscope low power objective. Reduction in the number of revertant colonies or diminution of the bacterial background lawn when compare with negative control is the constrain to determine Cytotoxicity.

Mutagenicity Test

Mutagenicity test was performed by following Plate incorporation method with 5 tester strains (TA 1537, TA 1535, TA 98, TA 100 and TA 102), at doses 5, 2.5, 1.25, 0.625, 0.312 mg/plate, in triplicates both in the absence and presence of metabolic activation system. Negative control plates are treated with 100 µL of DMSO/plate. Positive control plates for each strain both in the absence and presence of metabolic activation system are treated with known positive controls in triplicates. After treatment, plates were incubated in inverted position for a period of 48 hours at 37 ± 2°C. Colony counts were compared with negative control. Refer **Table 2** for Positive control details.

Criteria for determining a positive result is dose dependent increase or a reproducible increase at one or more concentrations, in the number of revertant colonies per plate in at least one strain in the presence or absence of metabolic activation system. Two fold (TA 98, TA 100, TA 102) or more than two fold (TA 1537, TA 1535) increase in revertant colonies at one or more concentrations corresponding to negative control will be considered as positive result. The results do not meet this criteria is considered as non-mutagenic in this test.

RESULTS

Genotyping: Tester strains are showing their genetic integrity and ready to use in the study. Refer **Table 3** for Genotyping result.

All the strains are showing histidine and biotin dependence (except TA 102) indicates the mutation in His gene and *uvrB* deletion. TA

102strain has shown biotin independence, no *uvrB* deletion. Zone of Inhibition indicates that strains are having leaky lipopolysaccharide envelope. Resistance to antibiotics (Ampicillin and Tetracycline) indicates that plasmid pKM101 and pAQ1 carrying by the strains TA 98, TA 100 and TA 102.

Cytotoxicity Test[23]

Reduction in the number of revertant colonies and diminution of the bacterial background lawn was not observed in all the test concentrations 0.039 to 5 mg/plate, both in the presence and absence of metabolic activation system. Refer **Table 4** for Cytotoxicity test result.

Cytotoxicity test results explains that up to 5 mg/plate jatamansi is non cytotoxic in this test. Based upon these results, 5mg/plate is decided as the highest dose for the mutagenicity test.

Mutagenicity Test

After incubation period, colonies per each plate was counted and mean and SD (standard deviation) calculated. Refer **Table 5** for Mutagenicity test result.

Substantial increase in revertant colony numbers was not observed in all tester strains up to the dose 5mg/plate, both in the presence and absence of metabolic activation when compare with negative control. Spontaneous revertant colony count in the negative control with in acceptable limits, as per historical data and data from strains supplier. Positive controls showed a distinct increase in induced revertant colonies in all the tester strains both in the presence as well as in the absence of metabolic activation system.

Statistical Analysis

The relationship between the dose concentrations and each individual strain was calculated using Karl Pearson's correlation coefficient and was tested using Student's t-test for their significance at $p < 0.05$. It was found that there is no significant increase or decrease in the colony count of each strain. A regression analysis was also performed using least squares method and found that the

regression coefficients were not differing significantly at $p < 0.05$ between the dose concentration and individual strain colony count values.

DISCUSSION

Mutations are the common cause of many human genetic diseases, Critical evidence that mutation plays a central role in cancer has come from molecular studies of oncogenes and tumor suppressor genes. Mutational alteration of proto-oncogenes lead to over expression of their growth-stimulating activity, whereas mutations that inactivate tumor suppressor genes (Hanahan and Weinberg, 2000). Bacterial reverse mutation test is widely used for the detection agents that cause such type of mutations leads to genetic disorders.

Jatamansi is widely using in ayurvedic and unani medicine because of its health benefits in several ways. A mutagenic property of such plant extract is evaluated in this assay. The uses of traditional herbal remedies as alternative medicine play a significant role in india, because of these plant products are effective and not harmful based on previous treatment data. This study results explains the genotoxicity of Jatamansi and will provide information and evidence for further research.

Biological and statistical results are considered for evaluation. This test results reveals that Jatamansi did not induced any of gene mutations in TA 1537, TA 1535, TA 98, TA 100 and TA 102 *salmonella typhimurium* tester strains at the test doses 5, 2.5, 1.25, 0.625, 0.312 mg/plate, both in the presence and absence of metabolic activation system.

CONCLUSION

Based on study results it is concluded that, Jatamansi is non mutagenic and non cytotoxic in *salmonella typhimurium* tester strains both in the presence and absence of metabolic activation system up to the dose 5mg/plate. Negative results in the bacterial reverse mutation test, suggesting that jatamansi is potentially safe to use them as medicinal plant supplement, even at high doses.

Table 1: It shows Mutations in tester strains

<i>Salmonella typhimurium</i>		
Strains	Genotype	Type of mutations indicated
TA 1537	His C 3076; rfa; uvrB	Frame shift mutations
TA 98	His D 3052; rfa; uvrB; R-factor	Frame shift mutations
TA 1535	His G 46; rfa; uvrB	Base-pair substitutions
TA 100	His G 46; rfa; uvrB; R-factor	Base-pair substitutions
TA102	His G 428; rfa; uvrB; R-factor	Transitions/Transversions

Table 2: It shows Positive control details

Strain*	Positive Control ($\mu\text{g}/\text{plate}$)	
	Absence of metabolic activation system (-S9)	Presence of metabolic activation[22] system (+S9)
TA 1537	9-aminoacridine (50)	2-aminoanthracene (10)
TA 1535	Sodium azide (5)	2-aminoanthracene (10)
TA 98	2-nitrofluorene (7.5)	Benzo (a) Pyrene (1.0)
TA 100	Sodium azide (5)	2-aminoanthracene (5)
TA 102	Mitomycin-C (0.5)	2-aminoanthracene (10)

**salmonella typhimurium*

Table 3: It shows Genotyping result

Test	TA 1537	TA 1535	TA 98	TA 100	TA 102
Histidine dependence	No Growth				
UvrB mutation	No Growth	No Growth	No Growth	No Growth	Growth
Histidine and Biotin dependence	Growth	Growth	Growth	Growth	Growth
Rfa mutation	Zone of Inhibition				
Ampicillin resistance	No Growth	No Growth	Growth	Growth	Growth
Tetracycline resistance	No Growth	No Growth	No Growth	No Growth	Growth

Table 4: It shows Cytotoxicity test result

Jatamansi concentration (mg/plate)	<i>Salmonella typhimurium</i>			
	+S9		-S9	
	Revertant colonies	Background lawn	Revertant Colonies	Background lawn
Negative control	154±6.5	NI	166±14.0	NI
0.0390	151±5.5	NI	145±16.5	NI
0.0781	157±14.2	NI	145±14.0	NI
0.1562	147±24.8	NI	152±20.0	NI
0.3125	154±14.5	NI	142±15.0	NI
0.625	171±7.0	NI	151±15.0	NI
1.25	141±9.0	NI	157±25.2	NI
2.5	142±11.0	NI	140±15.4	NI
5.0	145±16.0	NI	148±17.6	NI

+S9: In the presence of metabolic activation, -S9: In the absence of metabolic activation. Values indicate Mean + Standard Deviation.

Table 5: It shows Mutagenicity Test Result

DOSE* mg/ml	<i>In the absence of metabolic activation (-S9)</i>				
	TA 1537	TA 1535	TA 98	TA 100	TA 102
Negative control	6.0±1.0	13.0±2.0	25.0±1.0	149.3±16.9	315.7±18.6
0.3125	6.6±1.1	12.7±2.1	24.3±2.5	147.0±13.1	282.3±30.9
0.625	5.3±1.5	14.3±1.5	25.3±5.5	158.7±14.0	315.3±8.4
1.25	5.3±0.6	11.0±1.0	25±8.9	152.0±19.2	325.3±15.5
2.5	9.6±3.5	9.7±3.5	24.7±5.5	145.3±9.3	312.0±17.8
5.0	8.6±0.6	8.0±1.0	27.3±3.1	158.3±16.1	325.3±17.9
Positive control	155±32.6	274.7±26.8	395±41.6	951.3±71.0	1291.3±33.0
DOSE* mg/ml	<i>In the presence of metabolic activation (+S9)</i>				
	TA 1537	TA 1535	TA 98	TA 100	TA 102
Negative control	7.3±1.5	13.0±2.6	26.7±1.5	141.3±3.2	343.3±13.6
0.3125	7.7±3.1	10.0±2.0	26.0±2.0	149.0±4.6	341.7±21.5
0.625	6.7±2.1	10.7±2.1	25.3±2.5	144.3±7.2	345.3±22.3
1.25	6.3±2.1	12.0±2.0	22.7±10.7	153.0±20.3	374.7±8.7
2.5	7.0±1.7	11.3±3.1	23.0±6.2	137.0±10.0	340.0±18.0
5.0	7.7±2.1	11.7±3.0	27.0±1.7	136.7±33.1	323.3±32.1
Positive control	115±5.0	267.3±45.0	481.7±50.3	951.3±62.7	1224.0±174.1

Values indicate Mean + Standard Deviation. * Jatamansi.

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