Research Article

Genotoxicity Studies of Chandha Marudha Chendooram – A Siddha Medicine

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ABSTRACT

Chandha Marudha Chendooram (CMC) is a poly herbal Siddha formulation mainly indicated for arthritic patients has been screened for toxic effects according to the OECD guidelines. In bacterial reverse mutation assay, the bacteria (*S. typhimurium*) are exposed to the CMC, in the presence and absence of an exogenous metabolic activation system. These bacterial suspensions are then mixed with overlay agar and plated onto minimal medium. After incubation, the revertant colonies are counted and compared to the number of spontaneous revertants in the solvent/vehicle control plates. There was no biological significant increase in the number of revertant colonies observed in any strains, when compared with the concurrent negative control. Therefore, it is concluded that it is non-mutagenic in this bacterial reverse mutation test at the tested doses up to 5000 µg/plate in absence of metabolic activation and 1000 µg/plate in presence of metabolic activation under the conditions of testing employed.

Keywords: Genotoxicity, *S. Typhimurium*, Chandha Marudha Chendooram (CMC), Revertants.

INTRODUCTION

Chandha Marudha Chendooram (CMC) is a poly-herbal mineral formulation based on the Siddha system of traditional medicine, one of the Indian systems of medicine (ISM), and prescribed in southern parts of India as a remedy for all kinds of diseases. CMC is a composition of mercuric sulphide, mercurous chloride, mercuric chloride, elemental sulphur, red sulphide of mercury and egg white. Siddha practitioners prescribe CMC as a therapy for arthritis and fever. However, scientific evidence for the therapeutic efficacy of CMC in the treatment of arthritis is prospective but yet to be comprehensively concluded in regulatory perspectives. One of the challenges towards regulatory concerns is the fact that the complexity of the formulation does not facilitate investigations, especially *in vitro*. Besides the presence of heavy metals such as mercury, chloride and sulphur in the formulation of CMC, is basically toxic through the processing of the formulation based on the traditional practices utilizes their influences in treating arthritis. [1]

Considering the above facts *in vitro* challenges, there are only limited data available on the genotoxicity of this formulation and therefore it is deliberate to assess its toxicity, if any, using bacterial reverse mutation assay (*Salmonella typhimurium*) and profile its safety as an alternative medicine. These mutations acts as hot spots for mutagens that cause DNA damage via different mechanisms. This bacterial reverse mutation assay is used world-wide as an initial screening to determine the mutagenic potential OECD guidelines, 1997.[2] The test serves as a quick and convenient assay to estimate the toxicity potential of a compound since the assay on rodents are time consuming process and expensive. The principle of the test is very simple that it uses amino acid-dependent strains of *S.typhimurium*. In the absence of an external histidine source, the cells cannot grow to form colonies. Colony growth is resumed if a reversion of the mutation occurs, allowing the production of histidine to be resumed. Spontaneous reversions occur with each of the strains; mutagenic compounds can increase in the number of revertant colonies to the background level. A positive test indicates that the chemical compound is mutagenic.

The bacterial reverse mutation test uses amino acid requiring strains of *Salmonella typhimurium* (TA98, TA100, TA102, TA1535, TA1537) to detect point mutations, which involves substitution, deletion or addition of one or few DNA base pairs.[3,4] The revertant bacteria are detected by their ability to grow in the absence of the amino acid required by the parent tester strains.[5] Point mutations are the cause of many human genetic diseases. Hence it found to be an appropriate assay to evaluate the mutagenic potential of CMC through reverse mutation assay.

MATERIALS AND METHODS

Test System and Justification for the Selection

Histidine auxotrophic strains of *Salmonella typhimurium* viz.,

a) TA98 and TA1537 → Frame shift mutation

b) TA102, TA100 and TA1535 → Base substitution

This assay measures the ability of the CMC to induce reverse mutations at specific histidine loci in the above mentioned tester strains of *Salmonella typhimurium* which are known for their reliability and reproducibility in a short

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term mutagenicity assay and are also recommended by the OECD guideline.

Genotypic Characterization of Test System

Type of mutations in *Salmonella typhimurium* strains are summarized as follows:

Salmonella typhimurium		
Strains	**Genotype**	**Type of mutations**
TA1535	his G 46; rfa-, uvrB-	Base pair substitutions
TA1537	his C 3076; rfa-, uvrB-	Frame shift mutation
TA98	his D 3052; rfa-, uvrB-, pKM 101	Frame shift mutation
TA100	his G 46; rfa-, uvrB-, pKM 101	Base pair substitutions
TA102	his G 428; rfa-, pKM 101, pAQ1	Base pair substitutions

The genotype of the tester strains was confirmed on arrival for all the strains and before conduct of the study. Genotype of tester strains is confirmed according to procedure described in in-house standard operating procedure.

Outline of the Method

The principle of the Bacterial Reverse Mutation Test is; it detects mutations that revert the mutations already present in the test strains thereby restoring the functional capability of the bacteria to synthesize an essential amino acid. Such revertant bacteria are detected by their ability to grow in the absence of the amino acid (histidine) in the culture medium as required by the parent strain.

In this mutation assay, which is a plate incorporation mode of exposure, the bacterial suspensions are exposed to the CMC, negative and the positive controls in the presence and absence of an exogenous metabolic activation system. These bacterial suspensions are then mixed with overlay agar and plated immediately onto minimal medium viz., his^- for *Salmonella typhimurium*. After a suitable period of incubation, the revertant colonies are counted and compared with the number of spontaneous revertant colonies in the negative control plates.[6]

Microsomal Fraction (S9) Homogenate and Activation Mixture

Liver microsomal enzyme (S9) homogenate prepared in-house by inducing agent Aroclor was used in the study. During mutagenicity study, the efficiency of S9 homogenate was checked with 2-AA using strain TA100 in Trial I.

S9 mix was prepared immediately prior to use as follows:

Constituent	5%, v/v S9 mix (10 mL)	10%, v/v S9 mix (10 mL)
Co-factor mix	9.5 mL	9.0 mL
S9 fraction	0.5 mL	1.0 mL

During the experiment the S9 mix was kept in ice bath.

Preliminary Solubility Test for Selection of Vehicle and Precipitation Test

The solubility of the CMC was determined before performing the cytotoxicity test. The CMC was found insoluble in Milli-Q water (stock solution A) while it formed suspension at the maximum limit concentration of 50 mg/mL in DMSO (stock solution B). Therefore, DMSO was selected as the vehicle for treatment. A volume of 100 µL of stock solution B (50 mg/mL) was added to 2 ml of top agar, to assess the precipitation. Precipitation was observed on to the MGA plate at the maximum limit concentration of 5.0 mg/plate as observed by unaided eyes.

Preliminary Toxicity Test/Cytotoxicity test for Selection of Test Doses

Based on solubility and precipitation check, eight concentrations in absence and presence (5%, v/v S9 mix) of metabolic activation system were tested with duplicates each.[6]A preliminary toxicity test (cytotoxicity test) was conducted for the selection of test doses for the main study. The doses selected for the cytotoxicity test were negative control (0.0), T1 (39.0625), T2 (78.125), T3 (156.25), T4 (312.5), T5 (625), T6 (1250), T7 (2500) and T8 (5000 µg/plate). The tester strain, TA100 was inoculated from the master vial into a flask containing Oxoid nutrient broth no. 2 and the flask was incubated at 37±1ºC for 14 to 16 hrs at 120 rpm.

A CMC stock solution of 50000µg/mL was prepared by mixing 100 mg of CMC in DMSO and further diluted serially to get the required test concentrations of 25000 to 390.625 µg/mL in DMSO.

Mutagenicity Assay (Trial I and Trial II)

Based on cytotoxicity test results, the mutagenicity test was conducted as two independent experiments both in the absence and presence of metabolic activation system (5 and 10%, v/v S9 mix in Trial I and II, respectively).[7]

Test Doses

Based on the observations of the preliminary toxicity test, the test doses was selected for the initial mutation assay (Trial I) and confirmatory mutation assay (Trial II).

Controls

Negative Control

A volume of 100 µL DMSO was used as the negative control.

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Positive Controls

Following strain specific positive controls were used in each of the tester strains in the solvents shown below and delivered in 100 µL.

Strain	Activation	Positive controls	Solvent	Dose(µg/plate)
TA98	+	2-Aminoanthracene	DMSO	2.5
	-	2-Nitrofluorene		7.5
TA100	+	2-Aminoanthracene	DMSO	2.5
	-	Sodium azide	Milli-Q water	5.0
TA1535	+	2-Aminoanthracene	DMSO	5.0
	-	Sodium azide	Milli-Q water	0.5
TA1537	+	2-Aminoanthracene	DMSO	5.0
	-	9-aminoacridine		75.0
TA102	+	2-Aminoanthracene	DMSO	5.0
	-	Mitomycin C	Milli-Q water	0.5

+: With S9 mix; -: Without S9 mix

Statistical Analysis

The data was analyzed for differences among negative control, treatment and positive control groups using one-way ANOVA statistical test. Differences between individual treatment/dose level and negative control was tested by Dunnett's comparison at a 5% level ($p<0.05$) of significance. Statistical analysis was done for both trial I and trial II and the results showed that there were no statistically significant increases in the number of revertant colonies in any of the CMC treated strains under any of the conditions tested.

RESULTS AND DISCUSSION

Solubility and Precipitation Test

The solubility of the CMC was determined before performing the cytotoxicity test. The CMC was insoluble in Milli-Q water (stock solution A) while it formed suspension at the maximum limit concentration of 50 mg/mL in DMSO i.e., stock solution B. Therefore, DMSO was selected as the vehicle for treatment. A volume of 100 µL of stock solution B (50 mg/mL) was added to 2 mL of top agar, to assess the precipitation. Precipitation was observed on to the MGA plate at the maximum limit concentration of 5.0 mg/plate as observed by unaided eye.

Control Groups

The positive controls (reference substances) exhibited a significant increase in number of revertant colonies both in the presence and in the absence of metabolic activation when compared with the respective negative controls both in the Trial I and II.

Preliminary Toxicity/Cytotoxicity test

Cytotoxicity to the tester strain TA100 was tested at concentrations of 39.0625, 78.125, 156.25, 312.5, 625, 1250, 2500 and 5000 µg/plate both in the presence (5%, v/v S9 mix) and absence of the metabolic activation system.

Complete inhibition of lawn pattern with 100% reduction in revertant colonies were observed up to the lowest concentration, i.e.39.0625 µg/plate in the absence of metabolic activation system. Complete inhibition of lawn pattern with 100% reduction in revertant colonies were observed up to tested concentration, i.e.312.5 µg/plate in the presence of metabolic activation system. No inhibition of lawn pattern with 26.44% reduction in revertant colonies were observed at tested concentration, i.e.156.25 µg/plate while normal lawn pattern with no significant reduction in number of revertant colonies was observed at remaining tested concentrations i.e. 78.125 & 39.0625 µg/plate in the presence (5%, v/v S9 mix) of the metabolic activation system, when compared with concurrent negative control.

Based on these observations, highest dose of 50 µg/plate in the absence and 200 µg/plate in presence of metabolic activation system were selected in the mutagenicity tests (Trial I and II) for all the tester strains.

Viable Counts of Tester Strains in the Overnight Culture

Viable counts of all the tester strains were within the required range of 1-2x10^9 CFU/mL for the mutation assays.

Mutagenicity Assay

Trial I

In the absence of metabolic activation:

Tester strains TA1537, TA1535, TA98, and TA100: Complete inhibition of lawn pattern with 100% reduction in revertant colonies was observed at the highest two concentrations, i.e. 50 and 25 µg/plate. Partial inhibition with reduction (45% - 52%) in revertant colonies was observed at tested concentration, i.e.12.5 µg/plate. Partial inhibition with reduction (21% - 31%) in revertant colonies was observed at tested concentration, i.e.6.25 µg/plate. Normal growth with no reduction of revertant colonies was observed at the remaining tested concentrations 3.125 and 1.5625 µg/plate.

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Tester strain TA102: Normal growth with no reduction of revertant colonies was observed up to the highest tested concentration i.e. 50 µg/plate.

In the presence of metabolic activation:

Tester strains TA1537, TA1535, TA98, and TA100: Partial inhibition with reduction (46%-53%) in revertant colonies was observed at highest concentration, i.e. 200 µg/plate. Normal growth with no reduction of revertant colonies was observed from the tested concentrations of 100 to 6.25 µg/plate.

Tester strain TA102: Normal growth with no reduction of revertant colonies was observed up to the highest tested concentration i.e. 200 µg/plate.

Trial II

In the absence of metabolic activation:

Tester strains TA1537, TA1535, TA98, and TA100: Complete inhibition of lawn pattern with 100% reduction in revertant colonies was observed at the highest two concentrations, i.e. 50 and 20 µg/plate. Partial inhibition with reduction (32% - 40%) in revertant colonies was observed at tested concentration, i.e. 8.0µg/plate. Normal growth with no reduction of revertant colonies was observed at the remaining tested concentrations 3.2, 1.28 and 0.512µg/plate.

Tester strain TA102: Normal growth with no reduction of revertant colonies was observed up to the highest tested concentration i.e. 50 µg/plate.

In the presence of metabolic activation:

Tester strains TA1537, TA1535, TA98, and TA100: Partial inhibition with reduction (44%-53%) in revertant colonies was observed at highest concentration, i.e.200 µg/plate. Normal growth with no reduction of revertant colonies was observed from the tested concentrations of 80 to 2.048 µg/plate.

Tester strain TA102: Normal growth with no reduction of revertant colonies was observed up to the highest tested concentration i.e. 200 µg/plate. The results revealed that no biological relevant increase of revertant colonies was observed in all five strains with (5%, v/v S9 mix) and without S9 mix at any of the tested dose levels when compared to concurrent negative control. Positive control chemicals tested simultaneously produced more than a 3-fold increase in the mean numbers of revertant colonies for all the strains when compared to the respective negative control plates. Also there was no inhibition of background bacterial lawn in any of the negative control and positive controls plates.

The positive control showed a significant increase in the number of revertant colonies when compared to negative control values in both the trials for absence and presence of metabolic activation system.

DISCUSSION

The *Salmonella typhimurium* tester strains were found to be reliable and responsive to the different genotypic characterization tests like the amino acid requirement, *rfa* mutation, *uvrB* mutation and the R-factor plasmids.The test item, CMC did not produce either a 2-fold increase in the mean numbers of revertant colonies in the strains TA98, TA100 and TA102, or a 3-fold increase in the mean numbers of revertant colonies in the strains TA1535 and TA1537 either in the presence or absence of metabolic activation system when compared to the respective negative control plates.

Table 1: Summary Results of Bacterial Reverse Mutation Assay (Trial I)

Mean Count of His+ Revertant Colonies (Absence of Metabolic Activation System)															
Conc. Of Test item (µg/plate)	TA1537			TA1535			TA98			TA100			TA102		
	Mean	SD	F	Mean	SD	F	Mean	SD	F	Mean	SD	F	Mean	SD	F
NC (0.0)	6.50	3.54	-	15.50	2.12	-	20.00	1.41	-	120.00	5.66	-	286.50	12.02	-
T1 (1.5625)	7.00	4.24	1.08	16.00	4.24	1.03	17.50	0.71	0.88	129.00	2.83	1.08	287.50	4.95	1.00
T2 (3.125)	6.00	1.41	0.92	16.50	2.12	1.06	21.00	1.41	1.05	125.50	4.95	1.05	275.00	19.80	0.96
T3 (6.25)	4.50	0.71	0.69	11.00	1.41	0.71	14.00	2.83	0.70	95.00	8.49	0.79	284.00	12.73	0.99
T4 (12.5)	3.50	3.54	0.54	7.50	2.12	0.48	11.00	1.41	0.55	63.00	15.56	0.53	288.00	19.80	1.01
T5 (25)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	290.00	25.46	1.01
T6 (50)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	279.00	19.80	0.97
PC	147.00	15.56	22.62	180.50	2.12	11.65	261.50	9.19	13.08	770.00	115.97	6.42	930.50	72.83	3.25

Key: Test item: Chanda marudha chendooram, NC: Negative Control, PC: Positive Control {TA1537: 9-Aminoacridine (75 µg/plate); TA1535: Sodium Azide (0.5 µg/plate); TA98: 2-Nitrofluorene (7.5 µg/plate), TA100: Sodium Azide (5 µg/plate); TA102: Mitomycin C (0.5 µg/plate)}, F: Fold change (increase or decrease in the number of revertant colonies), * = S9 Efficiency check by using 2-Aminoanthracene (2AA) (2.5 µg/plate for the strains TA100), +/-: Significantly higher (+) / lower (-) than the control group

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Table 1: **(Contd...)**

Mean Count of His+ Revertant Colonies															
Trial I: Presence of Metabolic Activation System (5%, v/v S9 mix)															
Conc. Of Test item (µg/plate)	TA1537			TA1535			TA98			TA100			TA102		
	Mean	SD	F	Mean	SD	F	Mean	SD	F	Mean	SD	F	Mean	SD	F
NC (0.0)	8.50	3.54	-	16.00	4.24	-	18.50	2.12	-	127.00	2.83	-	290.00	19.80	-
T1 (6.25)	8.00	2.83	0.94	16.00	2.83	1.00	19.50	0.71	1.05	127.50	3.54	1.00	279.50	16.26	0.96
T2 (12.5)	9.00	5.66	1.06	18.00	1.41	1.13	14.00	1.41	0.76	123.00	7.07	0.97	295.50	21.92	1.02
T3 (25)	10.50	3.54	1.24	14.50	0.71	0.91	17.00	4.24	0.92	125.50	0.71	0.99	280.00	24.04	0.97
T4 (50)	8.50	0.71	1.00	17.00	1.41	1.06	20.00	1.41	1.08	127.50	3.54	1.00	296.50	16.26	1.02
T5 (100)	9.50	0.71	1.12	16.00	1.41	1.00	17.50	3.54	0.95	127.00	5.66	1.00	272.50	12.02	0.94
T6 (200)	4.50	2.12	0.53	7.50	4.95	0.47	10.00	1.41	0.54	69.00	18.38	0.54	288.00	19.80	0.99
PC	122.50	34.65	14.41	231.00	26.87	14.44	275.50	16.26	14.89	861.00	94.75	6.78	911.50	47.38	3.14

Key: Test item: Chanda marudha chendooram, NC: Negative Control, PC: Positive Control {2AA: 2-Aminoanthracene (2.5 µg/plate for the strains TA100 and TA98; 5.0 µg/plate for the strains TA1537, TA1535 and TA102)}, F: Fold change (increase or decrease in the number of revertant colonies), +/-: Significantly higher (+) / lower (-) than the control group

Table 2: Summary Results of Bacterial Reverse Mutation Assay (Trial II)

Mean Count of His+ Revertant Colonies															
Trial II: Absence of Metabolic Activation System															
Conc. Of Test item (µg/plate)	TA1537			TA1535			TA98			TA100			TA102		
	Mean	SD	F	Mean	SD	F	Mean	SD	F	Mean	SD	F	Mean	SD	F
NC (0.0)	7.50	0.71	-	16.00	2.83	-	20.00	2.83	-	125.50	6.36	-	293.50	24.75	-
T1 (0.512)	7.50	4.95	1.00	15.00	2.83	0.94	19.50	0.71	0.98	122.00	8.49	0.97	307.00	7.07	1.05
T2 (1.28)	7.00	1.41	0.93	15.00	1.41	0.94	19.50	4.95	0.98	125.50	0.71	1.00	273.00	15.56	0.93
T3 (3.2)	8.00	2.83	1.07	15.50	4.95	0.97	18.00	4.24	0.90	127.00	7.07	1.01	294.50	36.06	1.00
T4 (8)	4.50	2.12	0.60	10.00	1.41	0.63	12.50	2.12	0.63	85.00	9.90	0.68	293.00	19.80	1.00
T5 (20)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	289.50	7.78	0.99
T6 (50)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	293.50	17.68	1.00
PC	149.00	16.97	19.87	207.50	17.68	12.97	275.00	9.90	13.75	792.00	45.25	6.31	901.00	7.07	3.07

Key: Test item: Chanda marudha chendooram, NC: Negative Control, PC: Positive Control {TA1537: 9-Aminoacridine (75 µg/plate); TA1535: Sodium Azide (0.5 µg/plate); TA98: 2-Nitrofluorene (7.5 µg/plate), TA100: Sodium Azide (5 µg/plate); TA102: Mitomycin C (0.5 µg/plate)}, F: Fold change (increase or decrease in the number of revertant colonies); +/- : Significantly higher (+) / lower (-) than the control group

Table 2: (Contd...)

Mean Count of His+ Revertant Colonies															
Trial II: Presence of Metabolic Activation System (10%, v/v S9 mix)															
Conc. of Test item (µg/plate)	TA1537			TA1535			TA98			TA100			TA102		
	Mean	SD	F	Mean	SD	F	Mean	SD	F	Mean	SD	F	Mean	SD	F
NC (0.0)	7.00	2.83	-	17.00	4.24	-	20.50	0.71	-	126.50	2.12	-	295.00	16.97	-
T1 (2.048)	6.50	2.12	0.93	16.50	0.71	0.97	22.00	5.66	1.07	129.00	2.83	1.02	305.50	13.44	1.04
T2 (5.12)	7.00	4.24	1.00	15.50	3.54	0.91	19.50	4.95	0.95	123.50	3.54	0.98	302.00	31.11	1.02
T3 (12.8)	6.50	2.12	0.93	16.00	1.41	0.94	19.00	1.41	0.93	128.00	7.07	1.01	287.50	20.51	0.97
T4 (32)	7.50	4.95	1.07	17.50	4.95	1.03	19.50	3.54	0.95	124.50	6.36	0.98	299.50	12.02	1.02
T5 (80)	7.00	1.41	1.00	17.00	2.83	1.00	21.00	4.24	1.02	126.00	2.83	1.00	282.00	22.63	0.96
T6 (200)	3.50	2.12	0.50	8.00	2.83	0.47	11.50	0.71	0.56	68.00	8.49	0.54	296.00	29.70	1.00
PC	151.00	11.31	21.57	265.00	12.73	15.59	317.00	15.56	15.46	843.00	35.36	6.66	974.00	8.49	3.30

Key: Test item: Chanda marudha chendooram, NC: Negative Control; PC: Positive Control {2AA: 2-Aminoanthracene (2.5 µg/plate for the strains TA100 and TA98; 5.0 µg/plate for the strains TA1537, TA1535 and TA102)}, F: Fold change (increase or decrease in the number of revertant colonies); +/-: Significantly higher (+) / lower (-) than the control group

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CONCLUSION

It was clear from the above evidences that the CMC is having the property of non-mutagenic effect. The results of this study showed that the mean number of histidine revertants in the treatment groups was comparable to the mean number of revertants in the control group in all the five *Salmonella typhimurium* tester strains *viz.,* TA98, TA100, TA102, 1A1535 and TA1537 both in the absence and presence of microsomal enzyme (S9 fraction). Based on the results of these studies, It is concluded that CMC is non-mutagenic in this bacterial reverse mutation test up to the concentration of 5000µg/plate both in the absence and presence of metabolic activation system to all the five Salmonella typhimurium strains., at the tested doses and under the conditions of testing employed.

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