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Evaluation of carcinogenic potential of Surface adsorbed Hazardous chemicals on vegetables and fruits

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This is to certify that the research project entitled "Evaluation of carcinogenic potential of Surface adsorbed Hazardous chemicals on vegetables and fruits" is carried out in the department of Biochemistry Govt. Nagarjuna Postgraduate College of Science Raipur C.G during March 2017 to March 2020. This work is duly completed in this institute.

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CHAPTER-1

INTRODUCTION

The exponentially increase in the cases of the cancer in the small cities and the villages establishing challenges for the health and research organizations in the 21st century.

Vegetables and fruits are excessively treated with pesticides and ripening chemicals during farming and processing. Pesticides and ripening chemicals deeply adsorbed on the surface of vegetables and fruits and can't drain out by simple washing methods. These chemicals entered in the cell through digestive tract. Some of these chemicals are cyclic compound and mimic the nitrogenous bases of DNA. During the replication these chemicals get introduced in to the synthesizing DNA and causes Cancer by different molecular mechanism.

Analysis of potential of Carcinogenic chemicals through AMES test-

Ames test-

The Ames test is a simple and inexpensive screen for potential carcinogens. It assays the reversion rate of mutant strains of Salmonella typhimurium back to wild-type if the test chemical contains any carcinogenic chemicals. The following figure is explaining procedure of Ames test. Following steps will be performed to determine the carcinogenic potential of the chemicals adsorbed on the sample taken for the investigation.

Step-1

I. Histidine (his) auxotrophs of Salmonella typhimurium are used to tested for reversion in the presence of the chemical, by plating on media lacking the amino acid histidine.

II. Liver enzymes (the S9 extract) are mixed with the increasing test chemical-1 (see in washing step) concentration to determine whether the liver's detoxification pathways convert it to a mutagenic form and to find out the exact chemical concentration which is carcinogenic.

III. The above procedure will repeat with the Chemical-2 and chemical-3.

Step-2

Different his tester strains are available, to test for base-substitution and frameshift mutations. The bacterial strain will be cultured in the above differential media.

Step-3

More revertants in the region of the test chemical than in the untreated control indicate that it may be a mutagen, and further tests are indicated.

Step-4

The test can be made quantitative to produce a dose-response curve, allowing comparison of relative mutagenicity of different chemicals.

The Ames test is used routinely to screen industrial and agricultural chemicals, and shows a good correlation between mutagens and carcinogens.

Objectives-

1. To evaluate the carcinogenic potential of Surface adsorbed Hazardous chemicals.
2. To determine the easiest, safest and cheapest way to remove the adsorbed Hazardous chemicals.
3. To determine option of the chemicals can be used instead of practicing chemical which has reduced adsorbent and carcinogenic properties but same pesticidal effect.
4. To evaluate the possible health hazards on society by Surface adsorbed Hazardous chemicals.

CHAPTER-2

REVIEW OF LITERATURE

2.1 Cancer Epidemic in India

India had an estimated 1.16 million new cancer cases in 2018, according to a report by the World Health Organization (WHO), which said that one in 10 Indians will develop cancer during their lifetime and one in 15 will die of the disease.

The World Cancer Report said that according to the estimated cancer burden in India in 2018, there are about 1.16 million new cancer cases, 784,800 cancer deaths, and 2.26 million 5-year prevalent cases in India's population of 1.35 billion.

The report said that "one in 10 Indians will develop cancer during their lifetime, and one in 15 Indians will die of cancer." In India, the six most common cancer types were breast cancer (162,500 cases), oral cancer (120,000 cases), cervical cancer (97,000 cases), lung cancer (68,000 cases), stomach cancer (57,000 cases), and colorectal cancer (57,000). Together, these account for 49 per cent of all new cancer cases. Of the 570,000 new cancer cases in men, oral cancer (92,000), lung cancer (49,000), stomach cancer (39,000), colorectal cancer (37,000), and oesophageal cancer (34,000) account for 45 per cent of cases. The report added that of the 587,000 new cancer cases in women, breast cancer (162,500), cervical cancer (97,000), ovarian cancer (36,000), oral cancer (28,000), and colorectal cancer (20,000) account for 60 per cent of cases.

"During the past two decades, India has had one of the world's best performing and most stable economies, which has grown by more than 7 per cent annually in most years. "This economic development has given rise to vast socioeconomic changes, with an increasing risk of noncommunicable diseases, including cancer, and significant disparities in access to cancer prevention and control services," the report said (Word Health report 2019).

2.2 Chemical Carcinogenicity

Pesticide residues on vegetables and fruits continue to generate concern in the general population mainly about their potential long term adverse effects such as cancer. The pesticides currently in use include a wide variety of compounds belonging to different chemical classes. More than 800 chemicals marketed as multiple formulations, are used in the European Union, as insecticides, herbicides, fungicides. Pesticides have been considered potential chemical mutagens. Experimental data revealed that various agrochemical ingredients possess mutagenic properties inducing gene mutation, chromosomal alteration or DNA damage. The genotoxic potential for agrochemical ingredients is generally low: they give positive results in few genotoxicity tests. In human biomonitoring studies genetic damage associated with pesticides has been detected for high exposure levels and intensive use. The genetic effects depend on quantity and variety of chemical formulations consumed. Improvements of agricultural practices and of safety of work conditions has reduced the genotoxic hazard. The most recent studies failed to reveal genotoxic damage. This evidence suggests a negligible risk for general population exposed to very low levels of residues (Bolognesi *et al.*, 2000).

In India there are several reports and studies have been done and going on in different part of India. A large number of vegetables and fruits sold across the Capital Delhi contain dangerous pesticides that can cause serious neurological problems, kidney damage, skin diseases and cancer, a report submitted before the Delhi High Court said. The HC had acted suo motu on a report by NGO Consumer Voice, which had in 2010 found that 35 varieties of vegetables and fruits, picked from Delhi markets and tested for pesticide content, had toxins beyond the permissible limits. (*A Report from Hidustan Times (Delhi) 20014*).

A study from Malwa region of Panjab India has fund that the high use of pesticides, along with environmental and social factors, is responsible for the high concentration of pesticide residues in the food chain of this region. Moreover, many banned and restricted pesticides are still in use in this region, warranting strict periodical health checkups and other interventions (Mittal *et al., 2014*).

A study from Hyderabad also found exposure of urban populations to different classes of organophosphate, acephate, fenitrothion and phosalone pesticides due to the consumption of different types of vegetables. In this study researchers used to isolate eighteen organophosphate, acephate, fenitrothion and phosalone pesticides found in vegetable samples (eggplant, ladyfinger, cauliflower, cabbage, tomato and chili) at concentration of more than the permissible limit (Sinha *et al., 2012*).

A similar study from Uttar Pradesh A total of 244 samples of cereals (wheat flour, rice, and maize), pulses (arhar, moong, gram, lentil, and black gram), spices (turmeric, chili, coriander, and black pepper), vegetables (potato, onion, spinach, cabbage, brinjal, and tomato), fruits (mango, guava, apple, and grape), milk, butter, Deshi ghee, and edible oils (vegetable, mustard, groundnut, and sesame) was analyzed for the presence of organochlorine pesticide residues. The levels of hexachlorocyclohexane (HCH) and 2,2-bls(p-chlorophenyl)- 1,1,1-trlchloroethane residues detected high in wheat flour, oil, and fat samples analyzed (Kaphalia *et. al.,* 2020).

Another investigation from National Capital Region (NCR), India analyzed 20 different organochlorine pesticides; most of the organochlorine pesticides residues recorded in these study vegetable samples exceeded the maximum residue levels set by international and national regulatory agencies (Chourasiya *et. Al.,* 2015).

Another study from Patna (Bihar) found increased level of DDT and endosulfan than the permissible limit (Nath *et. Al.*, 2013).

2.3 Ames test

Ames test was introduced in 1975, by Bruce Ames a bacterial geneticist. It involves an experiment of growing bacteria on agar plates and comparing natural mutation rates to mutation rates of bacteria exposed to potentially mutagenic compounds or samples. In this test, an extract of rat liver called S-9 mix and containing of microsomal enzymes and cofactors, is often added to the bacteriological medium. The medium contains genetically modified Salmonella typhimurium strains. The presence of mutations in the histidine genes, causing defects in a metabolic pathway leading to the production of histidine, allows positive selection of histidine revertants on minimal agar plates lacking histidine. Only mutants that able to restore of this function occurred are able to form colonies on such plates. Usually the plates containing the tested compound and tester bacteria are incubated for 48 hours and bacterial colonies are counted.

Many studies (Ames *et al.*, 1975b; Levin *et al.*, 1982) revealed that there are many strains such as TA 98, TA 100, TA 104 and TA 102. In TA 100 and TA 1535 there is a *his* G46 mutation codes for the first enzyme of histidine biosynthesis (*hisG*) (Ames *et al.*, 1975b). In 1982 Barnes determined by DNA sequence analysis that this mutation, substitutes proline (-GGG-) for leucine (-GAG-) in the wild type organism (Barnes *et al.*, 1982). The tester strains TA 1535 and its R-factor derivative present in TA 100, detect mutagens which causes base-pair substitutions generally at one of these G-C pairs. The hisD3052 mutation in TA 1538 and TA 98 is in the *hisD* gene coding for histodinol dehydrogenase. TA 1538 and its R-factor derivative TA 98 detect various frameshift mutagens in repetitive sequences as 'hot spots' resulting in a frame

shift mutation (Walker and Dobson, 1979; Shanabruch and Walker, 1980). Levin *et al.* (1982) described a standard strain *Salmonella typhimurium* bacterium called TA 102 which was used to evaluate the effect of some compounds reacting with nucleotides AT.

Certain carcinogens present in active forms in biological reaction are easily catalyzed by cytochrome-P450. Metabolic activation system is absent in *Salmonella*, and in order to improve the potentiality of bacterial test systems, liver extracts of Swiss albino mice are used. This serves as a rich source in converting carcinogens to electrophilic chemicals that are incorporated to detect *in vivo* mutagens and carcinogens (Garner *et al.*, 1972; Ames *et al.*, 1973a). The crude liver homogenate as 9,000 *x g* S9 fraction contains free endoplasmic reticulum, microsomes, soluble enzymes and some cofactors set with S9 concentration to 10% (Franz and Malling, 1975). The oxygenase requires the reduced form of Nicotinamide Adenine Dinucleotide Phosphate (NADP) which is generally *in situ* by the action of glucose-6-phosphate dehydrogenase and reducing NADP both work as cofactors in assay (Prival *et al.*, 1984; Henderson *et al.*, 2000). While water is considered as a negative control, sodium azide, 2-nitrofluorine and mitomycin for TA 98, TA 100 and TA 102 without S9 metabolic activation and 2-anthramine with S9 hepatic fraction are used as positive controls for conducting the test. Before performing the experiment, fresh solutions must be prepared.

Genetic Approach: The Salmonella/E. coli tester strains: Several strains of Salmonella typhimurium have been used in Ames assay which requires histidine synthesis to assess the mutagenicity. In the histidine operon, each tester strain contains a different mutation. In addition to the histidine mutation, the standard tester strain of Salmonella typhimurium contains other mutations that greatly enhance their ability to detect the mutations. One of the mutations (rfa) causes partial loss of the lipopolysaccharides barrier that coats the surface of the bacteria and

increases permeability to large molecules such as benzo[a]pyrene allowing not to penetrate in the normal cell wall (Mortelman and Zeiger, 2000). The mutagens present in the tested samples give rise to induced revertants on a minimal medium (absence of histidine). They are further used to observe revertants in previously mutated strains (that are not able to grow in a medium without histidine). The other mutation (uvrB) is a deletion mutation in which deletion of a gene, coding for the DNA excision repair system, causing gradually increased sensitivity in detecting many mutagens (Ames *et al.,* 1973a). The reason behind this mutation is the deletion excising the uvrB gene emulsifying these bacteria requiring biotin for growth. The standard strains such as TA 97, TA 98, TA 100 and TA 102 contain the R-factor plasmid, pKM101. These R-factor strains are reverted by a number of mutagens that are detected weakly or not at all with the non R-factor parent strains (Ames *et al.,* 1975a).

Many studies (Ames *et al.,* 1975b; Levin *et al.,* 1982) revealed that development of plasmid pKM101 in TA 1535 and TA 1538 strains leads to complement other isogenic strains such as TA 98, TA 100, TA 104 and TA 102. The his G46 mutation in TA 100 and TA 1535 codes for the first enzyme of histidine biosynthesis (hisG) (Ames *et al.,* 1975b). This mutation, determined by DNA sequence analysis, substitutes proline (-GGG-) for leucine (-GAG-) in the wild type organism (Barnes *et al.,* 1982). The tester strains TA 1535 and its R-factor derivative present in TA 100, detect mutagens which causes base-pair substitutions generally at one of these G-C pairs. The hisD3052 mutation in TA 1538 and TA 98 is in the hisD gene coding for histodinol dehydrogenase. TA 1538 and its R-factor derivative TA 98 detect various frameshift mutagens in repetitive sequences as 'hot spots' resulting in a frame shift mutation (Walker and Dobson, 1979; Shanabruch and Walker, 1980).

CHAPTER-3

MATERIALS AND MATHODS

Materials and Methods

3.1 Sample collection- Three types of routinely supplied vegetables and fruits brought-

1. By the market
2. From the farmers
3. Unwashed sample from the fields.

3.2 Washing vegetables and fruits – The following washing techniques used for the present investigations-

1. Normal washing practicing at home- Normal washing procedure includes; 1 kg of sample washing in one litter of pure drinking water supplied at home. This washed out water with chemical designated as **Chemical-1**.
2. Extreme washing with normal water in three times- 1 kg of sample washing in one litter of pure water with consistent stirring for 10 minutes. This washed out water with chemical designated as **Chemical-2**.
3. Single washing with lukewarm water at 55^0C 1 kg of sample washing in one litter of pure lukewarm water with consistent stirring for 10 minutes. This washed out water with chemical designated as **Chemical-3**.

3.3 Mutagenicity Analysis-

Carcinogenic potential of above extracts (chemical I, II and III) were analyzed by AMES test as following –

Analysis of potential of Carcinogenic chemicals through AMES test-

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The Ames test is used routinely to screen industrial and agricultural chemicals, and shows a good correlation between mutagens and carcinogens.

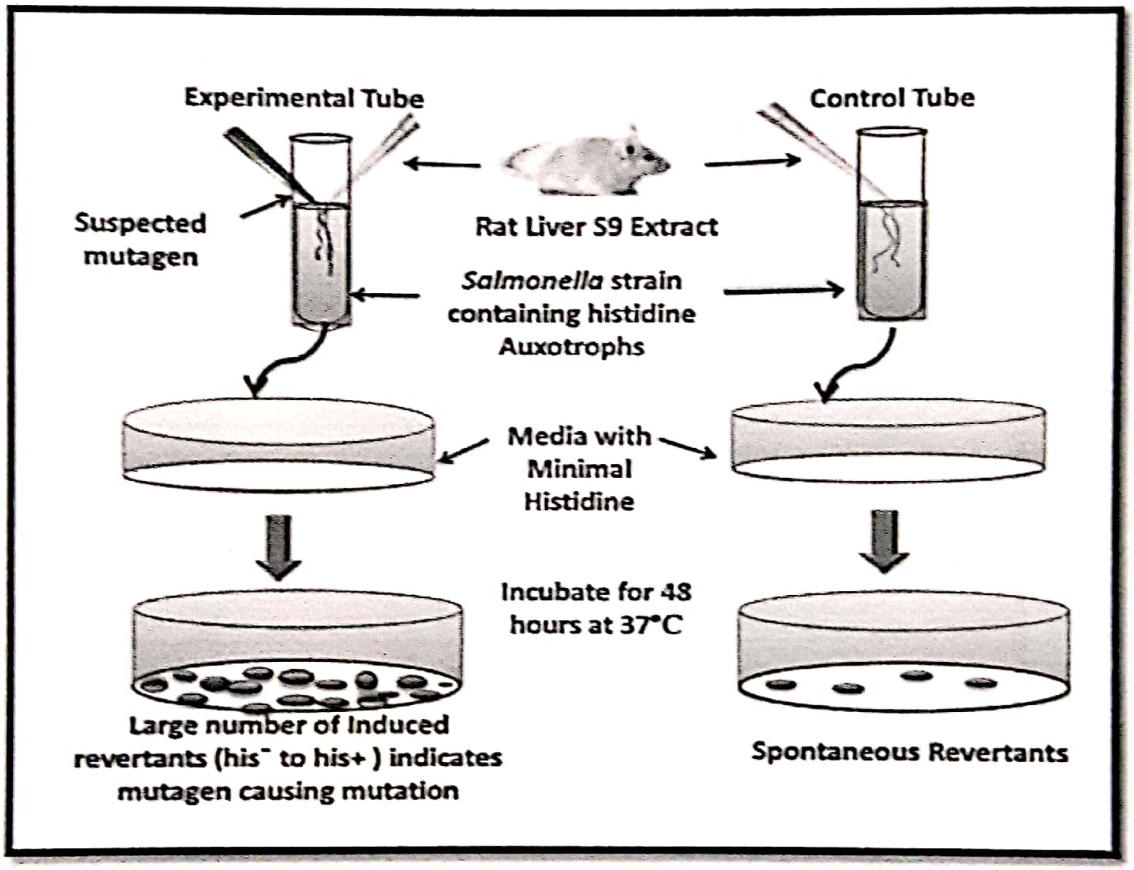

Figure 1. Genetic approach for assessing the mutagenicity in *Salmonella* strains (modified from https://en.wikipedia.org/wiki/Ames_test)

3.4 Materials and Reagents Required for Ames test

A. Materials

1. Tips (1,000 µl, 200 µl, 10 µl)
2. Sterile Petri plates
3. Erlenmeyer flask and beaker (10 ml, 250 ml, 500 ml)
4. Eppendorf tubes (1.5 ml, 2.0 ml)
5. Metal loop holder (metal loop Ch-2,)

6. L shaped spreader

B. Mutagens

C. Sodium azide

D. Reagents

1. Oxoid nutrient broth No. 2
2. 70% ethanol
3. Magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$)
4. Citric acid monohydrate
5. Potassium phosphate, dibasic (K_2HPO_4) (anhydrous)
6. Sodium ammonium phosphate tetrahydrate ($NaNH_4HPO_4 \cdot 4H_2O$)
7. D-biotin
8. L-histidine
9. Hydrochloric acid (HCl)
10. Potassium chloride (KCl)
11. Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)
12. Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$)
13. Disodium hydrogen phosphate (Na_2HPO_4)
14. NADP (sodium salt)
15. D-glucose-6-phosphate (monosodium salt)
16. Ampicillin trihydrate
17. Sodium hydroxide (NaOH)
18. Crystal violet
19. Agar-Agar
20. Nutrient broth
21. Tetracycline
22. Dimethylsulfoxide
23. Vogel-Bonner medium E (50x) (see appendix)

24. 0.5 mM histidine/biotin solution (see appendix)
25. Salt solution (1.65 M KCl + 0.4 M $MgCl_2$) (see appendix)
26. 0.2 M sodium phosphate buffer, pH 7.4 (see appendix)
27. 1 M Nicotinamide Adenine Dinucleotide Phosphate (NADP) solution (see appendix)
28. 1 M glucose-6-phosphate (see appendix)
29. Ampicillin solution (4 mg/ml) (see appendix)
30. Crystal violet solution (0.1%) (see appendix)
31. Minimal glucose plates (see appendix)
32. Histidine/Biotin plates (see appendix)
33. Ampicillin and tetracycline* plates (see appendix)
34. Nutrient agar plates (see appendix)
35. S9 mix (Rat Liver Microsomal Enzymes + Cofactors) (see appendix)
36. Sodium azide (see appendix)
37. Mitomycin (see appendix)
38. 2-Anthramine (see appendix)

3.5 Equipment

1. Orbital shaking incubator (Remi,)
2. Laminar Flow hood (Bio safety cabinet) (Jyoti scintefic Pvt Ltd.,)
3. Pipettes (Eppendorf)
4. Vortex mixer (Remi)
5. Hot water bath (Jyoti scintefic Pvt Ltd)
6. Autoclave (Jyoti scintefic Pvt Ltd)
7. Automatic Colony counter (Jyoti scintefic Pvt Ltd)

Procedure

1. Before performing the experiment, inoculate a single fresh colony of standard strains of S. typhimurium TA 98 in oxoid nutrient broth-2 and incubate for 10-12 h at 37 °C in an incubator shaker at 120 rpm to ensure sufficient aeration for 1 x 109 bacterial cells. Strain of S. typhimurium is grown separately in Erlenmeyer flasks (10 ml).

2. Prepare fresh mutagen for each experiment (see Recipes in appendix).

Negative control: Autoclaved distilled water Positive controls for TA 98 without S9 metabolic activation (S9 mix): sodium azide (1 µg/ml) For TA 98 with S9 metabolic activation (S9 mix).

3. Preparation of minimal glucose agar (MGA) plates: Mix the medium of minimal glucose agar plates (Recipe 9 Appendix) and pour 25 ml into each Petri dish. Prepare the plates freshly before use.

4. Label all minimal glucose agar plates and Eppendorf tubes prior to experiment.

5. To the 2 ml sterile Eppendorf tubes, add the following each:

   a. 0.1 ml fresh culture of Salmonella strains

   b. 0.2 ml of His/Bio solution

   c. 0.5 ml sodium phosphate buffer (absence of S9 mix) or 0.5 ml S9 (presence of S9 mix)

   d. 0.1 ml of test sample or 0.1 ml of positive or negative control

   e. Make up to 1 ml with autoclaved distilled water.

6. Mix the contents of Eppendorf tubes and pour onto Petri plates and spread using L-shaped spreader on the surface of MGA plates. Cover the Petri plates with sterile aluminum foil to protect the testing sample from photo reactive substances.

7. After incubation of 48 h at 37 °C, spontaneous revertants colonies appear and are clearly visible with unaided eyes. All plates are run in triplicates.

8. Revertants form a uniform lawn of auxotrophic bacteria on the surface the background of medium.

Data analysis

Statistical analysis-

The data obtained from Ames test would be analyzed for significant differences between control and test sample by ANOVA, and student's t- test through Prism 3.0.

CHAPTER-4

RESULTS & DISCUSSION

Results

In the present study there are different types of vegetables and fruits are sampled from three different sites they are the sample from city market, Sample Collected from Farmers and Unwashed sample from the fields to determine the exact content of the pesticides on the vegetables and the fruits. Vegetable which are used in this study are mainly leafy vegetables as well as as well as vegetables which are used as raw with the without cooking, like coriander, Capsicum, Chile and lady finger. Normally the vegetables and fruits which are used after peeling are excluded.

Each type of vegetable and fruits were washed separately and chemical I, II and III are obtained separately for each vegetable and fruits. Each chemical I, II and III are analyzed for carcinogenic potential through AMES test. Increasing concentration of chemical I, II and III were analyzed with S-9 and without S-9 plates. The numbers of revertant colonies are counted for each chemical I, II and III obtained from different vegetables and fruits from three different sites and mean ± SD is given in the following tables and result sections.

In the present study single vegetable bought from three different sites as mentioned in the method category, this single vegetable washed with Method 1 to obtain chemical –I this chemical is used to analyze carcinogenicity test with AMES method. Different amount of this chemical is used and revertent colony found are shown in table -1.

Results presented in table -1 showing that when increasing concentration of chemical-1 obtained from city market added in plate to analyze carcinogenic potential obtained revertant colonies was found insignificant when compared with negative control for both S-9 negative and S9 positive samples. These observations are consistent with sample collected from farmers.

This study found significant differences in number of riveted Colony between negative control and increasing concentration of chemical-1 obtained directly from field (Table-1). Whereas differences in S-9 +ve as well as S-9 –ve revertant colonies observed for chemical-I with different concentrations among three different sites were found also significant ($P<0.0001$). This result was fund consistent with chemical-II and III and showing that there are some carcinogenic substances in different amount is present on vegetables and fruits at three different sites. These observations are same as for chemical-1 shown in table- 1.

Obtained data were also analyzed to find out differences between negative control and increasing concentration of chemical-1 with student's t-test and the results are presented in table-2. This is study found that there is insignificant differences has been observed between negative control and chemical-1 (with and without S-9) obtained from city market as well as sample collected from farmers. The observations of Table -2 showing significant differences in revertent colonies obtained from negative control and chemical -1 of Unwashed sample from the fields, in concentration 20, 40, 60, 80 and 100 ml/plate.

Table-1 Showing results of carcinogenic potential of increasing concentration of Chemical I obtained samples of different sites

		Sample Collected from City market		Sample Collected from Farmers		Unwashed sample from the fields		
Dose (ml/plate)		TA98		TA98		TA98		
		S9(-)	S9(+)	S9(-)	S9(+)	S9(-)	S9(+)	ANOVA (One way)
Negative Control	Chemical I	32.42 ± 5.86	37.36 ± 5.32	42.11 ± 7.22	40.36 ± 5.43	32.42 ± 5.86	38.26 ± 5.71	Insignificant
20		32.92 ± 4.32	37.82 ± 3.11	43.54 ± 5.51	42.82 ± 4.31	66.45 ± 4.19	62.96 ± 4.49	P = 0.0030 for S9(-) P = 0.0030 for S9(+)
40		34.84 ± 4.33	37.44 ± 4.11	37.69 ± 4.48	33.34 ± 4.74	70.41 ± 4.23	73.74 ± 4.83	P = 0.0004 S9(-) P = 0.0004 S9(+)
60		32.45 ± 4.46	35.70 ± 4.48	33.73± 4.46	38.61± 4.64	89.61± 5.98	95.59± 5.96	P = 0.0028 for S9(-) P<0.0001) for S9 (+)
80		34.11 ± 3.12	33.59± 4.71	34.38± 4.27	34.11±4.67	89.34± 5.39	80.47±5.55	P<0.0001 S9(-) P<0.0001 for S9 (+)
100		38.39 ± 4.91	41.45± 4.12	44.45± 5.37	42.45± 5.19	99.15± 5.91	97.45± 5.71	P<0.0001 S9(-) P<0.0001 for S9 (+)
ANOVA		NS	NS	NS	NS	P<0.0001	P<0.0001	
Positive Control		425.67 ± 32.86	442.57± 35.16	429.82± 31.49	419.28± 30.66	429.52± 31.48	419.28± 30.38	Insignificant

The numbers indicate the means and standards deviation values.
Without (−) and with (+) S9 microsomal fraction of homogenized rat liver
Negative control: phosphate buffer.
Positive control: TA98, Sodium azide
Significantly different from the corresponding negative control values (ANOVA test, $p < 0.05$

Table-2 Showing results of t-test analysis of Chemical I obtained samples of different sites.

			Sample Collected from City market			Sample Collected from Farmers				Unwashed sample from the fields			
Dose (ml/plate)	Chemical I		TA98			TA98				TA98			
		S9(-)	t-test	S9(+)	t-test	S9(-)	t-test	S9(+)	t-test	S9(-)	t-test	S9(+)	t-test
Negative Control		32.42 ± 5.86	NS	37.36 ± 5.32	NS	42.11 ± 7.22	NS	40.36 ± 5.43	NS	32.42 ± 5.86		38.26 ± 5.71	
20		32.92 ± 4.32	NS	37.82 ± 3.11	NS	43.54 ± 5.51	NS	42.82 ± 4.31	NS	66.45 ± 4.19	P = 0.0002	62.96 ± 4.49	P<0.0001
40		34.84 ± 4.33	NS	37.44 ± 4.11	NS	37.69 ± 4.48	NS	33.34 ± 4.74	NS	70.41 ± 4.23	P<0.0001	73.74 ± 4.83	P<0.0001
60		32.45 ± 4.46	NS	35.70 ± 4.48	NS	33.73± 4.46	NS	38.61± 4.64	NS	89.61± 5.98	P<0.0001	95.59± 5.96	P<0.0001
80		34.11 ± 3.12	NS	33.59± 4.71	NS	34.38± 4.27	NS	34.11±4.67	NS	89.34± 5.39	P<0.0001	80.47±5.55	P<0.0001
100		38.39 ± 4.91	NS	41.45± 4.12	NS	44.45± 5.37	NS	42.45± 5.19	NS	99.15± 5.91	P<0.0001	97.45± 5.71	P<0.0001
Positive Control		425.67 ± 32.86		442.57 ± 35.16		429.82± 31.49		419.28± 30.66		429.52± 31.48		419.28± 30.38	

The numbers indicate the means and standards deviation values.
Without (−) and with (+) S9 microsomal fraction of homogenized rat liver
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Positive control: TA98, Sodium azide
Significantly different from the corresponding negative control values (ANOVA test, $p < 0.05$

Sample collected from same sites were undergone with different washing method as described in (chapter 3) methodology washed water or drained water were called chemical-II. This chemical -II was analyzed for its carcinogenic potential at four (20, 40, 60, 80 and 100 ml/plate) different concentrations and compared with negative control and the results are presented in table-3. The observations in table-3 showing that only unwashed sample collected from the fields were found significant differences in revertant colonies observed among negative control and different concentration of chemical-II.

To analyze carcinogenic potential of increasing concentration of chemical-III we applied Anova test to find significant differences in number of revertant colonies and the results are presented in Table 5. This study revealed that increasing concentration of chemical-III increases number of revertant colonies which are significantly different among negative controls at different concentration at level of $P = 0.001$ in all three simple sites with and without presence of S9. This result is showing that similar samples which are not showing any carcinogenic potential drained as chemical -I and II are showing carcinogenic potential when drained as chemical –III, it means that surface adsorbent hazardous chemicals remain without extensive and proper washing. Hazardous chemical which adsorbed on surface maybe carcinogenic, especially when eaten without cooking like coriander, chilies, reddish, etc. Results of table-5 also showing insignificant difference among revertant colonies of three different sample sides at the concentration level of 20, 40, 60, 80 and 100 ml/plate is showing significant differences at $P = 0.001$

Table-3 Showing results of ANOVA analysis of Chemical II obtained samples of different sites.

Dose (ml/plate)		Sample Collected from City market		Sample Collected from Farmers		Unwashed sample from the fields		ANOVA (One way)
		S9(-)	S9(+)	S9(-)	S9(+)	S9(-)	S9(+)	
Negative Control	Chemical II	32.42 ± 5.86	37.36 ± 5.32	42.11 ± 7.22	40.36 ± 5.43	32.42 ± 5.86	38.26 ± 5.71	
20		33.62 ± 4.12	37.32 ± 3.61	43.66 ± 5.53	40.74 ± 3.99	59.12 ± 4.11	60.94 ± 4.61	P = 0.0002 for S9 (-) P<0.0001 for S9 (+)
40		34.13 ± 4.55	35.29 ± 4.28	36.96 ± 5.03	37.40 ± 4.70	78.20 ± 4.31	79.29 ± 5.12	P<0.0001 for S9(-) P<0.0001 for S9 (+)
60		32.45 ± 4.46	35.70 ± 4.48	33.73± 4.46	37.01± 4.29	85.39 ± 5.44	80.19 ± 4.49	P<0.0001 for S9(-) P<0.0001 for S9 (+)
80		34.11 ± 3.12	33.59± 4.71	34.18± 4.36	38.39±4.39	91.34 ± 5.25	80.47 ± 5.55	P<0.0001 for S9(-) P<0.0001 for S9 (+)
100		48.39 ± 4.91	41.45± 4.12	43.34± 5.18	41.37± 5.38	97.15 ± 5.51	96.38 ± 5.28	P<0.0001 for S9(-) P<0.0001 for S9 (+)
ANOVA		NS	NS	NS	NS	P<0.0001	P<0.0001	
Positive Control		425.67 ± 32.86	442.57± 35.16	429.82± 31.49	419.28± 30.66	429.52± 31.48	419.28 ± 30.38	

The numbers indicate the means and standards deviation values.
Without (−) and with (+) S9 microsomal fraction of homogenized rat liver
Negative control: phosphate buffer.
Positive control: TA98, Sodium azide
Significantly different from the corresponding negative control values (ANOVA test, $p < 0.05$

Table-4 Showing results t- test of carcinogenic potential of increasing concentration of Chemical II obtained samples of different sites.

		Sample Collected from City market				Sample Collected from Farmers				Unwashed sample from the fields			
Dose (ml/plate)	Chemical II	TA98				TA98				TA98			
		S9(-)	t-test	S9(+)	t-test	S9(-)	t-test	S9(+)	t-test	S9(-)	t-test	S9(+)	t-test
Negative Control		32.42 ± 5.86	NS	37.36 ± 5.32	NS	42.11 ± 7.22	NS	40.36 ± 5.43	NS	32.42 ± 5.86		38.26 ± 5.71	
20		33.62 ± 4.12	NS	37.32 ± 3.61	NS	43.66 ± 5.53	NS	40.74 ± 3.99	NS	59.12 ± 4.11	P=0.0006	60.94 ± 4.61	P = 0.0001
40		34.13 ± 4.55	NS	35.29 ± 4.28	NS	36.96 ± 5.03	NS	37.40 ± 4.70	NS	78.20 ± 4.31	P<0.0001	79.29 ± 5.12	P<0.0001
60		32.45 ± 4.46	NS	35.70 ± 4.48	NS	33.73± 4.46	NS	37.01± 4.29	NS	85.39 ± 5.44	P<0.0001	80.19 ± 4.49	P<0.0001
80		34.11 ± 3.12	NS	33.59± 4.71	NS	34.18± 4.36	NS	38.39±4.39	NS	91.34 ± 5.25	P<0.0001	80.47 ± 5.55	P<0.0001
100		48.39 ± 4.91	P=0.0077	41.45± 4.12	NS	43.34± 5.18	NS	41.37± 5.38	NS	97.15 ± 5.51	P<0.0001	96.38 ± 5.28	P<0.0001
Positive Control		425.67 ± 32.86		442.57± 35.16		429.82± 31.49		419.28± 30.66		429.52 ± 31.48		419.28 ± 30.38	

The numbers indicate the means and standards deviation values.
Without (−) and with (+) S9 microsomal fraction of homogenized rat liver
Negative control: phosphate buffer.
Positive control: TA98, Sodium azide
Significantly different from the corresponding negative control values (ANOVA test, $p < 0.05$

Table- 5 Showing results ANOVA test of carcinogenic potential of increasing concentration of Chemical III obtained samples of different sites.

Dose (ml/plate)		Sample Collected from City market		Sample Collected from Farmers		Unwashed sample from the fields		ANOVA (One way)
		S9(-)	S9(+)	S9(-)	S9(+)	S9(-)	S9(+)	
Negative Control	Chemical III	32.42 ± 5.86	37.36 ± 5.32	42.11 ± 7.22	40.36 ± 5.43	32.42 ± 5.86	38.26 ± 5.71	
20		32.92 ± 4.32	37.82 ± 3.11	43.54 ± 6.51	42.82 ± 5.31	96.45 ± 6.19	92.96 ± 6.49	P<0.0001 S9(-) P<0.0001 S9 (+)
40		34.84 ± 6.33	37.44 ± 6.11	57.69 ± 7.48	53.34 ± 6.74	190.41 ± 7.23	183.74 ± 7.83	P<0.0001 S9(-) P<0.0001 S9 (+)
60		102.45 ± 8.46	115.70 ± 8.48	103.73± 7.46	98.61± 8.64	189.61± 7.98	195.59± 8.96	P<0.0001 S9(-) P<0.0001 S9 (+)
80		144.11 ± 11.12	163.59± 12.71	134.38± 11.27	154.11±12.67	289.34± 11.39	280.47±12.55	P<0.0001 S9(-) P<0.0001 S9 (+)
100		198.39 ± 13.91	201.45± 12.12	194.45± 13.37	182.45± 12.19	350.15± 13.91	347.45± 12.71	P<0.0001 S9(-) P<0.0001 S9 (+)
ANOVA		P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	
Positive Control		425.67 ± 32.86	442.57± 35.16	429.82± 31.49	419.28± 30.66	429.52± 31.48	419.28± 30.38	

The numbers indicate the means and standards deviation values.
Without (−) and with (+) S9 microsomal fraction of homogenized rat liver
Negative control: phosphate buffer.
Positive control: TA98, Sodium azide
Significantly different from the corresponding negative control values (ANOVA test, $p < 0.05$

Table-6 Results of t-test analysis of Chemical III

Dose (ml/plate)		Sample Collected from City market				Sample Collected from Farmers				Unwashed sample from the fields			
		S9(-)	t-test	S9(+)	t-test	S9(-)	t-test	S9(+)	t-test	S9(-)	t-test	S9(+)	t-test
Negative Control	Chemical III	32.42 ± 5.86		37.36 ± 5.32		42.11 ± 7.22		40.36 ± 5.43		32.42 ± 5.86		38.26 ± 5.71	
20		32.92 ± 4.32	NS	37.82 ± 3.11	NS	43.54 ± 6.51	NS	42.82 ± 5.31	NS	96.45 ± 6.19	P<0.0001	92.96 ± 6.49	P<0.0001
40		34.84 ± 6.33	NS	37.44 ± 6.11	NS	57.69 ± 7.48	NS	53.34 ± 6.74	NS	190.41 ± 7.23	P<0.0001	183.74 ± 7.83	P<0.0001
60		102.45 ± 8.46	P<0.0001	115.70 ± 8.48	P<0.0001	103.73± 7.46	P<0.0001	98.61± 8.64	P<0.0001	189.61± 7.98	P<0.0001	195.59± 8.96	P<0.0001
80		144.11 ± 11.12	P<0.0001	163.59± 12.71	P<0.0001	134.38± 11.27	P<0.0001	154.11±12.67	P<0.0001	289.34± 11.39	P<0.0001	280.47±12.55	P<0.0001
100		198.39 ± 13.91	P<0.0001	201.45± 12.12	P<0.0001	194.45± 13.37	P<0.0001	182.45± 12.19	P<0.0001	350.15± 13.91	P<0.0001	347.45± 12.71	P<0.0001
Positive Control		425.67 ± 32.86		442.57± 35.16		429.82± 31.49		419.28± 30.66		429.52± 31.48		419.28± 30.38	

The numbers indicate the means and standards deviation values.
Without (−) and with (+) S9 microsomal fraction of homogenized rat liver
Negative control: phosphate buffer.
Positive control: TA98, Sodium azide
Significantly different from the corresponding negative control values (ANOVA test, $p < 0.05$

When vegetables and fruits are washed with method III, chemical III is obtained. Different concentration (20, 40, 60, 80 and 100 ml/plate) of this chemical III is analyzed for carcinogenic potential against negative control with student's t-test and results are presented in table-6. This study revealed that chemical III is showing carcinogenic potential at concentration of 20, 40, 60, 80 and 100 mg/ml for samples collected directly from fields whereas samples collected from city markets and formers showing carcinogenic potential at concentration of 60, 80 and 100 mg/ml.

CHAPTER-5

SUMMARY AND CONCLUSIONS

Conclusions

Conclusions:

The present Study shows a simple wash can drain only loosely bound pesticides and not able to able to drain complete amount of pesticides.

When same sample washed extensively method -2 and 3 described in the section methodology more pesticide come out in the water and shows carcinogenicity in the experiment. Because when we again wash the same sample with simple washing method it shows no carcinogenicity in majority of samples (not all).

Samples collected from city market and direct from the farmers are also shows carcinogenicity in the extensive wash not in simple wash.

Limitations

Ames assay consists of *Salmonella typhimurium* strains and so it is not a perfect model for human. Mice liver S9 hepatic fraction is used to minimize the mammalian metabolic activations formed in the hepatic system so that the mutagenicity of metabolites can be assessed. There are several differences between human and mice metabolism which can affect the mutagenicity of testing substances. Major disadvantages of fluctuation test is slower and slightly more laborious than Ames protocol. The test is primarily used for testing aqueous samples containing low levels of mutagen and therefore, this test is well adapted for evaluating the mutagenicity of wastewater samples.

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CHAPTER-6

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CHAPTER-7

APPENDIX

Appendix

1. Vogel-Bonner medium E (50x)

For Minimal agar (Recipe 9)

Ingredients	Per 500 ml
Warm distilled H_2O (45 °C)	335 ml
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	5 g
Citric acid monohydrate	50 g
Potassium phosphate, dibasic (anhydrous) (K_2HPO_4)	250 g
Sodium ammonium phosphate ($NaNH_4HPO_4 \cdot 4H_2O$)	87.5 g

a. Salts are added to the warm water in a flask. Place the flask on a hot plate

b. After each salt dissolves entirely, transfer the solution into glass bottles and autoclave for 20 min at 121 °C

c. When the solution gets cool, cap the bottle tightly

d. Store the solution at 4 °C

2. 0.5 mM histidine/biotin solution

For mutagenic bioassay

Ingredients	Per 125 ml
D-Biotin (F.W. 247.3)	15.45 mg
L-Histidine·HCl (F.W. 191.7)	12.0 mg
Distilled H_2O	125 ml

3. Dissolve the biotin in hot distilled water. The solution is autoclaved for 20 min, at 121 °C and then stored at 4°C

4. Salt solution (1.65 M KCl + 0.4 M $MgCl_2$)

For S9 hepatic fraction

Ingredients	Per 250 ml
Potassium chloride (KCl)	30.75 g
Magnesium chloride ($MgCl_2 \cdot 6H_2O$)	20.35 g
Distilled H_2O to final concentration of	250 ml

5. All the components are dissolved in water. The solution is autoclaved for 20 min, at 121 °C and then stored at 4 °C
6. 0.2 M sodium phosphate buffer, pH 7.4

For S9 hepatic fraction

Ingredients	Per 250 ml
0.2 M sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H_2O$)	30 ml (6.9 g/250 ml)
0.2 M disodium hydrogen phosphate (Na_2HPO_4)	220 ml (7.1 g/250 ml)

7. Adjust pH to 7.4. Sterilize the buffer by autoclaving for 20 min at 121 °C
8. 1 M nicotinamide adenine dinucleotide phosphate (NADP) solution

For S9 hepatic fraction

Ingredients	Per 2.5 ml
NADP	191.5 mg
Sterile distilled H_2O	2.5 ml

9. NADP is dissolved in the distilled water and mixed by vortexing. Tubes are placed in an ice bath. The solution can be used for up to six months
10. 1 M glucose-6-phosphate

For S9 hepatic fraction

Ingredients	Per 5 ml
Glucose-6-phosphate (G-6-P)	1.41 g
Sterile distilled H_2O	5 ml

11. Glucose-6-phosphate is dissolved in the 5 ml distilled water and mixed by vortexing. Tubes are placed in an ice bath. The solution can be used for up to six months

12. Ampicillin solution (4 mg/ml)

Used in tests of ampicillin resistance

Master plates for R-factor strains

Ingredients	Per 500 ml
Ampicillin trihydrate	0.4 g
Sodium hydroxide (0.02 N)	50 ml

13. Ampicillin trihydrate is dissolved in the 50 ml of NaOH (0.02 N) and mixed by vortexing. Tubes are placed in an ice bath

14. Crystal violet solution (0.1%)

Used in tests for crystal violet sensitivity (to confirm rfa mutation)

Ingredients	Per 500 ml
Crystal violet	0.05 g
Distilled H_2O	50 ml

15. Minimal glucose plates

Used in Mutagenic bioassay

Ingredients	Per 500 ml
Agar	7.5 g
Distilled H_2O	465 ml

50x VB salts (Recipe 1)	10 ml
40% glucose	25 ml

16. Add agar in 465 ml of distilled water and autoclave for 20 min, at 121 °C. After cooling, add the salts and glucose gently

17. Histidine/Biotin plates (Master plates for non R-factor strains)

Used in tests for histidine requirement

Ingredients	Per 500 ml
Agar	7.5 g
Distilled H_2O	457 ml
50x VB salts	10 ml
40% glucose	25 ml
Sterile histidine (2 g per 400 ml H_2O)	5 ml
Sterile 0.5 mM biotin	3 ml

18. Dissolve agar in the given concentration in distilled water. Autoclave each solution separately for 20 min. After cooling of solution, add each salt gently

19. Ampicillin and tetracycline* plates

*Master plates for the cultivation of strains containing the plasmids pKM101 and pAQ1**

Ingredients	Per 500 ml
Agar	7.5 g
Distilled H_2O	405 ml
50x VB salts	10 ml
40% glucose	25 ml
Sterile histidine (2 g per 400 ml H_2O)	5 ml

Sterile 0.5 mM biotin	3 ml
Sterile ampicillin solution (8 mg/ml 0.02 N NaOH)	1.58 ml
*Sterile tetracycline solution (8 mg/ml 0.02 N HCl)	0.125 ml

20. Dissolve agar in the given concentration in distilled water. Autoclave each solution separately for 20 min. After cooling of solution, add each salt gently

**Note: TA 102 is resistant to tetracycline. The shelf life of the plates is two weeks at 4 °C.*

21. Nutrient agar plates

Used in tests for genotypes [Crystal violet sensitivity (rfa) and UV sensitivity (AuvrB)] and viability of bacteria

Ingredients	Per 500 ml
Nutrient agar	7.5 g
Distilled H_2O	500 ml

22. Dissolve agar in the given concentration in distilled water. Autoclave separately for 20 min. Pour the cooled solution into the Petri plates

23. S9 mix (Rat Liver Microsomal Enzymes + Cofactors)

Ingredients	Standard S9 mix Per 25 ml
Mice liver	1.0 ml (2%)
$MgCl_2$-KCl salts	0.5 ml
1 M glucose-6-phosphate	0.125 ml
0.1 M NADP	1.0 ml
0.2 M phosphate buffer, pH 7.4	12.5 ml
Sterile distilled H_2O	9.86 ml

24. *Note: Add each ingredient in the reverse order listed above (First water, and then phosphate buffer...). Avoid refreezing the S9 mix.*

25. Sodium azide

Used in Mutagenicity assay

Ingredients	Per ml
Sodium azide	10 µg
Autoclave distilled H_2O	990 µl (to make a total volume of 1 ml)

26. Working concentrations are prepared by taking 1, 2, 4 µl of 10 mg/ml

27. 2-Nitrofluorine

Used in Mutagenicity assay

Ingredients	Per ml
2-Nitrofluroine	10 µg
Autoclave distilled H_2O	990 µl (to make a total volume of 1 ml)

28. Working concentrations are prepared by taking 1, 2, 4 µl of 10 mg/ml

29. Mitomycin

Used in Mutagenicity assay

Ingredients	Per ml
Mitomycin	10 µg
Autoclave distilled H_2O	990 µl (to make a total volume of 1 ml)

30. Working concentrations are prepared by taking 1, 2, 4 µl of 10 mg/ml

31. 2-Anthramine

Used in Mutagenicity assay

Ingredients	Per ml

2-Anthramine	10 µg
Autoclave distilled H_2O	990 µl (to make a total volume of 1 ml)

32. Working concentrations are prepared by taking 1, 2, 4 µl of 10 mg/ml

Sterilization (safety considerations while working with *Salmonella*)

1. As *S. typhimurium* is a pathogenic bacterium, it is prudent to use precautionary measures every time and apply standard biosafety guidelines such as using plugged pipettes, proper sterilization by 70% ethanol and autoclaving all contaminated material.
2. Handling of chemicals and strains should be done in biosafety cabinet. Before and after the use, cabinet must be sterilized using 70% ethanol and exposed to 15 min UV.
3. Care must be taken to protect from chemical exposure by wearing gowns, eye glasses and gloves.
4. Before discarding, all contaminated material (*e.g.*, test tubes, pipettes and pipette tips, gowns and gloves) should be properly autoclaved.

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