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EVALUATION OF CERTAIN PESTICIDE MIXTURES FOR THEIR MUTAGENICITY USING AMES *Salmonella typhimurium* - REVERSE MUTATION ASSAY

B. Reddi Bhargavi¹, Ravi Kumar VELLANKI¹, Jagadeeswara Reddy Kanala^{2*}

¹Department of Biotechnology, Vignan University, Vadlamudi, Guntur-522213, AP, India

²Sugen Life Sciences Pvt.Ltd, Tirupati – 517502, AP, India

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ABSTRACT

In this study, the mutagenic effects of two different pesticide mixtures Deltamethrin 1% + Triazophos 35% EC (D+T) and Profenofos 40% EC + Cypermethrin 4% EC (P+C), used widely in agriculture, have been investigated by using bacterial reverse mutation test system namely, Ames/*Salmonella typhimurium* test. Five tester strains of *Salmonella typhimurium* (TA98, TA100, TA102, TA1535 and TA1537) were employed in current study. A range finding study was conducted to assess the cytotoxic effect of the pesticide mixtures by Pre-incubation method employing two tester strains TA98 and TA100. Seven test concentrations in duplicate viz., 78, 156, 313, 625, 1250, 2500 and 5000 µg/plate in the presence and absence of metabolic activation (10% S9 mix) was used in current study. Based on the cytotoxicity data, mutagenicity evaluation (Main Study by Pre-incubation method) was performed employing five tester strains (TA100, TA102, TA1535, TA98 and TA1537). Results of experiments were compared with positive and negative control groups' data. Compared to solvent control, no marked or consistent differences in the number of revertant colonies were observed in any concentration of D+T (EC), P+C (EC) in all the tester strains with and without exogenous mammalian S9 activation system. Based on study results it can be concluded that both D+T (EC) and P+C (EC) are non mutagenic and non-cytotoxic against *S. typhimurium* tester strains both in the presence and absence of metabolic activation system up to the dose 5000µg/plate.

* Corresponding author

E-mail: kjreddy32@gmail.com (Jagadeeswara Reddy Kanala)

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1 Introduction

Pesticides when used carefully and in appropriate dose can be useful in agriculture to increase crop yield but excess and random usage of these can damage human health and cause unintentional complications throughout the world (Akyil et al., 2017). Despite several advantages of using pesticides in agriculture, most of them pose a potential hazard to air, water and food that can lead to serious health problems for humans and ecosystems (Jamil et al., 2004). Adverse effects pesticides to humans can be determined different animal models, but it is always a difficult task to evaluate the impact of these complex chemicals on the ecosystem.

If the pesticides are applied in a mixture of two or three, it became more difficult to evaluate the adverse effect of these pesticides. A simple classification of complex mixtures does not warrant their effects on the various biological systems because a mixture of chemicals alters the toxicity of individual substances, this might happened due to the potential interactions between these chemicals. Further, active ingredients of pesticides can also exhibit different behaviors such as additives, synergistic, potentiating or antagonistic effects when separated or mixed (Llorente et al., 2012; Beyer et al., 2014).

There is a complicated nature of pesticides released into the environment, which makes the investigation very difficult to characterize their genotoxic and carcinogenic potential (Bull et al., 2006). Regulatory requirements for the use of pesticide mixtures are based on separate toxicological tests of the individual pesticides in the mixture and not on the mixture's combined toxic effects. Several research groups and international risk assessment bodies have addressed some general principles that demonstrate how substances with different toxicological properties can act either through a synergistic, additive or antagonistic mode of action. In practice, humans and animals are exposed to complex and variable chemical compound combinations (Rietjens & Alink, 2006).

In a multiple chemical exposure situation, the single chemicals may act independently as in a single exposure, or a number of chemicals may interact to modulate the effects of total multiple exposures. In general, approximately 95% of all chemical studies are conducted on individual chemicals (Carpenter et al., 2002). There are very few studies on the extent to which chemical mixtures have an impact, especially on the environment. Pesticides genetic toxicity is determined by several variables such as: (i) their biological accumulation or environmental degradation; (ii) their human metabolism; (iii) their reactivity to cellular components such as DNA, RNA, and proteins.

In a short-term test system, Jurs et al. (1983) published quantitative and qualitative research on the mutagenic potencies of certain

pesticides and other compounds. In order to compare the capabilities of chosen short-term assays to detect and confirm the genotoxic activity of pesticides, Waters et al. (1980) widely worked on the genotoxic activity shown by number of pesticides presenting various chemical structures in separate test systems. Three kinds of genetic DNA damage can be identified in the short-term bioassays: (i) gene or point mutations; (ii) primary DNA damage and repair; (iii) chromosomal changes (Ames, 1982).

The *Salmonella* mutagenicity test is the most commonly used short-term test for testing mutagens and environmental compounds (Ames et al., 1975). His operon contains various kinds of mutations in these *Salmonella* strains (Maron & Ames, 1983). The assay can determine if mutations were frame shifts or base pair replacements by its operon's reverse mutation. Such mutations lead in the formation of colonies on his "agar plates." However, the Ames test demonstrates that the frequency of spontaneous reverting colonies is high, if not slow (48 h), and that histidine contaminated samples are not appropriate for testing.

In view of the above, in present research, the bacterial reverse mutation test was conducted to evaluate the mutagenic potential of certain pesticide mixtures on *salmonella typhimurium* test strains by following pre-incubation method both in the presence and absence of metabolic activation system.

2 Materials and methods

2.1 Pesticide mixtures

The two pesticide mixtures viz., Deltamethrin 1% + Triazophos 35% EC and Profenofos 40% EC + Cypermethrin 4% EC used in the research were procured locally.

2.2 Culture

Salmonella typhimurium bacterial strains TA 98, TA 100, TA 102, TA 1535 and TA 1537 was obtained from Defense Research and Development Establishment (DRDE), Gwalior, India and were stored as frozen permanent stock in cryo vials with Oxoid nutrient broth no. 2 and 9% DMSO at -80°C. Prior to experiment overnight cultures were prepared by transferring a small quantity of frozen culture to a flask containing Oxoid nutrient broth no.2 and incubated in a shaking water bath at 100 strokes for 14 hours at 37± 2°C. 1-2 x 10⁹cfu/ml of cells were used for the study.

2.3 Positive control

To ensure both strain integrity and effectiveness of the metabolic activation system, strain specific positive controls (known mutagens) were included in each method both with and without S9 mix (Table.1). All known mutagens were obtained from Sigma, India.

Table 1 List of Positive controls

Method	Mutagen	CAS N°	Solvent	Conc. per plate (µg)	Responding <i>Salmonella</i> strains
Non-Activation (-S9) [Direct acting mutagens]	Mitomycin C	50-07-7	Water	0.5	TA102
	Sodium azide	26628-22-8	Water	1	TA1535, TA100
	2-Nitrofluorene	607-57-8	DMSO	10	TA98
	9-Aminoacridine	90-45-9	DMSO	50	TA1537
Activation (+S9)	Benzo(a)pyrene (Sigma, India)	50-32-8	DMSO	10	TA102, TA1535, TA100, TA98, TA1537

2.2 Confirmation of Genotypes

Genotyping of *Salmonella typhimurium* strains was performed prior to study initiation to ensure the genetic integrity. The presence of R-factor plasmids (ampicillin and tetracycline resistance) was confirmed by the method of ampicillin and tetracycline resistance followed by *rfa* mutation confirmed through sensitivity to crystal violet and presence of *uvrB* mutation was confirmed by demonstrating the sensitivity of the tester strains to UV suggested by Ames et al. (1975).

2.3 Solubility and Precipitation Test

Solubility of the pesticide mixtures was determined first by dissolving in distilled water followed by other well-known solvents not limited to DMSO, acetone and ethanol. 50 mg pesticide mixture was completely soluble in 1.0 ml of respective solvent, from this (50 mg/ml) 100 µl was mixed with top agar and added to minimal glucose agar (MGA) plate.

2.4 Preparation of the pesticide mixtures solution

The pesticide mixtures were prepared shortly prior to treatment of cultures and serial dilutions were done to achieve further lower concentrations. Consistency of the testing solutions was maintained during treatment by thorough vortexing of the preparations.

2.5 Cytotoxicity Test

The toxicity of each pesticide mixture was evaluated by performing cytotoxicity test with strains TA 98 and TA 100. Seven concentrations of pesticide mixtures 5000, 2500, 1250, 625, 313, 156 and 78 µg/plate were tested for toxicity in duplicate both in the absence and presence of metabolic activation system (S9 mix). 0.1 ml of DMSO/plate used as negative control. 0.1ml pesticide mixture at each dose level (treatment plates) or 0.1ml of DMSO (negative control plates), 0.5 ml of S9 mix (for test in the presence of metabolic activation) or 0.5 ml, 0.1 M sodium phosphate buffer (for test in the absence of metabolic activation), 0.1 ml bacterial suspension ($1-2 \times 10^9$ cfu/ml) were added to each tube and pre

incubated at 37°C for 15 minutes prior to mixing with the 2ml top agar. 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 $37 \pm 2^\circ\text{C}$. Number of revertant colonies per plate was counted by using colony counter and bacterial background lawn was observed with microscope low power objective.

2.6 Mutagenicity Test

Based on the cytotoxicity data mutagenicity test was performed by following pre-incubation method with 5 tester strains (TA 98, TA 100, TA 102, TA 1535 and TA 1537), at doses 5000, 2500, 1250, 625, 313 µg/plate, in triplicates both in the absence and presence of metabolic activation system. Negative control plates are treated with 0.1 ml 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 (Table 2). After treatment, plates were incubated in inverted position for a period of 48 hours at $37 \pm 2^\circ\text{C}$. Colony counts were compared with negative control.

2.7 Statistics: SPSS 16.0 software was used for statistical analysis.

3 Results

Ames test is one of the most reliable short-term bacterial test system has been used to detect mutagenic activity of pesticides via the detection of various types of gene mutations such as base-pair substitutions (TA100 and TA1535), frame-shift mutations (TA98 and TA97) and transition or transversion events (TA102).

3.1 Genotyping:

The genotypes of all the tester strains were assessed to be intact. All the strains showed histidine and biotin dependence (except TA 102) which indicate the mutation in His gene and *uvr B* deletion. TA102 strain 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 TA98, TA100 and TA102 (Table 2).

Table 2 Genotype confirmations of tester strains

Test	Strains				
	TA 98	TA 100	TA 102	TA 1535	TA 1537
Histidine dependence	-	-	-	-	-
UVrB mutation	-	-	-	-	-
Histidine and Biotin dependence	+	+	+	+	+
Rfa mutation	Z	Z	Z	Z	Z
Ampicillin resistance	+	+	+	-	-
Tetracycline resistance	-	-	+	-	-

-=No growth, +=Growth, Z=Zone of inhibition

Table 3 Cytotoxicity analysis of selected pesticides mixture on *S. typhimurium* strains TA98 and TA 100

Test Materials	Dose (µg/plate)	AHR ±SD*			
		TA 98 S9(+)	TA 98 S9(-)	TA100 S9(+)	TA100 S9(-)
Deltamethrin 1% + Triazophos 35% EC	78	39±5.39*	39±2.83*	129.5±0.71*	119±2.83*
	156	40±2.93*	42±1.87*	129±7.07*	124±7.07*
	313	48±0.71*	46±0.69*	120±1.41*	130±9.19*
	625	35±0.77*	40±1.98*	130±2.83*	123±5.66*
	1250	39±0.54*	45±3.82*	131±5.66*	120±9.90*
	2500	51±7.05*	38±5.32*	119.5±2.12*	114±7.07*
	5000	46±1.95*	48±0.71*	130±4.24*	124±3.54*
Solvent Control (DMSO)		54±4.24	53±5.66	183±5.66	162±4.24
Profenofos 40% EC + Cypermethrin 4% EC	78	42.5±7.78*	44.5±2.12*	139.5±9.19*	122.5±2.12*
	156	38.5±2.12*	42±4.24*	142±7.07*	132±5.66*
	313	36±8.49*	40±5.66*	148.5±9.19*	128.5±4.95*
	625	43.5±3.54*	31±1.41*	132.5±0.71*	122.5±4.95*
	1250	42.5±7.78*	43±2.83*	149±2.83*	111±2.83*
	2500	30.5±0.71*	46±1.41*	141±2.83*	118±4.24*
	5000	41±4.24*	43±1.41*	153.5±2.12*	118.5±9.19*
Solvent Control (DMSO)		47.5±2.12	47±1.41	150±1.41	135.5±4.95

AHR = Average Histidine Revertants, SD= Standard deviation * Statistically significant at $P < 0.05$; S9 (+) with S9 mix and S9 (-) without S9 mix

3.2 Cytotoxicity Test

For the initial assessment of the cytotoxic effect of the two pesticides mixtures, seven concentrations, viz., 78, 156, 313, 625, 1250, 2500 and 5000 µg/plate were chosen employing against two tester strains (TA98 and TA100) with the presence and absence of metabolic activation (S9 mix). After treatment, the non-interfering precipitate was observed in 5000 and 2500 µg/plate both in the cases of presence and absence of S9 mix. No cytotoxicity was observed by inhibition of the background lawn (Table 3). The

average revertant colony numbers in solvent control for pesticide mixture D+T (EC) was 54±4.24 for TA98 and 183±5.66 for TA100 in the presence of S9 mix where as 53±5.66 and 162±4.24 in the absence of S9 mix, respectively. Spontaneous revertant colonies obtained were within the normal values for the two strains examined. Some concentrations of D+T (EC) lowered the spontaneous reversion slightly. On the contrary, the plates with the pesticide mixture P+C (EC) were 47.5±2.12 for TA98 and 150±1.41 for TA100 in the presence of S9 mix and 47±1.41 and 135.5±4.95 in the absence of S9 mix, respectively. Cytotoxicity

test results explain that up to 5000µg/plate pesticide mixtures are non-cytotoxic in this test.

3.3 Mutagenicity Test

Based upon cytotoxicity results, 5000 µg/plate is decided as the highest dose for the mutagenicity test. 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.

In the mutagenicity confirmatory assay, with S9, compared to the solvent control, AHR of TA 98 and TA 1537 exposed to various concentrations of D+T (EC) did not change significantly, whereas AHR of TA 100, TA 102 and TA 1535 decreased in 1250 and 2500 µg/plate. Without S9, compared to the solvent control, AHR of all tester strains decreased in 5000 µg/plate (in 1537, AHR decreased in 2500 µg/plate also) AHR markedly increased in all the tester strains exposed to the positive control (Table 4).

As well as in P+C (EC) when treated with S9 did not change significantly, whereas AHR of TA 100, TA 102 and TA 1535 decreased in 1250 and 2500 µg/plate. Without S9, compared to the solvent control, AHR of all tester strains decreased in 313 to 5000 µg/plate. When treated with positive control AHR markedly increased in all the tester strains (Table 5).

4 Discussion

In the present study, two pesticides mixture which widely used in India are evaluated for their potential genotoxicity *in vitro* using Ames assay. Ames bacterial reverse mutation test is preferably used to detect the point and frame-shift mutations, which alter the nucleotide sequence of the DNA (Ames et al., 1975). There is no sufficient evidence in relation to mutagenicity of pesticide mixtures used in this study. This study will be the first report on detecting the mutagenic effects of D+T (EC) and P+C (EC) using Ames test in *S. typhimurium* strains *in vitro*. WHO (1990a) reported that deltamethrin alone is non mutagenic to Ames bacterial test system. Similarly, International program on chemical safety (INPCS) reported that triazophos was also non mutagenic to Ames bacterial test system (WHO, 1990b). However, as per joint FAO/WHO meeting on pesticide residues, Profenophos showed a negative result in the Ames assay (WHO, 2007). In WHO (2015) specifications report, cypermethrin is considered to be negative in the Ames assay. The results of current study revealed that combination of these pesticides is non-mutagenic at the experimental conditions employed.

Conclusion

There is no sufficient evidence in relation to D+T (EC) and P+C (EC) cytotoxicity and mutagenicity. This study is the first report on detecting the mutagenic effects of D+T (EC) and P+C (EC) using Ames test in *S. typhimurium* strains *in vitro*. Based on study results it can be concluded that, both D+T (EC) and P+C (EC) were non mutagenic and non-cytotoxic against *S. typhimurium* tester strains in the presence or absence of metabolic activation system up to the dose 5000µg/plate.

Table 4 Mutagenicity analysis of Deltamethrin 1% +Triazophos 35% EC (D+T) on *S. typhimurium* assay

Tester strains		Test concentration (µg/plate)					Solvent control (DMSO 0.1 ml/plate)	@positive control
		313	625	1250	2500	5000		
TA98	(+S9)	40±2.7*	41±4.55*	38±6.21	43±6.32*	41±3.02	43±3.21	154±9.71
	(-S9)	39±5.87*	45±3.8*	42±5.68*	35±5.41	42±7.3*	46±3.01	135±9.29
TA 100	(+S9)	127±7.09*	132±2.52*	131±3.00*	126±6.00	128±3.06	133±6.03	185±8.02
	(-S9)	110±5.13*	119±6.11*	124±7.00	125±1.53*	120±8.50	127±2.89	176±6.11
TA102	(+S9)	283±29.65*	364±36.54	312±62.69*	274±40.64	309±34.9	346±52.65	676±36.04
	(-S9)	280±24.1*	300±47.03*	291±44.6	288±88.62*	285±57.89*	297±56.86	650±38.59
TA 1535	(+S9)	46±3*	43±3.51*	45±2.52*	44±3.79	42±1.53	46±2.08	137±11.06
	(-S9)	46±2.52*	44±4.00*	45±1.53*	43±3.51	46±2.00*	47±1.53	136±8.62
TA 1537	(+S9)	10±3.51*	13±3.35*	14±1.57*	12±5.13	12±4.49	15±1.76	66.33±7.37
	(-S9)	9±2.86*	13±3.5*	15±1.31*	12±2.12	13±2.88	14±2.49	54±4.51

@ - Positive control with S9 - Benzo(a)pyrene (10 µg/plate) for all five strains (TA100, TA102, TA1535, TA1537 and TA98). @ - Positive control without S9 - Mitomycin C (0.5 µg/plate) - TA102, Sodium azide (1 µg/plate) - TA100 and A1535, 2 - Nitrofluorene (10 µg/plate) - TA98, 9 - Aminoacridine (50 µg/plate) - TA1537. AHR = Average Histidine Revertants, S.D. = Standard Deviation; S9 (+) with S9 mix and S9 (-) without S9 mix

Table 5 Mutagenicity analysis of Profenofos 40% EC + Cypermethrin 4% EC (P+C) using *Salmonella typhimurium* strain

Tester strains		Test concentration (µg/plate)					Solvent control (DMSO 0.1 ml/plate)	@ positive control
		313	625	1250	2500	5000		
TA98	(+S9)	44.9±5.19	37.96±3.27	37.62±8.22	39.9±6.43*	38.07±6.33	40.95±1.44	144±6.24
	(-S9)	40±6.93*	37±8.02	33±4.16	36±8.72	42±7.57	40±7.94	113±5.13
TA 100	(+S9)	126±4.73	128±7.02	128±8.50	130±5.20*	130±5.69*	134±3.06	184±8.19
	(-S9)	121±6.43	122±3.06	118±8.72	124±3.06*	121±5.00	127±3.61	174±11.15
TA102	(+S9)	291.33±23.02*	329.12±75.91	316.07±64.46	284±85.65	304.36±61.89	294.48±82.56	663.33±149.6
	(-S9)	312±38.53	284±66.50	354±44.61*	303±35.64	286±67.12	344±32.13	672±62.61
TA 1535	(+S9)	43±1.73	47±1.15*	45±3.79*	43±3.00	42±3.61	46±2.08	147±7.51
	(-S9)	43±4.16	37±3.51	42±4.51*	41±7.81	39±1.53	45±3.79	155±10.02
TA 1537	(+S9)	13.05±5.77*	11.14±4.11	10.71±3.89	12.4±5.36*	12.8±6.11*	12.64±5.46	58.67±3.06
	(-S9)	12.27±4.89	13.61±3.31	11.43±6.22	10.1±3.32*	9.81±4.51*	9.63±3.1	53.67±8.02

@ - Positive control with S9 -Benzo(a)pyrene (10 µg/plate) for all five strains (TA100, TA102, TA1535, TA1537 and TA98). @ - Positive control without S9 - Mitomycin C (0.5 µg/plate) -TA102, Sodium azide (1 µg/plate) - TA100 and A1535, 2 - Nitrofluorene (10 µg/plate) - TA98, 9 - Aminoacridine (50 µg/plate) - TA1537. AHR = Average Histidine Revertants, S.D. = Standard Deviation; S9 (+) with S9 mix and S9 (-) without S9 mix

Conflict of Interest

Authors hereby declare no conflict of interest.

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