MUTAGENICITY STUDY OF VIGRX CAPSULES AS PER OECD GUIDELINE NO. 471 - BY *SALMONELLA TYPHIMURIUM* REVERSE MUTATION ASSAY

STUDY NO: VLTO-100221

Study Completion Date: 15.06. 2010

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MUMBAI – 400 053
INDIA

STATEMENT OF COMPLIANCE

To the best of our knowledge and belief, this Study entitled "Mutagenicity study of VigRX capsules by *Salmonella typhimurium*, Reverse Mutation Assay" was performed under my supervision in compliance with the test guidelines laid down in OECD – 471". The objectives laid down in the study protocol were achieved.

No unforeseen circumstances were observed which might have affected the quality or integrity of the study.

Jayesh Chaudhary
MD, Vedic Lifesciences Pvt. Ltd.

Deepali Jadhav
Executive,Preclinical

CERTIFICATE

We certify that the work reported here is a true and authentic report of the study entitled, ‘‘Mutagenicity study of VigRX capsules by *Salmonella typhimurium*, Reverse Mutation Assay as per the OECD guidelines – 471”, based on the experiment conducted in one of the partnered Toxicology Laboratory Services of VEDIC LIFESCIENCES PVT LTD (B-203 Morya Landmark I, Off New Link Road, Andheri (W), Mumbai - 400 053,) India. The results presented here are faithful reflection of data collected during the study.

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QUALITY ASSURANCE STATEMENT

The study entitled “Mutagenicity study of VigRX capsules by *Salmonella typhimurium*, Reverse Mutation Assay” has been inspected in the spirit of OECD Guidelines 471

This study was inspected and findings reported to Management and to the Study Director.

Inspections were performed according to the Standard Operating Procedures of the Quality Assurance Unit. The report was audited against the approved study plan and pertinent raw data and accurately reflects the raw data.

STATEMENT OF CONFIDENTIALITY

This report which contains **CONFIDENTIAL** and **PROPRIETARY** information of **LEADING EDGE MARKETING.** Will not be disclosed to anyone except the employees of this company wherever necessary or to persons authorized by law or judicial judgment without the expressed or written approval of Sponsor.

DECLARATION

The Study Director hereby declares that the work was performed under his supervision and in accordance with the described procedures. It is assured that the reported results faithfully represent the raw data obtained during the experimental work. No circumstances have been left unreported which may have affected the quality or integrity of the data or which might have a potential bearing on the validity and reproducibility of this study.

The Study Director accepts overall responsibility for the technical conduct of the study as well as the interpretation, analysis, documentation and reporting of the results.

1. STUDY DETAILS

1.1 TITLE : Mutagenicity study of VigRX capsules by *Salmonella typhimurium* Reverse Mutation Assay (Ames test)

1.2 STUDY NUMBER : VLTO – 100221

1.3 TESTING FACILITY : VEDIC LIFESCIENCES PVT. LTD,
203, Morya Landmark-I,
Off Link Road, Andheri (w),
Mumbai – 400 053, India

1.4 SPONSOR'S CONTACT PERSON : DM CONTACT MANAGEMENT
100-645 Tyee Road
Victoria, Bc V9a6x5
Canada

1.5 STUDY SCHEDULE :

Study Initiation date : 26.04.2010

Metabolic activation –S9 preparation : 27.04.2010 to 05.05.2010

Solubility and precipitation test : 06.05.2010

Initial cytotoxicity test and colony counting : 08.05.2010 to 10.05.2010

Trial 1: test item exposure : 15.05.2010
(With or without S9 activation)

Trial 1 : Colony counting : 17.05.2010
(With or without S9 activation)

Trial 2 : Test item exposure : 22.05.2010
(With or without S9 activation)

Trial 2 : Colony counting : 24.05.2010
(with and without S9 activation)

2. MONITORING PERSONNEL

Sr. No.	Designation	Personnel	Signature with date
1.	Executive Preclinical	DEEPALI JADHAV VEDIC LIFESCIENCES PVT.LTD MUMBAI	
2.	Managing Director	JAYESH CHAUDHARY VEDIC LIFESCIENCES PVT.LTD MUMBAI	

3. SUMMARY

The test item VigRX capsules supplied by DM contact management, Canada was assessed for its mutagenic effects using *Salmonella typhimurium*. The study was conducted using *Salmonella typhimurium* strains: TA98, TA100, TA102, TA1535, and TA1537. The test item was tested at the concentration of 150, 375, 750, 1500 and 3000 µg/plate using dimethyl sulphoxide (DMSO) as solvent based on the initial cytotoxicity test. The study was conducted, with and without the metabolic activation (S9) prepared from Aroclor 1254 induced in rat liver. The control, solvent control and appropriate positive controls (2-nitrofluorene, sodium azide and 9-aminoacridine, mitomycin C for trials "without metabolic activator" and 2-aminoanthracene for trials "with metabolic activator") were tested simultaneously. Two trials were carried out for this study in triplicates. Data were statistically analyzed and expressed as mean ± SD.

From the experimental results obtained, the mean numbers of revertant colonies in the above mentioned concentrations were comparable to those of the control and solvent control, in both the trials, in the presence and absence of metabolic activation. There was no significant increase in number of revertant colonies at all concentrations tested. The number of revertant colonies in the positive controls has shown 3.0 to 21.9 fold increase under identical conditions.

Conclusion

From the results obtained, the test item VigRX capsules is found to be non-mutagenic at the highest concentration 3000 µg/plate in Bacterial Reverse Mutation Test in the tester strain *Salmonella typhimurium* strains: TA98, TA100, TA102, TA1535, and TA1537.

4. OBJECTIVE

To assess the potential of test item that can induce point gene mutations, viz., substitution, addition or deletion of one or a few DNA base pairs in the *Salmonella typhimurium* mutation. The experiment was performed by plate incorporation method.

5. MATERIALS AND METHODS

5.1 TEST ARTICLES

The following information was provided about the test article.

Test article	: VigRX capsules
Characteristics	: Transparent capsule with pale brown conent.
Batch No.	: NA
Date of Manufacture	: NA
Date of Expiry	: NA
Purity	: NA
Sponsor	: DM CONTACT MANAGEMENT 100-645 Tyee Road Victoria, Bc V9a6x5 Canada

5.2 TEST SYSTEM

Tester Bacteria	: *Salmonella typhimurium*
Tester Strain Used	: TA 1537, TA 98.
Base substitution	: TA 100, TA 102 and TA 1535.
Source	: Moleular Toxicology, Inc.,157 ,Industrial park, Dr. Biine, NC 28607,USA

5.3 Genetic Characterization of Tester Strains

Strains were maintained as master plates and periodically checked for viable counts and genetic characteristics as mentioned below:

a. Histidine requirement
b. rfa mutation
c. uvr B mutation
d. R-factor (pKM 101 plasmid)
e. Spontaneous reversion

5.4 Metabolic Activation (S9) Details

Preparation of S9 homogenate (Metabolic activator)

Liver microsomal enzymes were prepared from male Wistar rats induced with single exposure of Aroclor 1254 at a concentration of 500mg/kg body weight. After 5 days of Aroclor dosing, animals were fasted over night and sacrificed on 6^{th} day. Livers were excised under aseptic condition. The preparation of the microsomal enzyme fraction was carried out with sterile glassware and solutions under ice cold condition. The liver was suspended in 3 volumes of 0.15 M KCl (3 ml/g of liver) and minced with sterile scissors. The minced liver was homogenized in a tissue homogenizer and was centrifuged at 9000 x g for 10 minutes in a refrigerated centrifuge. The supernatant was decanted and aliquots transferred into cryovials which were frozen and stored at -70^{o}C.

Details of S9 homogenate

Date of preparation: 02.05.2010

Date of characterization: Sterility check: 02.05.2010; Activity check: 03.05.2010

Sterility check for S-9 fraction

Sterility was checked by streaking the supernatant fluid on nutrient agar plates and incubated at 37^{o}C for 24 hours.

Activity check for S-9 homogenate

Activity of the S-9 homogenate was determined using a pro-mutagen 2-aminoanthracene and Benzo(a)pyrene with the tester strain TA100.

5.5 Sterility Test of the Test item

Test item was checked for its microbial load by pour plate technique

5.6 Solubility Test of the Test item

The solubility test of test item was carried out with water and DMSO**.**

5.7 Test Item Preparation

Test item stock solutions preparation: 1.25 g of the test substance was suspended in DMSO and volume was made up to 25 ml corresponding to 50 mg/ml stock.

5.8 Precipitation Test

The test item was dissolved in DMSO and serially diluted to get concentrations of 1000, 2000, 3000, 4000 and 5000 µg/ml and precipitation test was carried out for the same.

5.9 Duration of Culture

Cultures from genotyped master plates of each strain were grown in oxoid nutrient broth No. 2 for 16-18 hours at 37°C ± 1°C in the shaking water bath set at approximately 68 shakes / minute and were assessed using viable count analysis.

5.10 Initial Cytotoxicity Test

Based on the results of the precipitation test, the following concentrations were selected for the initial cytotoxicity test (µg/plate):

a. 1000 b. 2000 c. 3000 d. 4000 e. 5000

Initial toxicity test was conducted using overnight TA100 tester strain, both in the presence and the absence of metabolic activator, in triplicate, along with concurrent solvent control (DMSO). The test item was judged as toxic based on decrease in the number of revertants/plate and/or the bacterial background lawn intensity.

6.0 PLATE INCORPORATION METHOD FOR THE MUTAGENICITY ASSAY

Test System Identification:

Based on the initial cytotoxicity test results, the following concentrations were selected for the main study (µg/plate):

a. 150 b. 375 c. 750 d. 1500 e. 3000

The plates were labeled with the type of study number, strain, metabolic activation (with or without), trial number and concentration of test item.

Plating:

Five concentrations of the test item were plated, with each of the following tester strains: TA98, TA100, TA102, TA1535 and TA1537 with and without metabolic activator. Two trials were conducted with three replicates per strain for each concentration of test item, both in the presence and absence of the metabolic activation.

7.0 OBSERVATIONS

Effect on bacterial background lawn:

The condition of the bacterial background lawn was evaluated for evidence of the test item toxicity using the code system.

Number of revertants:

Revertant colonies for a given strain within the test item dilution series were counted manually.

8.0 DATA ANALYSIS

Data was analyzed for differences among control, solvent control and positive control groups using ANOVA. Differences between the control, solvent control and treatment groups were tested by Dunnett's test at a 5% level ($p \leq 0.05$) of significance.

9.0 ARCHIVING

All test article, reference sample, study protocol, raw data and other documents generated during the course of this study together with a copy of final report will be stored in the archives of Vedic Lifesciences Pvt. Ltd., India for a period of one year from the date of submission of final report.

10.0. RESULTS AND DISCUSSION

10.1 Genetic Characterization of Tester Strains

All the tester strains fulfilled the quality check criteria.

10.2 Metabolic activation

Sterility check for S-9 fraction:

Sterility was checked by streaking the supernatant fluid on nutrient agar plates and incubated at 37^{o}C for 24 hours. It was found sterile.

Activity check for S-9 homogenate:

Activity of the S-9 homogenate was determined using a pro-mutagen 2-aminoanthracene and Benzo(a)pyrene with the tester strain TA100 and it was found to be active.

10.3 Sterility Test

The test item was found to be sterile at tested conditions.

10.4 Solubility Test

The test item was found to be suspended in DMSO at 50 mg/ml concentration.

Solvent	:	DMSO
Batch No	:	R237F09
Make	:	Rankem
Storage	:	Room temperature

10.5 Precipitation Test

It was found that the test item did not cause any precipitation at the concentration 5000 µg/plate in Minimal Glucose agar.

10.6 Initial Cytotoxicity Test

In the test, with and without S9 activation, at the tested concentrations of 1000, 2000, 3000, 4000 and 5000 µg/plate the mean number of revertant colonies and the bacterial background lawn were compared to that of the solvent control. The test item was found to be cytotoxic at and above 4000 µg/plate with decrease in number of revertant colonies and depletion in bacterial background lawn as compared to that of the solvent control. Refer Table 1, Appendix 1

10.7 Plate Incorporation Method for the Mutagenicity Assay

Trial I:

The concentrations tested at 150, 375, 750, 1500 and 3000 µg/plate showed very close resemblance to the solvent control DMSO in both with and without metabolic activation. There was no significant increase in number of revertant colonies and no change in bacterial background lawn as compared to that of the solvent control, among the tester strains. The specific positive controls tested simultaneously produced approximately 3.0 to 21.9 fold increase in mean number of revertants as compared to the solvent control. Refer Table 2, Appendix 2.

Trial II:

Similar results were observed in the second trial, the concentrations tested at 150, 375, 750, 1500 and 3000 µg/plate showed very close resemblance to the solvent control DMSO in both with and without metabolic activation. There was no significant increase in number of revertant colonies and no change in bacterial background lawn as compared to that of the solvent control, among the tester strains. The mean number of revertant colonies/plate and bacterial background lawn in the solvent control for the tested strains were comparable to that of solvent control. The specific positive controls tested simultaneously produced approximately 3.0 to 21.9 fold increase in the mean number of revertants as compared to the solvent control. Refer Table 3, Appendix 3.

Statistical analysis of the combined results from both trials indicated no significance in all the five concentrations tested which was compared to the respective solvent control in any of the five *Salmonella* strains.

11.0 INTERPRETATION

The test item VigRX capsules was assayed for Bacterial Reverse Mutation Test at the concentration of 150, 375, 750, 1500, 3000 µg/plate using *Salmonella typhimurium* strains; TA98, TA100, TA102, TA1535 and TA1537. In the two trials conducted, with metabolic activation (S9) and without metabolic activation, the number of revertant colonies in the five different test item concentrations did not increase significantly over the solvent control, while, the positive controls tested simultaneously showed a 3.0 to 21.9 fold increase in the number of revertant colonies/plate. This was observed for all the five tester strains.

12.0 CONCLUSION

From the results obtained, the test item VigRX capsules is found to be non-mutagenic at the highest concentration 3000µg/plate in Bacterial Reverse Mutation Test in the tester strain *Salmonella typhimurium* strains: TA98, TA100, TA102, TA1535, and TA1537.

TABLE – 1: SUMMARY OF INITIAL CYTOTOXICITY TEST

Treatment		Control		Vehicle Control		Test item (µg/plate)									
Test concentration (µg/plate)		100µl of autoclaved distilled water		100µl of DMSO		1000		2000		3000		4000		5000	
---		Mean ± SD	Lawn Intensity	Mean ± SD	Lawn Intensity	Mean ± SD	Lawn Intensity	Mean ± SD	Lawn Intensity	Mean ± SD	Lawn Intensity	Mean ± SD	Lawn Intensity	Mean ± SD	Lawn Intensity
No. of Revertants / Plate *Salmonella typhimurium* TA 100	With S-9	100.7 ± 0.6	4+	99.3 ± 0.6	4+	100.7 ± 0.6	4+	97.3 ± 2.1	4+	93.0 ± 1.0	4+	83.3 ± 2.5	3+	81.7 ± 1.2	3+
	Without S-9	100.0 ± 1.0	4+	100.7 ± 0.6	4+	99.7 ± 0.6	4+	95.3 ± 1.5	4+	93.7 ± 1.5	4+	84.7 ± 2.5	3+	83.3 ± 1.5	3+

Lawn intensity:

4+= Thick lawn: Distinguished by a healthy (Normal) background lawn comparable to solvent control plates.

3+= Slightly thin lawn: Distinguished by a noticeable lawn (Slightly thinning of the background lawn reduced) compared to solvent control plates.

TABLE – 2: SUMMARY OF COLONY COUNTS OF REVERTANTS OF TRIAL – I

Treatment	Test concentration (µg/plate)	No. of Revertants (Mean of 3 plates)									
		With S-9					Without S-9				
		TA 98	TA 100	TA 102	TA 1535	TA 1537	TA 98	TA 100	TA 102	TA 1535	TA 1537
Control	100µl of autoclaved distilled water	17.0 ± 1.0	99.0 ± 1.7	251.3 ±3.2	19.7 ± 0.6	7.3 ± 1.5	15.0 ±1.0	100.0 ±1.0	258.3 ±0.6	17.3 ±1.2	6.7 ±0.6
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Vehicle Control	100µl of DMSO	17.0 ± 2.0	100.7 ± 0.6	246.0 ±3.6	18.7 ± 2.1	8.0 ± 1.0	15.3 ±1.2	99.3 ±1.2	258.0 ±1.7	18.7 ±1.5	8.7 ±0.6
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Test Item	150	16.3 ± 1.5	99.0 ± 1.0	250.3 ± 0.6	17.0 ± 1.0	7.7 ± 0.6	14.3 ±0.6	101.0 ±1.0	256.3 ±2.1	16.7 ±0.6	8.7 ±0.6
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Fold increase	1.0	1.0	1.0	0.9	1.0	0.9	1.0	1.0	0.9	1.0
	375	18.7 ± 0.6	98.3 ± 1.2	248.0 ± 1.0	19.3 ± 0.6	7.7 ± 1.5	15.0 ±1.0	101.0 ±1.7	252.0 ±2.0	18.7 ±1.5	8.3 ±1.2
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Fold increase	1.1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	750	17.0 ± 1.0	99.7 ± 0.6	250.0 ± 1.0	17.0 ± 2.0	7.3 ± 1.2	15.7 ±0.6	101.3 ±0.6	254.7 ±1.5	16.3 ±0.6	8.3 ±0.6
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Fold increase	1.0	1.0	1.0	0.9	0.9	1.0	1.0	1.0	0.9	1.0
	1500	17.0 ± 2.0	98.3 ± 0.6	248.0 ± 2.0	18.0 ± 1.0	8.0 ± 1.7	15.0 ±1.0	100.3 ±0.6	257.7 ±2.1	17.7 ±0.6	8.7 ±0.6
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Fold increase	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.9	1.0
	3000	16.3 ± 1.2	99.0 ± 1.0	250.3 ± 1.2	17.7 ± 1.5	7.0 ± 1.0	15.3 ±0.6	99.7 ±1.2	256.7 ±1.5	19.0 ±1.0	8.3 ±1.2
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Fold increase	1.0	1.0	1.0	0.9	0.9	1.0	1.0	1.0	1.0	1.0
Positive Control	100µl	195.3 ± 1.5	315.7 ± 3.1	757.0 ± 3.6	186.3 ± 4.5	123.0 ±2.0	219.0 ±2.0	315.0 ±1.0	780.3 ±6.1	199.0 ±3.0	182.3 ±2.1
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Fold increase	11.5	3.1	3.1	10.0	15.4	14.3	3.2	3.0	10.7	21.0

Values of Revertants are in Mean±SD

TABLE – 3: SUMMARY OF COLONY COUNTS OF REVERTANTS OF TRIAL - II

Treatment	Test concentration (µg/plate)	No. of Revertants (Mean of 3 plates)									
		With S-9					Without S-9				
		TA 98	TA 100	TA 102	TA 1535	TA 1537	TA 98	TA 100	TA 102	TA 1535	TA 1537
Control	100µl of autoclaved distilled water	17.7 ±0.6	100.3 ±0.6	257.3 ±2.1	16.0 ±1.0	7.7 ±0.6	18.7 ±0.6	101.3 ±1.2	258.7 ±1.2	17.0 ±1.0	7.3 ±1.5
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Vehicle Control	100µl of DMSO	19.0 ±1.0	99.3 ±0.6	248.3 ±1.5	18.7 ±0.6	8.0 ±1.0	20.7 ±1.2	103.0 ±1.0	259.0 ±1.0	17.7 ±0.6	9.0 ±1.0
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Test Item	150	18.7 ±0.6	98.0 ±1.0	259.3 ±1.2	19.0 ±1.0	7.3 ±1.2	19.0 ±1.0	101.3 ±1.5	254.7 ±0.6	19.0 ±1.0	8.7 ±0.6
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Fold increase	1.0	1.0	1.0	1.0	0.9	0.9	1.0	1.0	1.1	1.0
	375	18.7 ±1.2	99.0 ±1.0	260.0 ±2.0	20.7 ±0.6	9.0 ±1.0	20.0 ±1.0	100.0 ±1.0	258.0 ±1.0	19.3 ±1.5	8.3 ±1.5
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Fold increase	1.0	1.0	1.0	1.1	1.1	1.0	1.0	1.0	1.1	0.9
	750	17.7 ±1.5	100.0 ±1.0	249.0 ±1.0	18.7 ±0.6	8.0 ±1.0	17.7 ±1.5	100.7 ±2.1	257.3 ±2.5	17.0 ±1.0	8.3 ±0.6
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Fold increase	0.9	1.0	1.0	1.0	1.0	0.9	1.0	1.0	1.0	0.9
	1500	17.7 ±0.6	97.7 ±1.5	249.7 ±3.5	17.0 ±1.0	7.7 ±1.5	18.7 ±1.2	99.3 ±0.6	254.3 ±2.1	17.0 ±1.0	8.7 ±0.6
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Fold increase	0.9	1.0	1.0	0.9	1.0	0.9	1.0	1.0	1.0	1.0
	3000	18.0 ±1.0	98.3 ±1.5	249.0 ±2.0	18.0 ±1.0	8.3 ±0.6	19.3 ±0.6	99.0 ±1.0	257.3 ±2.1	17.3 ±0.6	8.0 ±1.0
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Fold increase	0.9	1.0	1.0	1.0	1.0	0.9	1.0	1.0	1.0	0.9
Positive Control	100µl	198.3 ±2.5	328.0 ±2.6	767.3 ±9.7	198.3 ±6.1	121.0 ±2.0	222.7 ±2.5	322.7 ±4.0	768.7 ±5.1	203.0 ±4.4	197.0 ±2.6
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Fold increase	10.4	3.3	3.1	10.6	15.1	10.8	3.1	3.0	11.5	21.9

Values of Revertanats are in Mean±SD

APPENDIX – 1: INDIVIDUAL PLATE COLONY COUNTS OF INITIAL CYTOTOXICITY TEST

Treatment			Control			Vehicle Control			Test item (µg/plate)												
Test concentration (µg/plate)			100µl of autoclaved distilled water			100µl of DMSO			1000			2000			3000			4000			5000		
Plate No.			1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
No. of Revertants/Plate	*Salmonella typhimurium* TA 100	With S-9	101	100	101	99	99	100	101	100	101	99	98	95	93	92	94	81	83	86	81	83	81
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	3+	3+	3+	3+	3+	3+
		Without S-9	101	99	100	101	100	101	99	100	100	95	97	94	94	92	95	85	87	82	82	85	83
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	3+	3+	3+	3+	3+	3+

APPENDIX – 2: INDIVIDUAL PLATE COLONY COUNTS OF REVERTANTS OF TRIAL - I

Treatment	Test concentration (µg/plate)	Plate No.	No. of Revertants/Plate									
			With S-9					Without S-9				
			TA 98	TA 100	TA 102	TA 1535	TA 1537	TA 98	TA 100	TA 102	TA 1535	TA 1537
Control	100µl of autoclaved distilled water	1	17	98	255	19	7	15	99	259	18	6
		2	16	101	249	20	6	16	101	258	16	7
		3	18	98	250	20	9	14	100	258	18	7
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Vehicle Control	100µl of DMSO	1	15	101	245	18	9	16	100	257	20	8
		2	17	101	250	17	7	16	100	257	17	9
		3	19	100	243	21	8	14	98	260	19	9
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Test item	150	1	15	99	250	16	7	14	102	257	17	9
		2	16	98	251	17	8	14	100	254	16	9
		3	18	100	250	18	8	15	101	258	17	8
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	375	1	19	99	249	19	9	16	103	252	20	9
		2	18	97	247	20	6	14	100	250	19	9
		3	19	99	248	19	8	15	100	254	17	7
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	750	1	16	100	251	15	6	15	102	255	16	9
		2	18	99	249	17	8	16	101	256	17	8
		3	17	100	250	19	8	16	101	253	16	8
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	1500	1	19	98	246	19	9	15	100	260	18	9
		2	17	99	250	17	6	14	100	257	17	9
		3	15	98	248	18	9	16	101	256	18	8
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	3000	1	17	100	251	19	7	15	99	258	19	9
		2	15	98	251	16	8	15	101	255	18	7
		3	17	99	249	18	6	16	99	257	20	9
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Positive Control	100µl	1	195	315	760	191	125	217	315	787	199	180
		2	197	313	758	182	121	219	314	779	202	183
		3	194	319	753	186	123	221	316	775	196	184
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+

APPENDIX – 3: INDIVIDUAL PLATE COLONY COUNTS OF REVERTANTS OF TRIAL - II

Treatment	Test concentration (µg/plate)	Plate No.	No. of Revertants/Plate									
			With S-9					Without S-9				
			TA 98	TA 100	TA 102	TA 1535	TA 1537	TA 98	TA 100	TA 102	TA 1535	TA 1537
Control	100µl of autoclaved distilled water	1	17	101	255	16	7	18	102	258	16	6
		2	18	100	258	17	8	19	100	258	17	7
		3	18	100	259	15	8	19	102	260	18	9
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Vehicle Control	100µl of DMSO	1	20	99	248	19	9	20	104	260	18	10
		2	19	100	247	18	7	22	103	259	17	8
		3	18	99	250	19	8	20	102	258	18	9
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Test item	150	1	19	98	260	20	6	19	101	254	19	9
		2	18	97	258	19	8	18	100	255	20	8
		3	19	99	260	18	8	20	103	255	18	9
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	375	1	18	100	262	20	10	21	99	257	21	7
		2	20	98	258	21	9	19	100	258	19	8
		3	18	99	260	21	8	20	101	259	18	10
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	750	1	16	101	250	19	7	16	103	260	18	9
		2	19	99	248	18	9	19	100	257	16	8
		3	18	100	249	19	8	18	99	255	17	8
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	1500	1	17	98	250	17	6	20	99	256	18	9
		2	18	96	246	16	9	18	100	252	17	8
		3	18	99	253	18	8	18	99	255	16	9
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	3000	1	19	100	249	18	9	19	98	259	17	9
		2	18	98	247	19	8	19	99	258	18	7
		3	17	97	251	17	8	20	100	255	17	8
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Positive Control	100µl	1	196	330	765	205	119	220	319	770	208	195
		2	198	329	759	197	123	223	322	773	200	200
		3	201	325	778	193	121	225	327	763	201	196
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+

APPENDIX – 4: STERILITY STATUS OF THE STUDY

Sl.No.	Sterility Controls	Trial I	Trial II
1	Autoclaved Distilled Water	No microbial contamination	No microbial contamination
2	DMSO	No microbial contamination	No microbial contamination
3	S-9 Mix	No microbial contamination	No microbial contamination
4	Minimal Glucose Agar Plates	No microbial contamination	No microbial contamination
5	Strain Inoculation broth	No microbial contamination	No microbial contamination
6	PBS	No microbial contamination	No microbial contamination
7	Overlay Agar	No microbial contamination	No microbial contamination