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Research Paper

Genotoxic evaluation of Rasagenthi Mezhugu, a Siddha formulation, using *Salmonella typhimurium* strains

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Abstract

Rasagenthi Mezhugu, a Siddha formulation is proven for its medicinal properties in the treatment of various diseases. However, due to limited data on the genotoxicity of this formulation, it is aimed to assess its genotoxicity using bacterial reverse mutation assay in *Salmonella typhimurium* tester strains (TA97a, TA98, TA100, TA102 and TA1535) for the detection of gene mutation according to the guidelines of OECD 471(1997). All these strains carry a mutation in one of several genes, which governs the biosynthesis of histidine. In addition to the histidine mutation, these *Salmonella typhimurium* strains contain other mutation that greatly increases their ability to detect mutagens. The results of the 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.*, TA 97a, TA 98, TA 100, TA 102 and TA 1535 both in the absence and presence of mammalian microsomal enzyme (S9 fraction). Therefore, it is concluded that Rasagenthi Mezhugu is non-mutagenic up to the concentration of 5000 μg/plate both in the presence and absence of metabolic activation system to all the five *Salmonella typhimurium* tester strains and therefore could serve as a potential formulation for the treatment of various diseases, especially its therapeutic indication against cancer treatment.

Keywords: Rasagenthi Mezhugu, Genotoxic, *Salmonella typhimurium,* mutagenesis assay

Introduction

Rasagenthi Mezhugu (RGM) is a herbo-mineral formulation based on the Siddha system of traditional medicine, one of the Indian systems of medicine (ISM), and is prescribed in the southern parts of India as a remedy for all kinds of cancers including diseases closely resembling to HIV clinically[1]. Rasagenthi Mezhugu is a composition of 38 herbal plants and 8 metals including elemental states of Mercury and, Sulphur, Mercurous chloride, Arsenic trisulphide, Iron, Zinc, Copper suphate and Lead monoxide[2]. Siddha practitioners prescribe RGM as a therapy for different types of cancers. However, scientific evidence for the therapeutic efficacy of RGM in the treatment of cancers 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, lead, and arsenic, in the formulation of RGM, is basically toxic though the processing of the formulation based on the traditional practices utilizes their influences in killing the cancerous cells and at the same time nullifying their toxicities[3].

Considering the above facts of *in vitro* challenges and also the prudent processing methods using metals to have a therapeutic, and probably hormetic effect against cancer cells, there are only limited data available on the genotoxicity of this formulation and therefore it is intended to assess its genotoxicity, if any, using bacterial reverse mutation assay (*Salmonella typhimurium*) and profile its safety as an alternative medicine. These mutations act as hot spots for mutagens that cause DNA damage via different mechanisms. This bacterial reverse mutation assay is used world-wide as an initial screen to determine the mutagenic potential OECD Guidelines, 1997[4]. The test serves as a quick and convenient assay to estimate the carcinogenic potential of a compound since standard carcinogen assays on rodents are time-consuming (taking two to three years to complete) 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 cause an increase in the number of revertant colonies relative to the background level. A positive test indicates that the chemical is mutagenic and therefore may act as a carcinogen, since cancer is often linked to mutation.

The bacterial reverse mutation test uses amino acid-requiring strains of *Salmonella typhimurium* (*S. typhimurium*) to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs[5,6]. The revertant bacteria are detected by their ability to grow in the absence of the amino acid required by the parent tester strain. Point mutations are the cause of many human genetic diseases and there is substantial evidence that point mutations in oncogenes and tumour suppressor genes of somatic cells are involved in tumour formation in humans and experimental animals. Hence, it is found to be an appropriate assay to evaluate the mutagenic potential of RGM through Ames reverse mutation assay.

Materials and Methods

Mutagenicity Assay

Genotoxic studies are carried out by mutagenesis assay in *Salmonella typhimurium* tester strains (TA97a, TA98, TA100, TA102 and TA1535) for the detection of gene mutation according to the guidelines of OECD Guidelines (1997). These tester strains were obtained from Xenometrix, Discovery Partners International, San Diego, USA and stored in liquid nitrogen. The description of *Salmonella typhimurium* tester strains are described (Table 1).

All these strains carry a mutation in one of several genes, which governs the biosynthesis of histidine. In addition to the histidine mutation, these *Salmonella typhimurium* strains contain other mutation that greatly increases their ability to detect mutagens. When the *Salmonella* tester strains are grown on a minimal media agar plate containing a trace of histidine, only those bacteria that revert to histidine independence are able to form colonies. The number of spontaneously induced revertant colonies per plate is relatively constant. However, when a mutagen is added to the plate, the number of revertant colonies per plate is increased, usually in a dose-related manner.

Many carcinogenic chemicals become biologically active only after liver metabolism. Bacteria do not have metabolic systems compatible with vertebrates. Therefore, for detection of metabolism activated substances, the use of an exogenous metabolic activation system (rodent liver enzyme, S9) was prepared and used in the study. The prepared S9 enzyme was checked for 3 parameters, viz., sterility, total protein content and efficacy of S9.

Sterility was checked by inoculating 0.1 ml of the S9 on a minimal glucose agar plate containing histidine and biotin. The plates were incubated overnight at 37 ± 1ºC in an incubator. The preparation was found to be sterile. Protein was estimated and was found to be 4.8 g/dl. The prepared S9 was compared for its efficacy by plating with an indirect mutagen and it was found to induce a higher number of revertant colonies (> 10 fold) when compared with control. Table 2 gives the cell density of *Salmonella typhimurium* strains cultures used in the study.

Table 1: Description of *Salmonella typhimurium* tester Strains

Strain	Type of Mutation	Main DNA target @	His mutation locus (1)	LPS or rfa mutation (2)	Uver B (3)	R-factor plasmid pKM101 (4)	R-factor plasmid pAQ1 (5)	Full Genotype
TA 97a	Frame-shift substitution	GC	hisD 6610	Yes	Yes	Yes	No	rfa Dgal chl DbiouvrB (pKM101)
TA 98	Frame-shift substitution	GC	hisD 3052	Yes	Yes	Yes	No	rfa Dgal chl DbiouvrB (pKM101)
TA 100	Base pair substitution	GC	hisG 46	Yes	Yes	Yes	No	rfa Dgal chl DbiouvrB (pKM101)
TA 102	Frame-shift substitution	AT	hisG 428	Yes	No	Yes	Yes	hisD(g) 8476 rfa galE (pAQ1) (pKM101)
TA 1535	Base pair substitution	GC	hisG 46	Yes	Yes	No	No	rfa DgalE chl DbiouvrB

The key for the identification of genotype are as follows: rfa - Deep rough; D - Deletion of gene following this symbol; gal - UDP galactose 4-epimerase; chl D - nitrate reductase (resistance to chlorate); bio – biotin; uvrB - UV endonuclease component B; pAQ1 - a plasmid containing the hisG 428 gene; pKM101- a plasmid carrying uvrAB that enhances error prone repair; (1) - Histidine mutation incorporated; (2) - LPS mutation at the lac locus for deficiency in bacterial cell wall lipo polysaccharide (LPS) barrier; (3) - Ultra violet light damage repair gene B (uvr B) detection; (4)- R-factor plasmid pKM101- Extra nuclear genetic that enhances error prone repair and (5) R-factor plasmid pAQ1: It confirms tetracycline resistance gene.

Table 2: Cell density of *Salmonella typhimurium* strains – cultures

Strain	Cell Density / ml	
	Experiment 1	Experiment 2
TA 97a	1.2×10^9	1.2×10^9
TA 98	1.1×10^9	1.24×10^9
TA 100	1.2×10^9	1.1×10^9
TA 102	1.12×10^9	1.2×10^9
TA 1535	1.1×10^9	1.1×10^9

Solubility Test
The Rasagenthi Mezhugu (RGM) was checked in DMSO and was found to be of maximum solubility at 25 mg/ml.

Genotype Test
The genotype of the tester strains was confirmed as per the procedures described in the OECD test guideline, 471 (1997).

Ames Test
Minimum glucose agar (MGA) plates were prepared by pouring 20 to 25 ml of a medium containing 1.5% agar, 5.0% of 40% glucose solution, 2% Vogel Bonner medium and 1% of 0.5 mM histidine/biotin solution. The prepared MGA Plates were inverted and incubated overnight in an incubator at 37 ± 1ºC for sterility check. Top agar, containing 0.6% of agar and 0.5% of NaCl, was melted in a conical flask, dispensed as 2 ml portions into capped test tubes and autoclaved for 30 minutes at 121ºC. For the assay, sterilized top agar tubes were held at 45 ± 2 ºC in a water bath. The frozen permanents were stored in liquid Nitrogen and the master plates at 4ºC for each experiment, well isolated colony of the individual strains was inoculated into a 10 ml nutrient broth number 2 and incubated overnight at 37 ± 1ºC in a water bath shaker at not more than 120 rpm.

Preliminary cytotoxicity study
Rasagenthi Mezhugu was evaluated for preliminary cytotoxicity (Dose Range Finding) study at the dose levels viz., 39.1, 78.1, 156.3, 312.5, 625, 1250, 2500 and 5000 µg/plate and includes negative control too. Evaluation of cytotoxicity was done with and without S9 mix with 10% v/v S9 mix (**Table 3)**.

Table 3: Preliminary cytotoxicity and findings of dose ranges for the Strain: TA 100

Concentrations (µg/plate)	Revertant Colonies / Plate (Mean (n=3) ± SD)	
	Absence of S9 Mix	Presence of S9 Mix
NC (DMSO)	212 ±23	227 ± 7
39.06	226 ± 21	229 ± 11
78.12	223 ± 16	226 ± 20
156.25	227 ± 8	224 ± 24
312.5	233 ± 16	236 ± 19
625	125 ± 16	136 ± 10
1250	188 ± 15	201 ± 14
2500	182 ± 12	188 ± 17
5000	183 ± 8	175 ± 9

µg - microgram; NC- Negative control; n - No. of triplicates; NC - Negative Control; DMSO - DiMethyl Sulfoxide (NC)

Results and Discussion

The dose range finding study indicated that no evidence of cytotoxicity was observed up to 5000 µg/plate with respect to negative control, both with and without metabolic activation, therefore 5000 µg/plate was selected as the highest concentration for the main study**.** The lower concentrations selected were 2500, 1250, 625, and 313 µg/plate presence and absence of metabolic activation system.

Mutagenicity Study
Normal background lawn was observed in all the plates as that of negative control plates. All the five strains of *Salmonella typhimurium* exposed to Rasagenthi Mezhugu at dose levels of 5000, 2500, 1250, 625, and 313 µg/plate showed comparable number of revertant colonies with respect to negative control both in the presence and absence of metabolic activation system. The repeat experiment using 20% S9 mix also revealed more or less similar results (Table 4). All strains used in the study exhibited marked increase (>2 fold) in the number of revertants when treated with positive control agents (Table 4). These results, thus, confirmed the sensitivity of the tester strains to mutagens and thereby the validity of the assay.

Table 4: Mutagenicity study results with their mean plate count and positive control

Conc. (μg/plate)	Revertant Colonies / Plate (Mean (n=3) ± SD)									Conc. (μg / plate)	Revertant Colonies / Plate (Mean (n=3) ± SD)					
	TA97a			TA98			TA100				TA102			TA1535		
	-S9	+S9 (10%)	+S9 (20%)	-S9	+S9 (10%)	+S9 (20%)	-S9	+S9 (10%)	+S9 (20%)		-S9	+S9 (10%)	+S9 (20%)	-S9	+S9 (10%)	+S9 (20%)
NC (DMSO)	192 ± 9	187 ± 12	186 ± 6	39 ± 2	37 ± 4	41 ± 6	182 ± 7	187 ± 4	180 ± 4	NC (DMSO)	300 ± 5	303 ± 6	301 ±4	15 ± 4	18 ± 2	16 ± 4
312.5	182 ± 9	194 ± 6	183 ± 5	38 ± 3	35 ± 8	41 ± 4	181 ± 3	186 ± 5	185 ± 6	312.5	298 ± 12	290 ± 3	298 ± 8	12 ± 2	20 ± 2	18 ± 3
625	193 ± 7	193 ± 1	180 ± 2	37 ± 6	38 ± 2	44 ± 4	183 ± 3	186 ± 3	184 ± 3	625	293 ± 5	290 ± 1	301 ± 4	18 ± 3	19 ± 3	18 ± 2
1250	184 ±7	191 ± 5	182 ± 5	39 ± 3	34 ± 6	42 ± 4	182 ± 8	184 ± 6	185 ± 6	1250	294 ± 6	296 ± 8	300 ± 1	16 ± 2	20 ± 1	14 ± 4
2500	1191 ± 9	187 ± 4	187 ± 7	41 ± 2	38 ± 3	38 ± 2	193 ± 7	185 ± 7	182 ± 3	2500	293 ± 5	299 ± 3	302 ± 3	18 ± 3	19 ± 2	15 ± 4
5000	185 ± 12	180 ± 1	181 ± 4	37 ± 2	35 ± 1	44 ± 4	191 ± 9	194 ± 6	186 ± 5	5000	294 ± 6	300 ± 4	295 ± 5	18 ± 3	14 ± 4	14 ± 3
PC SA	NA	NA	NA	NA	NA	NA	2269 ± 72	NA	NA	PC SA	NA	NA	NA	1444 ± 413	NA	NA
PC 4NQN	1886 ± 43	NA	NA	807 ± 14	NA	NA	NA	NA	NA	PC 4NQN	NA	NA	NA	NA	NA	NA
PC MMC	NA	NA	NA	NA	NA	NA	NA	NA	NA	PC MMC	3355 ± 62	NA	NA	NA	NA	NA
PC 2AF	NA	1979±63	2193 ± 25	NA	1534± 59	1500 ± 20	NA	2703± 16	2726± 47	PC 2AF	NA	3133 ± 116	3170 ± 51	NA	647 ± 65	621 ± 45

μg - microgram; SD - Standard Deviation; NC - Negative control; PC - Positive Control; 4NQN - 4-Nitroquinoline-1-Oxide; SA- Sodium Azide; MMC - MitoMycin C; 2AF - 2-Aminofluorene; NA- Not Applicable; n - No. of triplicates; DW- Distilled Water (Sterile); Conc. – Concentration; .DMSO = Di Methyl Sulfoxide.

Ames Mutagenic Assay

Rasagenthi Mezhugu was evaluated in the *Salmonella* plate incorporation assay to determine its ability to induce reverse mutation at selected histidine loci in five strains of *Salmonella typhimurium* (in absence of metabolic activation system as well as in presence of 10% and 20% v/v exogenous metabolic activation system (Table 5).

Table 5: Results of Genotyping

Name of the Test	Bacterial Strains				
	TA 97a	**TA 98**	**TA 100**	**TA 102**	**TA 1535**
Histidine requirement	Growth	Growth	Growth	Growth	Growth
rfa Mutation	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive
uvr B Mutation	No growth	No growth	No growth	Growth	No growth
Ampicillin resistance	Resistant	Resistant	Resistant	Resistant	Sensitive
Tetracycline resistance	Sensitive	Sensitive	Sensitive	Resistant	Sensitive

The results of the Ames mutagenic assay indicated 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.*, TA 97a, TA 98, TA 100, TA 102 and TA 1535 both in the absence and presence of mammalian microsomal enzyme (S9 fraction).

The concomitant positive control groups induced 10 to 96 fold revertants without metabolic activation and 10 to 41 fold revertants with metabolic activation (10%) in assay 1 and 11 to 39 fold revertants with metabolic activation in assay 2 (20%), when compared to the respective solvent control group.
There are a few studies conducted using some of the ingredients of RGM through Ames mutation assay. They include *Myristica fragrans*, suggesting its potential in cancer prevention and treatment but remaining as anti-mutagenic[7,8]. Similarly, it is demonstrated[9] that lead as plumbagin was not a mutagen by using the *S. typhimurium* and in various *Escherichia coli* strains[10]. *Cuminum cyminum* extracts showed protective effect against several mutagens and proved to be devoid of any inherent mutagenic potential[11,12.] Further, the antimutagenic activity of aqueous extract and hydrolyzable tannins from *Terminalia chebula* in *Salmonella typhimurium* was also documented[13].

In line with the above, it is clear from the experimental evidences conducted on the Rasagenthi Mezhugu, with all its ingredients based on the traditional medicinal practices, it could be stated that it is also having the property of non-mutagenic effect.

Conclusion

It was clear from the above evidences that the Rasagenthi Mezhugu is having the property of non-mutagenic effect. The results of the 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.*, TA 97a, TA 98, TA 100, TA 102 and TA 1535 both in the absence and presence of mammalian microsomal enzyme (S9 fraction). It is concluded that Rasagenthi Mezhugu is non-mutagenic up to the concentration of 5000 μg/plate both in the presence and absence of metabolic activation system to all the five *Salmonella typhimurium* tester strains. Based on the results of these studies, it is concluded that Rasagenthi Mezhugu is non-mutagenic up to the concentration of 5000 μg/plate both in the presence and absence of mammalian microsomal enzyme (S9 fraction) to all the five *Salmonella typhimurium* strains used in the study.

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