STUDIES ON THE CLASTOGENICITY AND MUTAGENICITY OF IRRADIATED PAPAYA

by

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An Undergraduate Thesis Submitted to
The Division of Natural Sciences and Mathematics
College of Arts and Sciences
University of the Philippines, Manila
Padre Faura, Manila

As Partial Fulfillment of the Requirements
for the Degree of
BACHELOR OF SCIENCE IN BIOLOGY

March 1989

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ACKNOWLEDGEMENT

I would like to express my deepest gratitude to the the following people who have helped me in the completion of my thesis:

Professor Malou Nicolas for her assistance in the Micronucleus Test and her valuable advice in the interpretation of my data.

Professor Amelia Asencion for all the suggestions and assistance she has provided in the planning and experimental stages of my thesis as well as the preparation of the manuscript.

Ariel Porcalla for his assistance especially in the scoring of the micronuclei and in the typing of the manuscript and for simply being a true friend whose companinnship never fails to provide me an assurance that everything will be fine especially when they all seem to be going wrong.

Philomel Cruz for helping me with my statistical analysis

Tammy de la Rosa, Ruwell Loyola and Gizbert Hermoso for assisting me in my experimentation and keeping me company whenever I have to go home late at night.

Vincent Aluquin and Glenn Pacio for being ever available whenever I needed extra hands.

Chut Lim and Lilyanne Dimaano for teaching me some techniques in Microbiology.

Miriam Marzan for supplying me with some of the materials I needed for the Micronucleus Test.

Manang Tess, Butch and Margie for providing me with the laboratory materials.

My parents, Mr. and Mrs. Angelito Yango for the love they have given me in all the nineteen years of my existence.

And to Him, the Almighty, for blessing me with a loving family and true friends, and for loving so much inspite of all my shortcomings, I am most truly grateful.

A B S T R A C T

The mutagenicity and clastogenicity of gamma irradiated papaya extract at dose levels of 50, 100, and 150 kR were investigated using the Rec-Assay and Micronucleus test.

Rec-Assay, an *in vitro* microbial assay using Rec^+ and Rec^- strains of *Bacillus subtilis* showed significant mutagenic properties for 150 kR irradiated papaya extract even after 8 days of storage. Such mutagenic properties were not observed in papaya extracts subjected to lower irradiation dosages of 0, 50, and 100 kR gamma rays.

Micronucleus test, an *in vivo* analysis for clastogenicity using mice as test organisms showed significant clastogenic properties for 150 kR irradiated papaya extract after 8 days of storage. Such clastogenic properties were not observed in papaya extracts subjected to lower irradiation dosages of 0, 50, and 100 kR gamma rays.

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STUDIES ON THE CLASTOGENICITY AND MUTAGENICITY OF IRRADIATED PAPAYA

I. INTRODUCTION

The growing world population has been accompanied by the problem of food supply. This problem has laid tremendous pressure on technology to come up with ways and means of increasing food production as well as developing techniques concerning food preservation. Indeed, the urgency of the problem concerning food supply and preservation has been great that in response, technology came up with what seemed to be then as perfect solutions. Take for example the case of DDT and other broad spectrum insecticides which were produced in response to the era of industrialized agriculture. These chemicals were supposed to solve all pest problems forever but as now too evident, this resulted to a severe backlash that has damaged the environment. The chlorinated hydrocarbon insecticides, now among the world's most widely distributed synthetic chemicals are contaminating a substantial part of the biosphere. Their broad toxicity indicates a potential for biological effects on many kinds of organisms including man (Odum, 1971).

The point, however, is not to condemn technology for after all, it is the product of man's unquenchable thirst for knowledge; and because of which, we all enjoy many comforts in life. The point is, man should be careful in the use of his technology especially in trying to control his environment. He should consider not just its short-term effects but also its long-term effects for after all, he will be the one who will bear the consequences of a battered environment.

Over the centuries, man's technology has developed tremendously. One technology that has seen great developments is food preservation. Among the chief methods of food preservation currently employed are keeping out of reach of microorganisms, maintenance of anaerobic conditions in sealed, unaerated containers, use of high and low temperatures, irradiation, drying, use of chemical preservatives, mechanical destruction of microorganisms by grinding or subjecting to high pressure, and combination of two or more of the above methods (Frazier, 1978).

The most economical method of food preservation is the administration of chemical preservatives. However, scientific inquiry has revealed substantial evidences that these chemicals when used improperly can cause adverse effects to human beings and animals. The question of safeness of chemical preservatives is not only confined to toxic manifestations but it also includes its genetic effects to man. The widely used meat preservative nitrite, commonly added in the form of sodium or potassium nitrite, was shown to induce cancer in test animals and can increase mutation frequency of Salmonella strains upon metabolic activation by mammalian enzyme systems (Couch and Freidman, 1974). This happens when nitrites chemically react with secondary and tertiary amines to form nitrosoamines which are known to be carcinogens (Sylianco, 1974).

A possible alternative to the administration of chemicals as food preservatives is the use of ionizing radiation. Irradiation of foods increases its storage life and inhibits sprouting. The method is also effective in the disinfestation, sterilization, and pasteuriza-

tion of selected commondities. For example, treatment with gamma irradiation under 50 kR, has been shown to disinfest as well as improve the storage life of fresh pineapple without producing undesirable changes in the fruit color, flavor, and sweetness (Makinen, et. al., 1967). Considering the importance of exporting agricultural products in our country's economy, such technology could provide great help in improving the quality of our agricultural products as well as in minimizing loss by prolonging their storage life.

However, before we begin to conclude that the problem of food preservation has at last been solved by irradiation techniques, it is imperative that all aspects of this technology be carefully studied in order to avoid any disastrous occurrence.Of particular concern to scientists is the possible effects of these irradiated foods on the DNA of the organisms that consume it. There are very convincing evidences that there are substances with cytotoxic or mutagenic agents found in the tissues of food treated with ionizing radiation. Some examples of food types presently being preserved through irradiation are vegetables, grain crops, fruits and fruit juices, meat, fish, and other products of plant and animal origin. Their mutagenic effects possess serious considerations in the use of irradiation techniques in food preservation. Many studies have been and are currently being undertaken in order to establish the fitness of irradiated food for human consumption (Barna, 1976).

Having these considerations in mind, this study aims to investigate and come up with additional data on mutagenic and clastogenic potential of irradiated food to provide addtional basis in establishing how safe or unsafe food irradiation is to human beings.

II. REVIEW OF LITERATURE

Despite the wide application of gamma irradiation of food for the improvement of storage life, inhibition of sprouting, and sterilization, it is of utmost importance that questions concerning its safety be settled.

In 1947, Stones, Wyss and Haas demonstrated that the ultraviolet irradiated nutrient broth had mutagenic properties in Staphylococcus aureus. On the other hand, Swaminathan, et. al. (1963) have obtained small but statistically significant increases of mutation rates in Drosophila melanogaster raised on irradiated food, indicating that in certain circumstances, marginal effects in mutation rate may be produced (Chopra, 1965). In a similar experiment by Parkash in 1965, he observed that the irradiation of DNA (a component of most foodstuffs) prior to its addition to a Drosophila culture medium produces a pronounced increased in mutation in the larvae compared with the unirradiated (and non-mutagenic) DNA sample (Chopra, 1965).

Other experiments confirm the mutagenic and clastogenic properties of irradiated food. Vijayalaxmi and Sadasivan in 1975 confirmed that feeding of freshly irradiated wheat leads to an increased number of polyploid cells in the rat bone marrow. Furthermore, their experiment showed that when irradiated wheat was stored for a period of 12 weeks before being fed to rats, no increase in incidence of polyploid cells was seen. This results suggested that during irradiation of wheat, some toxic substances are formed, the concentration of which falls during storage.

The identity of compounds producing toxic manifestations in irradiated carbohydrate solutions was exper-

imented on by Schubert, et. al. (1967) using microbiological assay procedure. They found out that under certain conditions, deleterious compounds are produced in irradiated sucrose solutions. These compounds appear to be hydroxyalkyl peroxides (HAP), derived from the interaction of radiolytic H_2O_2 with carbonyl compounds produced in the radiolysis of sucrose. They have shown that the growth of Salmonella typhimurium was inhibited by irradiated, buffer-free oxygenated solutions of sucrose and to a far lesser extent, by solutions of sucrose irradiated in helium.

Several organisms ranging from bacteriophages to mammals have shown an impairment of growth when cultured on irradiated substrate or fed with irradiated food. As with direct irradiation, growth stimulation has also been occassionally reported instudies of indirect effects (Kesavan and Swaminathan, 1970).

Blank and Arnold in 1935 showed that irradiation of nutrient agar with ultraviolet light produced a non-volatile, thermostable, toxic substances that was capable of diffusing through the medium and preventing the growth of Bacillus subtilis. It was further shown that the growth of Bacillus subtilis is prevented by adding to the medium an ultraviolet irradiated solution of any one of a number of different carbohydrates.

Baumgartner in 1936 found that the irradiation of a 1% sucrose solution or culture medium caused a drop in pH from 7.0 to 3.5-4.0, causing the inhibition of growth in Bacillus subtilis and Escherichia coli. However, if the medium was neutralized before use, growth was not inhibited. If the sucrose solution was irradiated in the presence of excess calcium carbonate, theus preventing pH changes upon irradiation, no inhibition of growth was observed. It was proposed that about half the acidity

formed by the irradiation of a 1% sucrose solution was due to formic acid (Keesavan and Swaminathan, 1970).

Molin and Ehrenberg in 1964 reported a strong anti-bacterial action of gamma-irradiated glucose on Pseudomonas sp. 128. Irradiation of the water solution of glucose was more effective than the irradiation of modifications of crystalline glucose. Part of the anti-bacterial activity of the irradiated solution persisted after storage for 6 days. The antibacterial activity increased with decreasing pH of the irradiated solution and was eliminated by the addition of catalase. Despite a high natural catalase activity, Pseudomonas sp. 128 was sensitive to hydrogen peroxide; as little as 1-5 ug/ml caused some mortality.

Kuzin in 1962 reported that extracts from leaf tissue from irradiated plants inhibit the reproduction of Escherichia coli cells. The cells retained the ability to form colonies when spread on agar. The extract from irradiated (15 Kr) leaves reduced the colony forming ability by more than 50 percent. Similarly, a pronounced inhibitory effect of potato extracts irradiated at 10-15 Kr on Escherichia coli (Kesavan and Swaminathan, 1970).

Chopra in 1965, studied the effect of irradiated culture media on the growth and mutation rates of Bacillus subtilis and Escherichia coli. In experiments with B. subtilis, Haye's minimal medium irradiated with 226 kR of x-rays was seeded with bacteria at cell densities as close as 10^6 per ml. as possible and incubated at $37^{\circ}C$ In several experiments, viable counts declined with time of incubation in irradiated medium to a minimum of 2.5×10^3 after 8 hours of incubation.

Similar experiments regarding the cytotoxicity and inhibitory effects of irradiated medium on eukaryotes

were also conducted. Even as early as 1927, Woodrow, et. al. showed that the growth rate of Saccharomyces cereveseae was decreased when grown in U. V.-irradiated water solution of the same compound. This inhibition was attributed to the formation of non-volatile toxic substances formed by the action of U. V. light on sucrose (Kesavan and Swaminathan, 1970).

Evans, et. al.(1942) reported that X-radiation of sea water caused an immediate reduction in fertilizing capacity of Arabacia sperms. They attributed this to the formation of toxic-free radicals then designated as "activated water molecules". Evans also obtained experimental evidence that crude extracts of sea urchin sperm containing catalase could prevent the death of sperm immersed in irradiated sea water.

Wagner, et. al. (1950) found that irradiated broth and hydrogen peroxide were mutagenic to Neurospora cras a. The importance of organic peroxides formed indirectly or directly by irradiation of the medium or cell is indicated by the results of Dickey, et. al. (1949) who demonstrated the effectivity of certain organic peroxides in causing reverse mutations at an adenineless locus in Neurospora.

Similar studies on medium effects were made by Jensen, et. al. in 1951 using the Neurospora back mutation technique with an adenineless strain. They observed a rather strong mutagenic effect which appeared to result from the reaction of hydrogen peroxide, or its decomposition products, with cell components. Irradiation with U. V. of a medium containing formaldehyde before the addition of conidia greatly increased the mutation rate in Neurospora. It was suggested that the treatments were mutagenic because free radicals, formed by decomposition, reacted with molecules within or near the gene.

On the effects of irradiated medium on the growth and development of higher plants, Ehrenberg (1961) studied the effect of irradiated glucose and glycine (50×10^6R of gamma rays) on barley seeds. He found that the irradiated substrate gave a mutation rate of 1% while the unirradiated substrate gave 0.4%. Similarly, other experiments have shown that Alium cepa grown on X-irradiated (66 Kr) distilled water exhibit an increase in the incidence of chromosome and chromatid breaks (Natarajan and Swaminathan, 1958). Inhibition of mitosis and root growth was likewise observed in unirradiated barley embryos grown in irradiated potato mash exposed to 20 to 80 Kr of gamma rays and X-rays (Swaminathan, et. al., 1962).

The mutagenic effects of irradiated medium and other substances (DNA, sucrose) have been extensively studied in Drosophila melanogaster, since elegant stocks are available for scoring accurately sex-linked as well as autosomal lethal mutations. The mutagenic effect of irradiated basic media (containing water, glucose, agar, yeast powder and propionic acid) to D. melanogaster was first reported by Swaminathan et. al. in 1962. The Muller-5 technique was employed following the feeding of wild type (oregon-K) males from egg to adult stage with irradiated basic media (150 Kr gamma rays). These males were then mated to Muller-5 females reared on unirradiated medium. The irradiated medium was found to induce a 3-4 fold increase over the spontaneous (unirradiated medium) rate of sex-linked recessive lethals (results were significant at P=0.01). The irradiated medium was also found to increase the frequency of visible changes, but most of them were not heritable.

The Rec-Assay method is an *in vitro* microbial assay which uses recombination deficient strain of *Bacillus subtilis* M45 Rec^- and recombination proficient strain of *B. subtilis* H17 Rec^+. Recombinant-negative strains of *B. subtilis* (arg-tyr), due to their inability to repair damaged DNA through recombination are highly sensitive to radiation and other chemical mutagens. On the other hand, recombinant-positive strains (rec-arg-tyr) are more resistant to mutation due to their ability to recombine. From this, it can be concluded that agents exhibiting lethality on Rec^- over Rec^+ may have damaging effects on cellular DNA and are therefore mutagenic. The strain M45 of *B. subtilis* is sensitive to frameshift and base-pair substitution mutagens (Kada, 1974).

The method used in the detection of chromosomal aberrations is the Micronucleus Test. It is an *in vivo* method which was developed by Schmid in 1974. When test animals are treated with chromosome-breaking agents, the cells from the bone marrow of these animals contain micronucleus as long as they have completed mitosis under theinfluence of the mutagen (Schmid, 1975).

The Micronucleus Test is based on the following principles. In anaphase, chromosome fragments and acentric chromatids lag behind when the centric elements move towards the spindle poles. After telophase, the undamaged chromosomes and centric fragments give rise to regular daughter nuclei. Although several lagging elements are included in the daughter cells, a considerable portion is transferred into secondary nuclei which are much smaller than the principal nucleus, hence they are known as micronuclei. If the functioning of the spindle fibers are impaired, similar events happen. The only difference

is that the main nucleus is often replaced by a whole group of small nuclei which are considerably larger than the typical micronuclei (Schmid, 1976; Tsuchimoto and Matter, 1979).

Micronuclei can be found in the myelocytes, myeloblasts and erythroblasts. Erythroblasts expel their nucleus a few hours after the last mitosis. The micronuclei remain in the cytoplasm of the young erythrocyte where they can easily be recognized. For the duration of their adolescence, approximately 24 hours, the young erythrocytes stain bluish, they are polychromatic whereas the older forms stain red. The micronuclei are typically round with a diameter of about 1/20 to 1/5 of an erythrocyte. Most of the micronucleated cells contain only one micronucleus, but some may contain two or more. Others have almond-shaped micronucleated polychromatic erythrocytes (Schmid, 1975; Matter, 1976; Dag and Rainel, 1980).

III. MATERIALS AND METHODS

A. Irradiation of Papaya

Three 50 ml. samples of homogenized extracts were obtained from a single fruit of ripe papaya. The extracts were exposed to irradiation at these dose levels - 50, 100, and 150 Kr gamma rays in the Cobalt-60 facility of the Philippine Atomic Energy Commission (now known as the Philippine Nuclear Research Institute) in Diliman, Quezon City.

Each extract was labelled with the corresponding dosage given and stored in the freezer.

B. Rec-Assay Method

I. Bacterial Test Systems

This assay employs recombination-deficient and recombination-proficient strains of Bacillus subtilis (Marburg), M45 Rec$^-$ and H17 Rec$^+$ respectively. They were kept in nutrient agar stabs at 4^{o}C.

II. Media

A. B-2 Broth: 10 grams meat extract, 10 grams peptone dry powder and 5 grams NaCl were mixed in one liter of distilled water. The pH was adjusted to 7.

B. B-2 Soft Agar: 1.5% agar (w/v) was added to the B-2 Broth

III. Experimental Procedure

Cultures of tester strains of Bacillus subtilis M45 Rec$^-$ and H17 Rec$^+$ were prepared by dipping a sterile inoculating loop into a nutrient agar stab. The adhering cells were then transferred into a sterile 20-ml test tube containing 5 ml of the B-2 Broth. These cultures were incubated overnight at 37^{o}C to allow the bacteria to produce approximately 10^8 cells per .1 ml of culture.

Ten ml. o B-2 Soft Agar was placed on a 20 ml test tube. After sterilization, half of the liquid was poured into a previously sterilized petri plate. The remaining half of the liquid agar was then cooled to a temperature low enough for microorganisms to survive and high enough for the agar not to solidify. Cultures of a tester strain was then aseptically dipped into the agar using an inoculating loop. Then the agar was poured evenly on the hardened bottom agar. Constant agitation of the top agar (the agar with the microbial strain) was done in order to insure that the agar would remain liquid up to this point.

Once the top agar has been poured into the petri plate, the petri plate was set aside for the agar to solidify.

A sterile paper disk of 16 mm in diameter, earlier spotted with about 0.5 ml of the test sample, was placed on the center of the hardened top agar surface.

The plates were kept at cold storage (4-5°C) for 24 hours and followed by an incubation period at 37°C for about 20 hours. The length of the inhibition zone was measured starting from the edge of the paper disk.

Blank and control plates were done, using distilled water and formaldehyde which is a known mutacarcinogen, respectively, as test compounds. Five replicates for each set-up were performed.

C. The Micronucleus Test

I. Test Animals

Albino mice, both male and female were used for the test whose ages were between 7 to 12 weeks old.

II. Test Compounds: Irradiated papaya (50, 100, 150Kr)
0.5 ml/20 gms body weight

Test Chemicals: Tetracycline HCl
55 mg/kg body weight

III. Experimental Procedure

For each dose of the test sample, five mice of both sexes were treated orally by using a gavage. The oral administration was given twice within a 24-hour interval. Six hours after the second treatment, the mice were killed by cervical dislocation of the neck. From each of the freshly-killed mice, both femora were removed and stripped clean of all muscle fibers. The proximal end of the femur was shortened carefully, exposing the marrow canal.

A 5-ml test tube was filled with one-ml of fetal calf serum. About 0.20 ml of fetal calf serum was drawn into syringe fitted with a needle. The needle was inserted into the proximal end of the bone marrow. By careful aspirations and flushings into the test tube containing

the fetal calf serum, the bone marrow was flushed out through the opening of the marrow canal and centrifuged at 1,000 rpm for five minutes. The supernatant was gently poured out and the remaining cells in the sediment was smeared on a slide. Three slides permouse were prepared and were air-dried overnight.

On the following day, the slides were stained in the following manner; 3 minutes in undiluted May-Grunwald solution with distilled water and 10 minutes in 15% Giemsa stain solution diluted with distilled water.

The slides were screened and analyzed under the light microscope. For each slide, 1000 polychromatic erythrocytes were scored for the presence of micronuclei. The scored elements were micronucleated cells.

IV. RESULTS AND DISCUSSION

Tables 1 and 2 shows the results of the mutagenicity and clastogenicity of irradiated papaya using the Rec-Assay and the Micronucleus Test. No zone of inhibition was observed for the treatment with distilled water for both the Rec^- and the Rec^+ strains. The same result was also observed for the papaya treatment of 50 and 100 Kr gamma rays. On the other hand, treatment with formaldehyde caused formation of zines of inhibition for both Rec^+ and Rec^- strains, the former exhibiting a mean of 1.78 cm. and the latter a mean of 2.12 cm. The papaya extract irraidated at a dose levll of 150 Kr caused a zone of inhibition only for the Rec^- strain with a mean of 1.46 cm and apparently no effect on the Rec^+ strain.

The effect of formaldehyde on the Rec$^-$ strain showed a significantly greater effect on the Rec$^+$ strain. This was manifested by the greater diameter of zones of inhibition observed on all the plates containing Rec$^-$ strains as compared to the Rec$^+$ strain.

For the 150 Kr irradiated papaya, it was shown that the diameters of the zones of inhibition observed on the Rec$^-$ plates were significantly greater than the Rec$^+$ plates which actually never developed zones of inhibition.

Comparing the effects of formaldehyde with the 150 Kr irradiated papaya, it can be safely concluded that the effect of formaldehyde is significantly greater than the effect of the 150 Kr irradiated papaya. This holds true for both Rec$^-$ and Rec$^+$ strains of Bacillus subtilis.

Evidently, water and the two dose levels of irradiated papaya (50 and 100 Kr) had no effect on both the Rec$^+$ and the Rec$^-$ strains as manifested by their failure to effect any zone of inhibition.

The results confirm the mutagenic effect of irradiated papaya at dose level of 150 Kr administered to a bacterial system. The significant difference between the zones of inhibition in the Rec$^-$ and Rec$^+$ strain suggests the mutagenic properties of the extract. Although no qualitative and quantitative analyses were done in order to identify the mutagenic chemicals present in the extract, conclusions can be easily drawn from the nature of the bacterial strains themselves and their reactions to the extract. Bacillus subtilis H17 Rec$^+$ exhibits less susceptibility to killing with radiations and other chemical substances that may cause mutations. The primary reason behind this is that the strain has a recombinational repair system, that is, it has the ability to repair damaged DNA through cellular recombinational functions. On the other hand, M45 Rec$^-$ has no such

Table 1: Zones of inhibition produced on culture strains of *Bacillus subtilis* (H17 Rec^+ and M45 Rec^-) by distilled water, 37% formaldehyde, and irradiated papaya of dose levels 50, 100, and 150 Kr..

* measured in cm. radius.

Extract	Zones of inhibition H17 Rec^-	Zones of inhibition M45 Rec^+
Distilled water		
Plate 1	0	0
Plate 2	0	0
Plate 3	0	0
Plate 4	0	0
Plate 5	0	0
37% formaldehyde		
Plate 1	2.0	1.8
Plate 2	2.1	1.7
Plate 3	2.2	1.7
Plate 4	2.2	1.8
Plate 5	2.1	1.9
Papaya 150Kr		
Plate 1	1.5	0
Plate 2	1.4	0
Plate 3	1.4	0
Plate 4	1.6	0
Plate 5	1.4	0
Papaya 100Kr		
Plate 1	0	0
Plate 2	0	0
Plate 3	0	0
Plate 4	0	0
Plate 5	0	0
Papaya 50Kr		
Plate 1	0	0
Plate 2	0	0
Plate 3	0	0
Plate 4	0	0
Plate 5	0	0

Table 2: Number of micronucleated erythrocytes per 2,000 cells obtained from mouse bone marrow in five set-ups for Micronucleus Test.

Mouse Number	* TREATMENT a	b	c	d	e
1	5	28	34	6	7
2	9	34	32	8	7
3	7	33	27	10	9
4	6	32	30	7	8
5	8	35	28	9	6
Total	35	162	151	40	37
Mean($\bar{x}$)	7	32.4	30.2	8	7.4
Variance (s^2)	2.5	7.3	8.2	2.5	1.3

* treatments

a. distilled water
b. tetracycline.HCl
c. papaya 150 Kr
d. papaya 100 Kr
e. papaya 50 Kr

recombinaitonal repair system so that it is extremely susceptible to damage or killing with radiation or certain chemicals.

This consideration shows us that the increased lethal activity of 150 Kr irradiated papaya on Rec^- over Rec^+ may be due to the damaging cellular DNA properties of the extract which evidently affects the less susceptible Rec^- strain. As a matter of fact, no zone of inhibition was observed in the Rec^+ strain suggesting that the strain was able to repair whatever damage the papaya extract has caused to the Rec^- strain.

The significantly greater lethality of formaldehyde over the 150 Kr irradiated papaya suggest that the former is a stronger mutagen than the latter. In fact, data showed (Table 1) that formaldehyde was able to produce an effect not only to the Rec^- strain but also to the Rec^+ strain although the zones of inhibition developed in the Rec^+ strain were still significantly lower than that in the Rec^- strain.

Formaldehyde is a known mutacarcinogen. It reacts readily with the free amino group of adenine where the reaction is one on mucleophilic addition to the positive carbonyl carbon of formaldehyde. The action finally provides a methylene bridge between the two adenine molecules that are properly oriented. Bis-adenosine derivatives will be formed. Normal base-pairing characteristics will be altered if a covalent crosslink is present such as the case of two adenines reacting with formaldehyde. Misreading or mispairing during replication or transcription may occur if the defect is not repaired (Sylianco, 1981).

The best characterized strains of recombination deficient mutants of Bacillus subtilis are H17 Rec^+ and M45 Rec^-. They are sensitive to base-pair and frameshift

substitution mutagens indicating that the nature of mutation effected by the papaya would be base-pair or frame-shift.

While the 150 Kr irradiated papaya showed significant mutagenic properties, 50 and 100 Kr treatments showed no such activity. However, before one makes hasty conclusions that lower dose levels of radiation are non-mutagenic, further research must be undertaken. Also, it must be noted that the papaya extracts were actually tested one week after their irradiation so that the potency of the lower dosages could have diminished significantly by that time.

The micronucleus test was the second test employed to investigate the clastogenic properties of irradiated papaya on a mammalian system using albino mice as test organism. Tetracycline, was used as a positive control and distilled water as negative control.

Scoring young erythrocytes for the presence of micronuclei per 2000 cells showed that distilled water exhibited a mean of 7 micronucleated cells/2000 cells while tetracycline had a mean of 32 micronucleated cells/2000 cells. Irradiated papaya of dosages 0, 50, and 100 Kr showed slightly higher number of micronucleated erythrocy es but they are not significantly different from the negative control. There is, however, a significant increase in the number of micronucleated erythrocytes observed in treatment with 150 Kr irradiated papaya, with a mean of 30 micronucleated cells/2000 cells. Furthermore, it can be observed that the mean of micronucleated erythrocyte scored for tetracycline and 150 Kr are almost the same (Table 2).

Tetracycline HCl is a known clastogen due to its ability to cause chromosomal breaks. These breaks or fragments of chromosome lags behind normal chromosomes

during mitosis so that a considerable portion of such fragmented chromosomes is transferred into secondary nuclei which are much smaller than the principal nucleus, hence, they are known as micronuclei. The clastogenic property of tetracycline is due to its polycyclic structure which by intercalation with normal DNA produces subtle but critical distortion in the helix sufficient to cause steric hindrance towards interactions to the nearest neighbor site (Sylianco, 1981).

From the results, it is clear that the 150 Kr irradiated papaya exhibits clastogenic actions as it significantly increased the number of micronucleated erythrocytes almost comparable to the number of micronucleated erythrocytes produced by tetracycline HCl. Such action could be due to chemicals or even free radicals produced by the irradiation of the papaya extract. No clastogenic activity was observed for 50 and 100 Kr and again, this could be due to the decline of their potency during storage.

The question of just what it is in the irradiated papaya extract that contributed to its mutagenicity and clastogenicity can not be answered directly as there was no chemical analyses performed to identify the substance. However, it is believed that this can be attributed to the free radicals formed in the extract during irradiation as well as secondary, tertiary and subsequent reaction products such as hydrogen peroxide, formic acid, glyxal, hydroxyalkyl peroxide and deoxycompounds arising from the radiolysis of carbohydrate and water (Keesavan and Swaminathan, 1970). The physical event and several several steps of the chemical change that take place in the irradiated medium and the reaction products, when enabled to diffuse in an active state into the metabolic pathway of unirradiated organism, could produce changes

in its DNA which could lead to aberrations in the chromosomal structure.

V. SUMMARY AND CONCLUSION

Papaya extracts subjected to gamma irradiation 50 Kr, 100 Kr and 150 Kr were tested in bacterial and mammalian host systems for their mutagenic and clastogenic properties after 8 days of storage in a freezer. Results obtained from the bacterial test system showed that papaya extract irradiated to a dose of 150 Kr could cause mutation in M45 Rec^- strain of Bacillus subtilis. No mutation was observed on the H17 Rec^+ strain using 150 Kr irradiated papaya extract.

Papaya extracts of lower dose level - 50 and 100 Kr did not cause mutation in both the Rec^+ and Rec^- strain of Bacillus subtilis after 8 days of storage.

Results obtained from mammalian system showed that irradiation of papaya extracts could cause chromosomal breakage in young erythrocytes extracted from the bone marrow of albino mice. Such clastogenicity however, was demonstrated only in those mice fed with 150 Kr irradiated papaya extract. Mice fed with extracts treated with lower levels of gamma rays (50 and 100 Kr) failed to show a significant increase in their micronucleated erythrocytes.

It is therefore concluded that 150 Kr irradiated papaya is effective in causing base-pair or frame-shift mutation as well as chromosomal breakage even after 8 days of storage.

VI. LITERATURE CITED

1. Barna, J. 1976. Review on International Wholesomeness Testing of Irradiated Foods and Feeds from 1926 to 1976. Publishing House of the Hungarian Academy of Sciences. 24.

2. Baumgartner, J. G. 1936. Ultraviolet irradiated carbohydrates and bacterial growth. Journal of Bacteriology. 32: 75-77 .

3. Blank, I. H. and W. Arnold. 1935. The inhibition of growth of Bacillus subtilis by UV-irradiated carbohydrates. Journal on Bacteriology. 30: 507-511. Thru Kesavan, P. C. and M. Swaminathan. 1971. Cytotoxic and mutagenic effects of irradiated substrate and food materials. Radiation Botany. 11: 253-281.

4. Chopra, V. L. 1965. Test on Drosophila for the production of mutations by irradiated medium or irradia- DNA. Nature. 208: 699-700.

5. Chopra, V. L. 1965. The effects of irradiated culture medium on bacteria. Microbiology Genetic Bulletin. 23: 8-9.

6. Couch, D. B. and M. Friedman. 1974. Suppression of dimethylnitrosoamine mutagenicity by sodium nitrite. Mutation Research. 26: 361-376.

7. Dag, J. and C. Rainel. 1980. The micronucleus test as part of a short term mutagenicity test program for the prediction of carcinogenicity evaluated by 143 agents tested. Mutation Research. 75: 191-212.

8. Dickey, F. H., G. H. Cleland and C. Lots. 1949. The role of organic peroxides in the induction of mutations. Prooeedings of the National Academy of Science. USA. 35: 581-583.

9. Ehrenberg, A. 1961. Research on free radicals in enzyme chemistry and in radiation biology. Academic Press, New York. 337-350.

10. Ehrenberg, A. and L. Ehrenberg. 1958. The decay of x-ray induced free radicals in p ant seeds and starch. Arkiv Fyisk. 14: 133-141.

11. Evans, T. C., J. C. Slaughter, E. B. Little and G. Failla. 1942. Influence of the medium on radiation injury of sperm. Radiology. 39: 663-680.

12. Frazier, W. C. and D. C. Westhoff. 1978. Food Microbiology. McGraw Hill Inc., New York. 24.

13. Jensen, K. A., I. Kirk, G. Kolmard and M. Westergard. 1951. Chemically induced mutations in Neurospora. Cold Spring Harbor Symposium on Quantum Biology. 16: 245-261.

14. Kesavan, P. C. and M. Swaminathan. 1970. Cytotoxic and mutagenic effects of irradiated substrates and food materials. Radiation Botany. 11: 253-282.

15. Kada, Tsuneo. 1974. Screening of pesticides for DNA interactions by Rec-Assay and mutagenesis testing and frameshift mutagens detected. Mutation Research. 26: 243-248.

16. Kuzin, A. M. 1962. On the role of the disturbance of metabolic processes in the radiation damage of the cells. International Journal on Radiation Biology. 6: 211-220.

17. Matter, B. E. 1976. Failure to detect chromosome damage in bone marro cells of mice and chinese hamsters exposed in vivo to some ergot derivatives. Journal on Internal Medicine Research. 4: 382-392.

18. Makinen. T.,M. Upadhya and J. Brewbaker. 1967. Cytotoxin effects on extracts from gamma irradiated pineapples. Nature. 214: 413.

19. Molin, N. and L. Ehrenberg. 1964. Abti-bacterial action of irradiated glucose. International Journal on Radiation Biology. 8: 223-231.

20. Natarajan, A. and M. Swaminathan. 1958. Indirect effects of radiation and chromosome breakage. Indian Journal of Genetics. 18: 220-223.

21. Odum, E. P. 1971. Fundamentals of Ecology. W. B. Saunders Co., West Washington Square, Phil delphia. 64.

22. Parkash, O. 1965. On the radiomimetic effect of irradiated DNA on Drosophila melano aster. Naturwissenchafter. 6: 142.

23. Schmid, W. 1975. The micronucleus test. Mutation Research. 31: 9-15.

24. Schmid, W. 1976. The micronucleus test for cytogenetic analysis. Thru Hollander, A. (ed.). Chemical Mutagens. New York. Plenum Press. 324-326 .

25. Tsuchimoto, T. and B. E. Matter. 1979. In vivo cytogenetic screening methods for mutagens, with special reference to the micronucleus test. Archives of Toxicology. 42: 239-248.

26. Schubert, J., J. A. Watson and P. R. White. 1967. Hydroxyalkyl peroxides and toxicity of irradiated sucrose. International Journal of Radiation Biology. 13: 485-489.

27. Stones, W. S., U. Wyss, and F. Haas. 1947. The production of mutations in Staphylococcus aureus by irradiation of the substrates. Proceedings of the National Academy of Science. USA. 33: 59-66. Thru Luber, R. P. 1981. Studies on Mutagenicity of irradiated mangoes using mice, bacteria, and Drosophila melanogaster as test organisms. M. S. Thesis, University of the Philippines. 34.

28. Sylianco, Clara Y. L. Structure and Function of Biomolecules. 1st edition. Aurum Technical Books. 62 .

29. Sylianco, Clara Y. L. 1981. Modern Biochemistry. Aurum Technical Books. Quezon City. 45.

30. Swaminathan, M. S., V. Chopra and S. Bhaskaran. 1962. Cytological aberrations observed in barley embryos cultured in irradiated potato mash. Radiation Research. 16: 182-188.

31. Swaminathan, M. S., S. Nirula, A. T. Natarajan and R. P. Sharma. 1963. Mutations : incidence in Drosophila melanogaster reared on irradiated medium. Science. 141: 637-638.

32. Vijayalaxmi, A. and C. Sadasivan. 1975. Chromosomal aberrations in rats fed with irradiated wheat. International Journal on Radiation Biology. 27:283.

33. Wagner, R. P., C. H. Haddox and W. S. Stone. 1950. The effect of irradiated medium, cyanide and peroxide on the mutation rate in Neurospora. Genetics. 35: 237-248.

34. Woodrow, J. W., A. C. Bailey and E. I. Fulmer. 1927. The effect of ultraviolet radiation upon yeast culture media. Plant Physiology. 2: 171-176.

VII. FIGURES AND PLATES

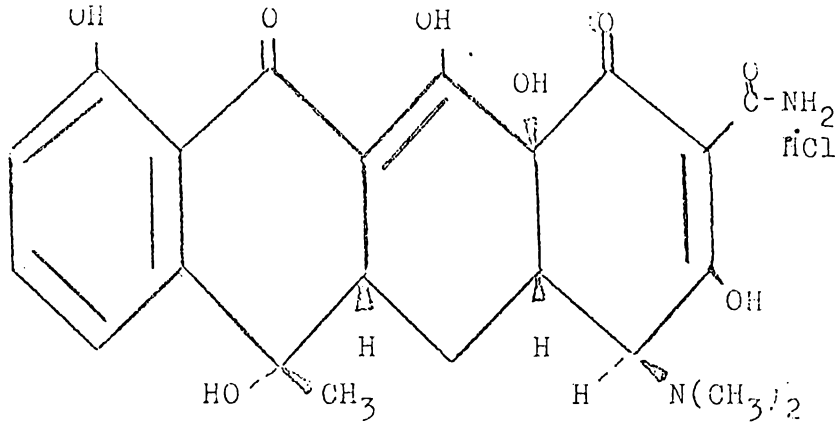


Figure 1: Tetracycline.HCl
(Source: Lehninger, 1979)

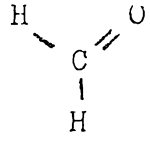
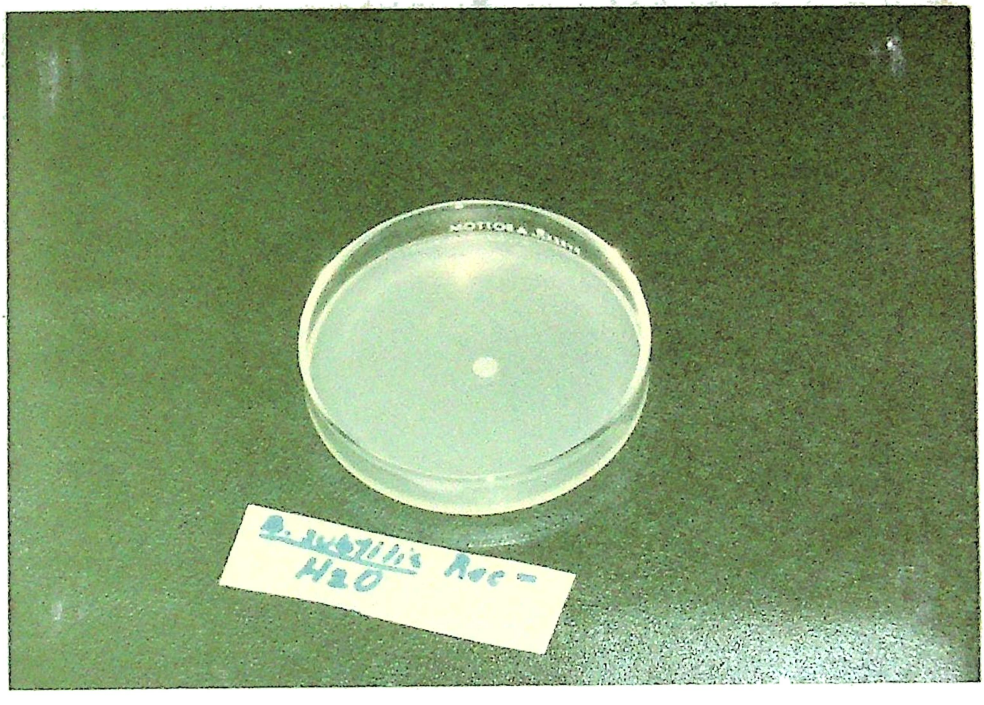


Figure 2: Formaldehyde
(Source: Morrison and Boyd, 1974)

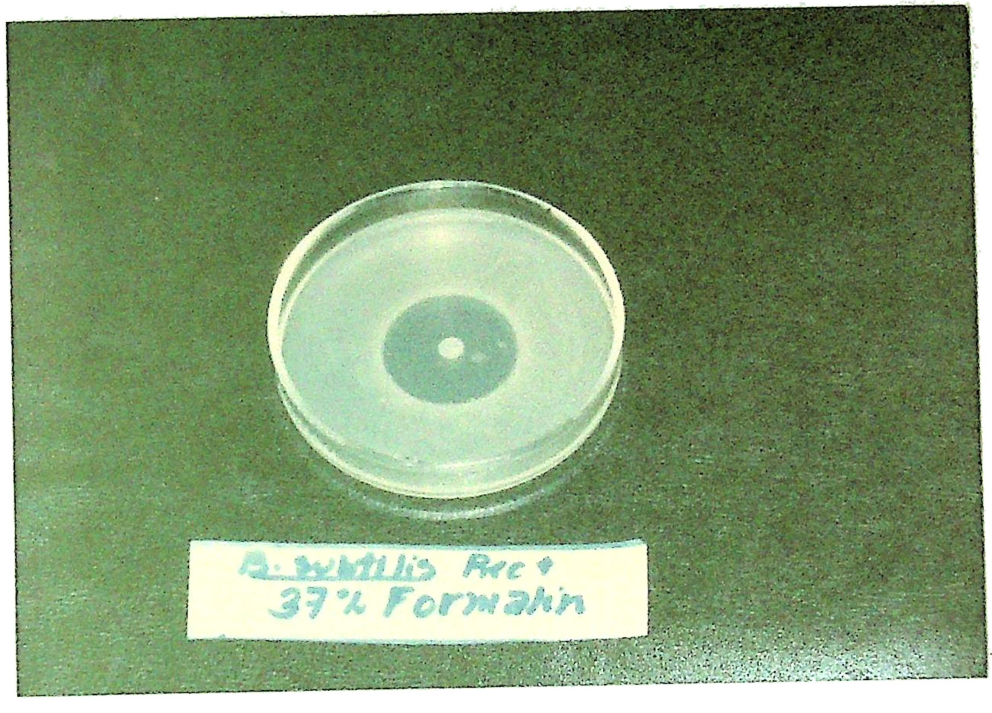
Negative Control Plate

Plate 1. Zone of Inhibition Produced on Bacillus subtilis Rec^+ by distilled water.



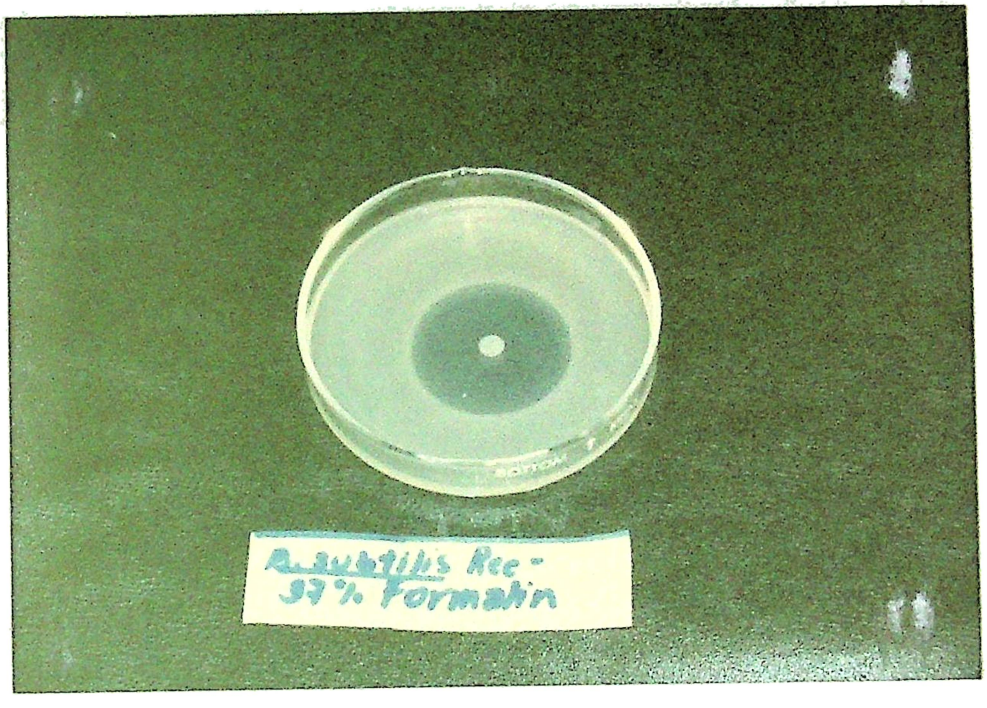
Negative Control Plate

Plate 2. Zone of Inhibition Produced on Bacillus subtilis Rec by distilled water.



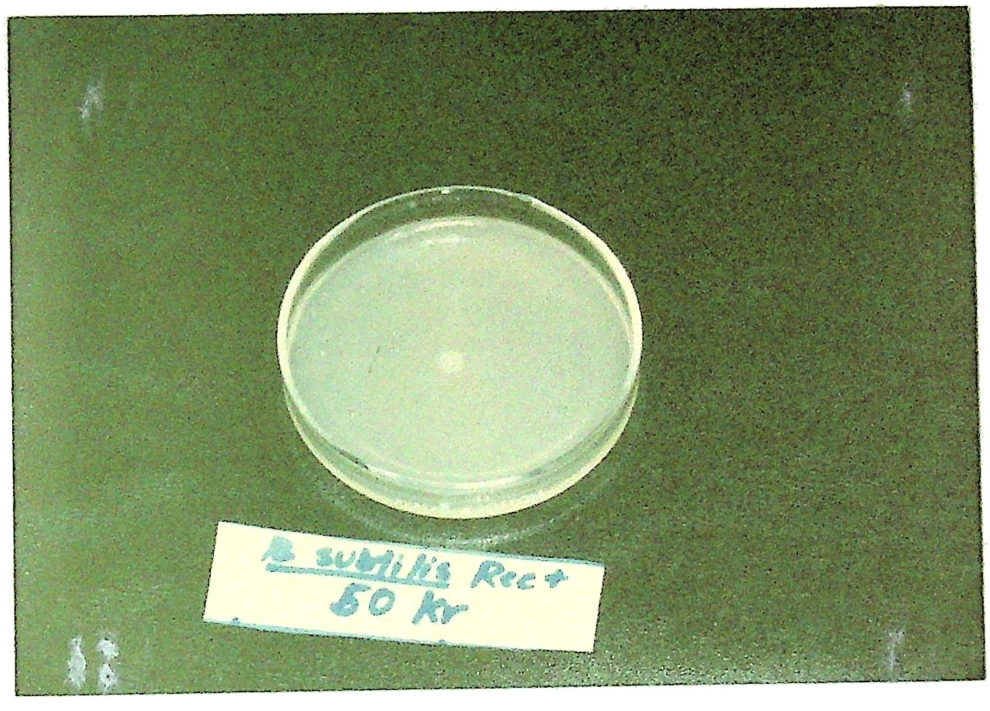
Positive Control Plate

Plate 3. Zone of inhibition produced on Bacillus subtilis Rec^{+} by 37% Formaldehyde



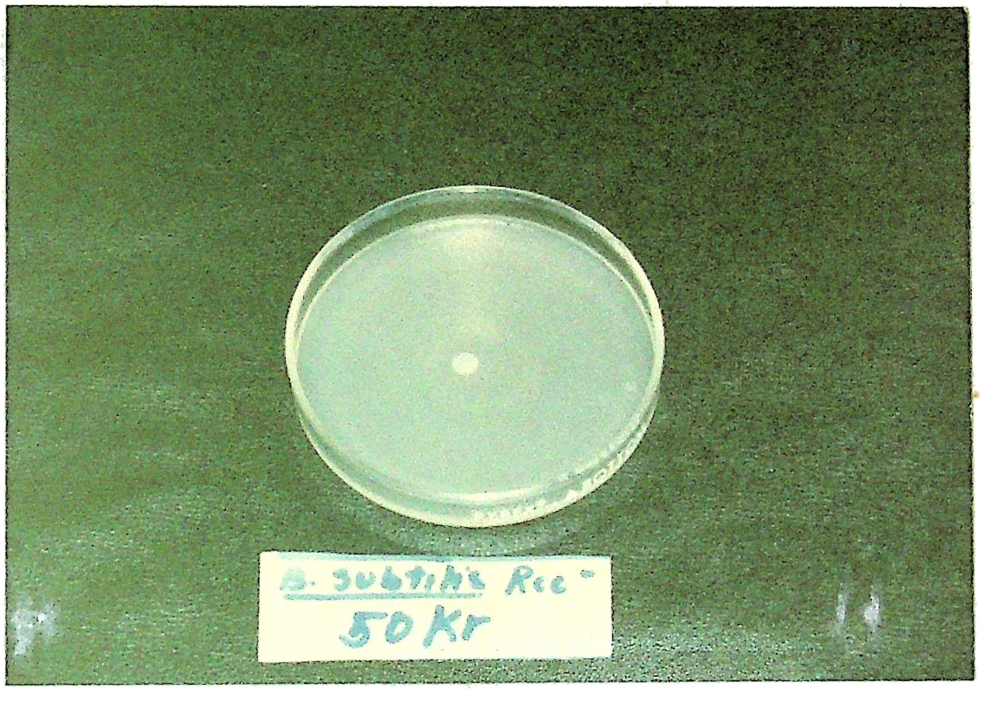
Positive Control Plate

Plate 4. Zone of Inhibition Produced on Bacillus subtilis.Rec- by 37% Formaldehyde



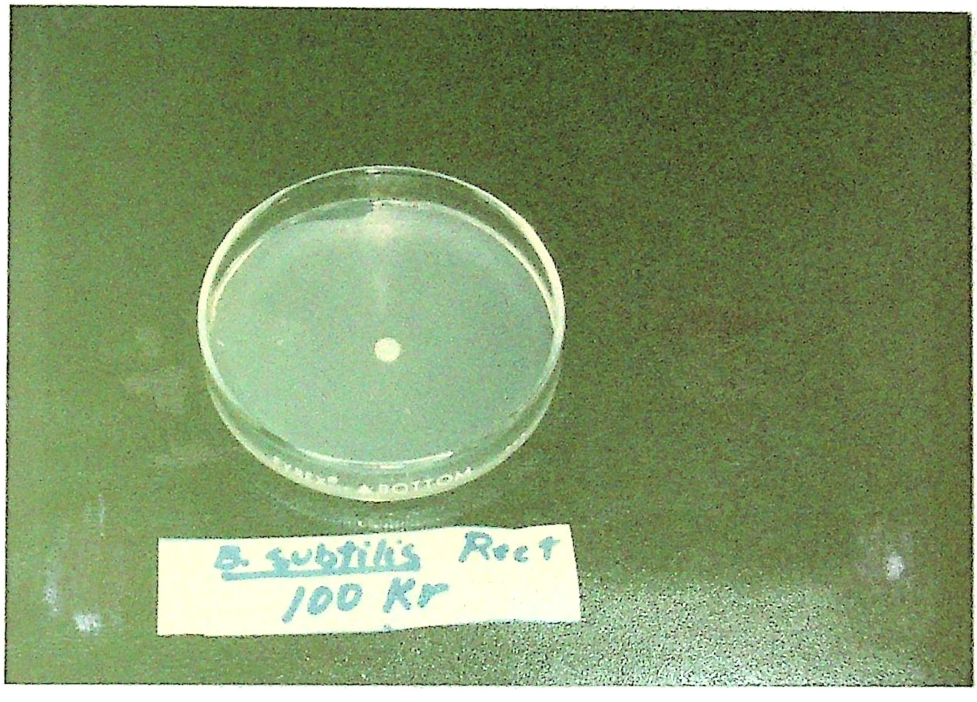
Experimental Plate

Plate 5. Zone of Inhibition Produced on Bacillus subtilis Rec^{+} by Papaya Extract Irradiated at a Dose Level of 50Kr



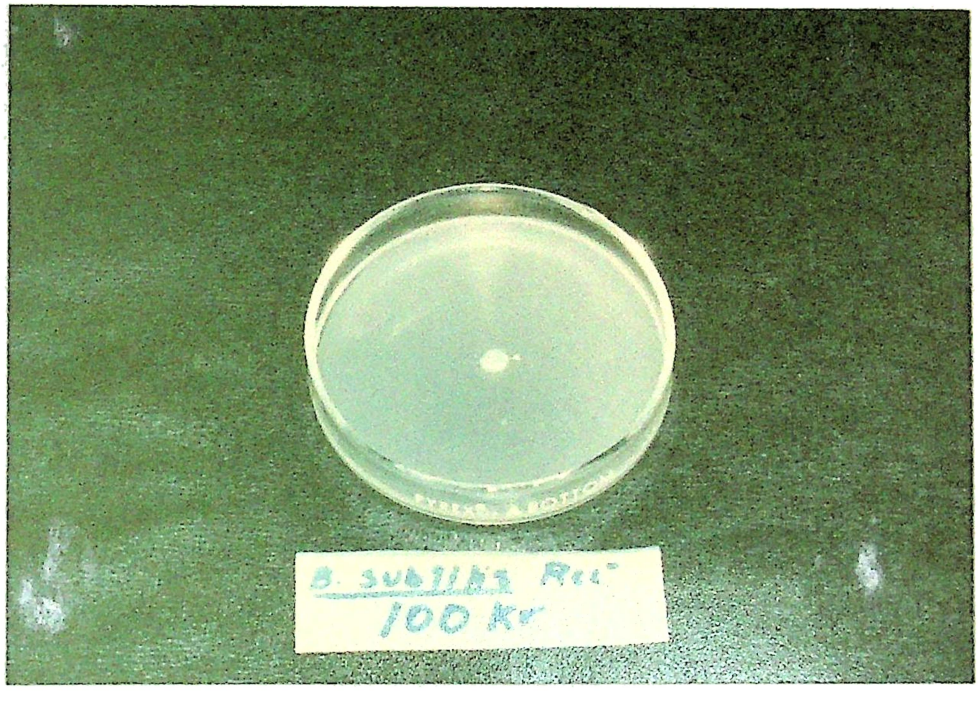
Experimental Plate

Plate 6. Zone of Inhibition Produced on Bacillus subtilis Rec⁻ by Papaya Extract Irradiated at a Dose Level of 50 Kr



Experimental Plate

Plate 7. Zone of Inhibition Produced on Bacillus subtilis Rec^+ by Papaya Extract Irradiated at a Dose Level of 100Kr

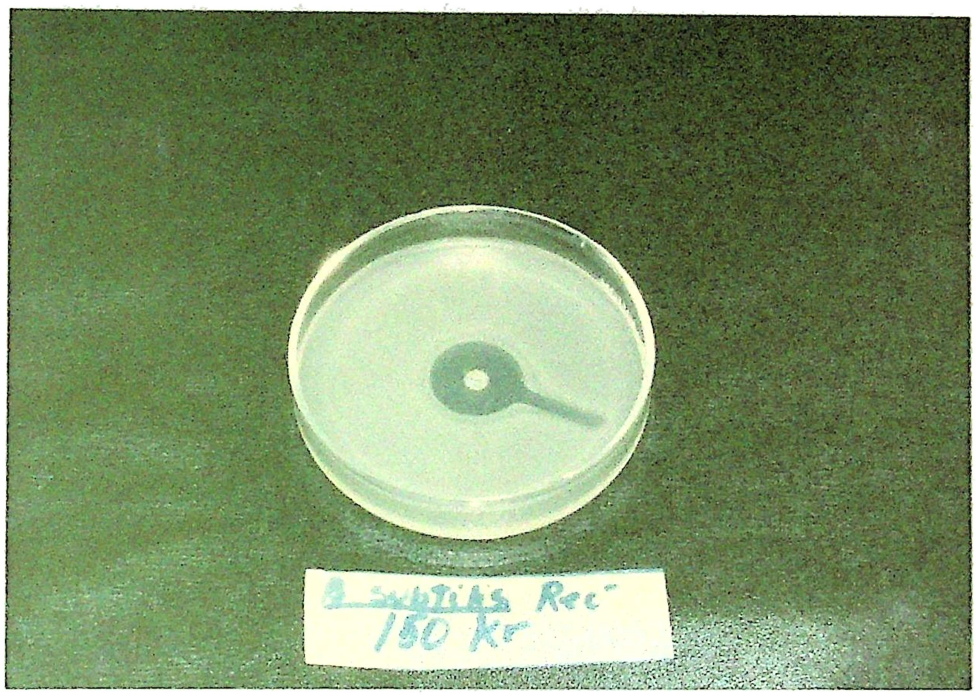


Experimental Plate

Plate 8. Zone of Inhibition Produced on *Bacillus subtilis* Rec by Papaya Extract Irradiated at a Dose Level of 100Kr

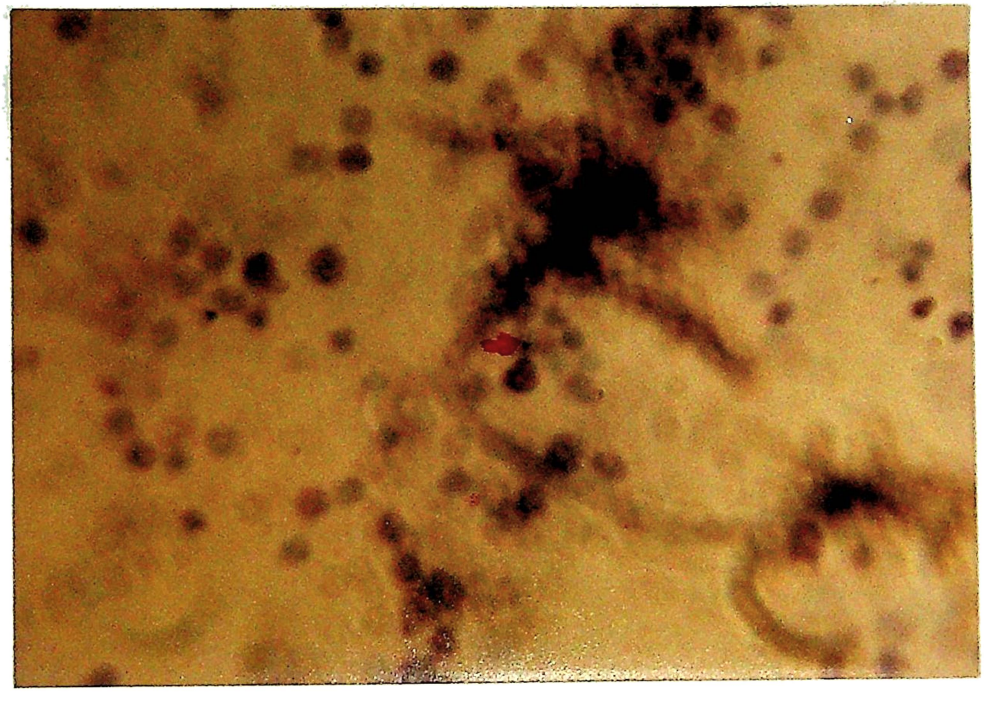
Experimental Plate

Plate 9. Zone of Inhibition Produced on Bacillus subtilis Rec$^+$ by Papaya Extract Irradiated at a Dose Level of 150Kr.



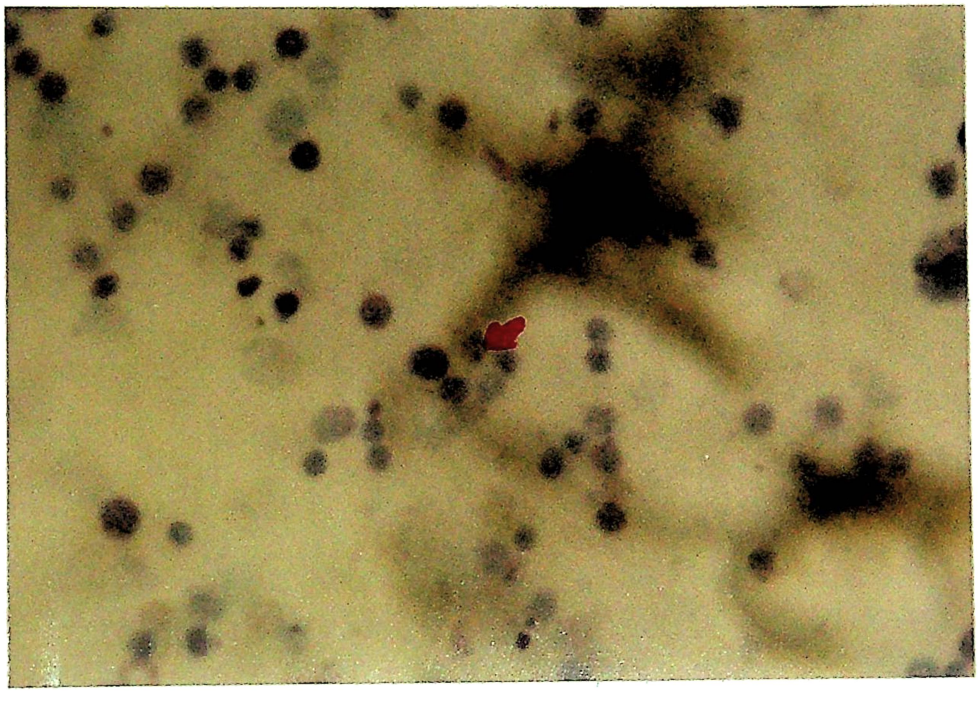
Experimental Plate

Plate 10. Zone of Inhibition Produced on *Bacillus subtilis* Rec⁻ by Papaya Extract Irradiated at a Dose Level of 150kr.



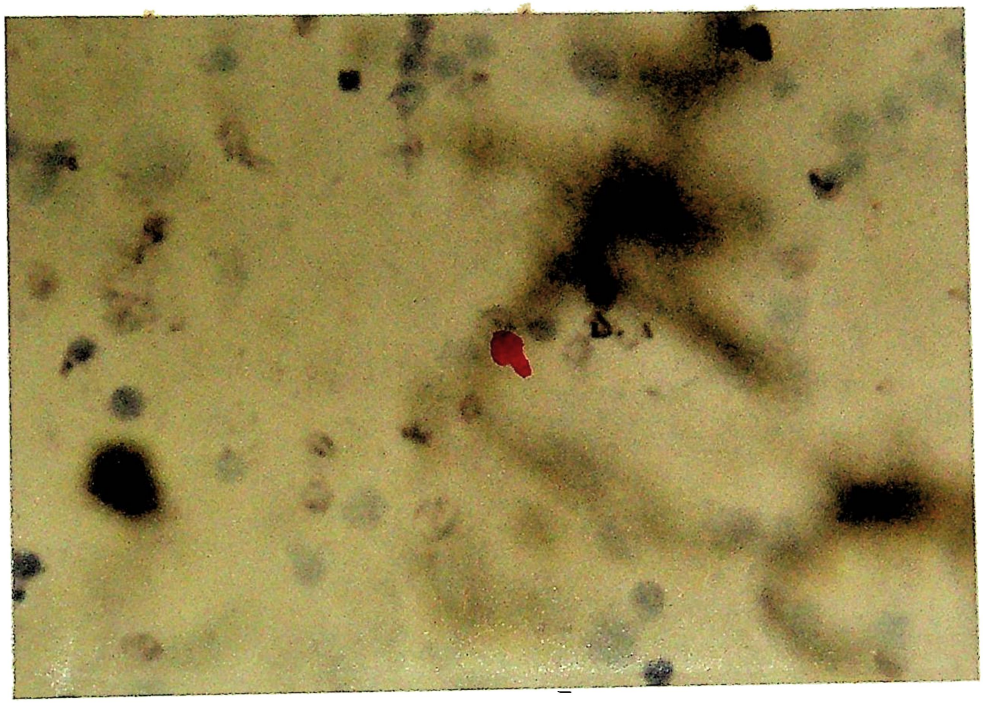
Negative Control

Photomicrograph 1. Bone marrow cells extracted from mouse treated with distilled water. Arrow shows micronucleated polychromatic erythrocyte.



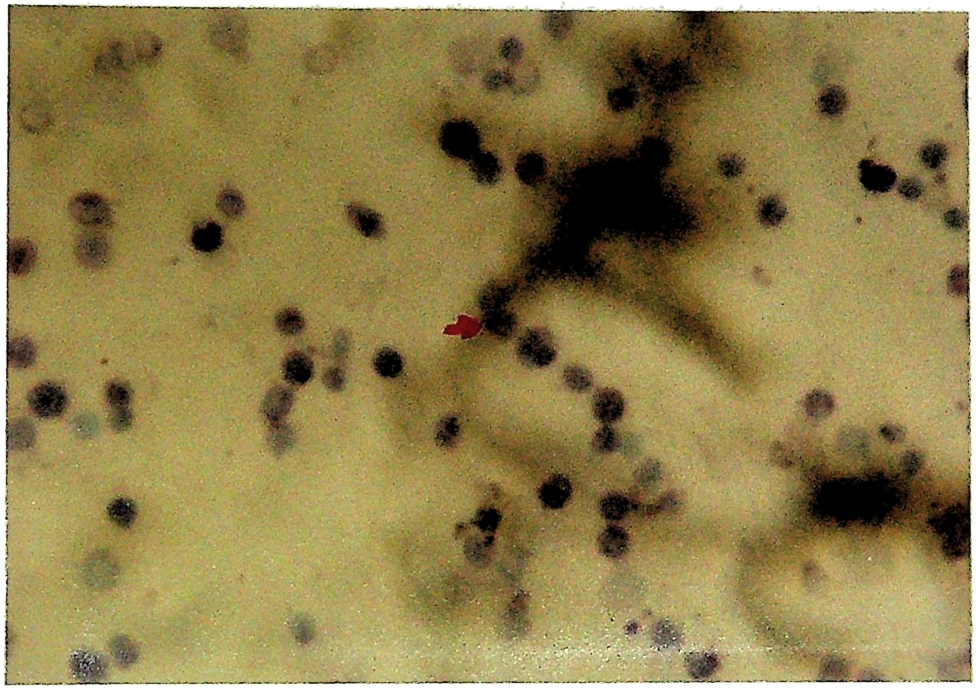
Positive Control

Photomicrograph 2. Bone marrow cells extracted from mouse treated with Tetracycline.HCl. Arrow shows micronucleated polychromatic erythrocyte.



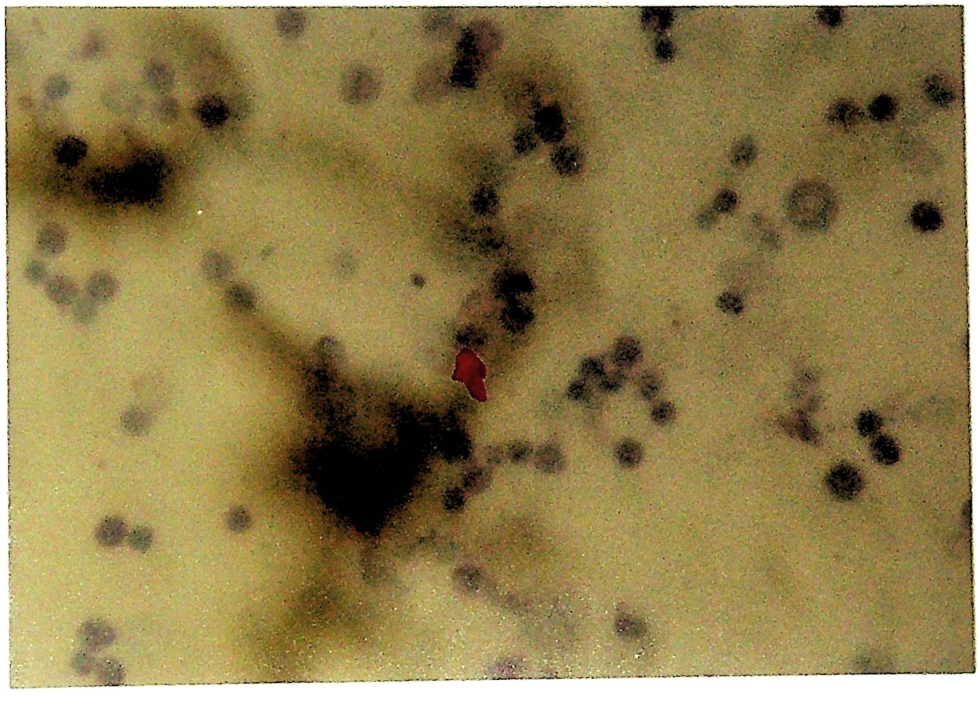
Experimental Treatment

Photomicrograph 3. Bone marrow cells extracted from mouse treated with papaya extract irraidated at a dose level of 50Kr. Arrow shows micronucleated polychromatic erythrocyte.



Experimental Treatment

Photomicrograph 4. Bone marrow cells extracted from mouse treated with papaya extract irradiated at a dose level of 100Kr. Arrow shows micronucleated polychromatic erythrocyte.



Experimental Treatment

Photomicrograph 5. Bone marrow cells extracted from mouse treated with papaya extract irradiated at a dose level of 150Kr. Arrow shows micronucleated polychromatic erythrocyte.

VIII APPENDICES

Appendix A: Preparation of stains for the Micronucleus Test

Giemsa Stain - Giemsa crystal - 4 grams
Glycerine - 250 ml.
Methyl alcohol - 250 ml.

May-Grunwald Stain - May-grunwald - 1 gram
Methyl alcohol - 400 ml.

Appendix B: Statistical Analysis for Rec-Assay Using a Factorial Design.

Summary Table:

Extract	Summation of zones of inhibition Rec$^+$	Rec$^-$	Total
distilled water	0	0	0
37% formaldehyde	8.9	10.6	19.5
papaya - 150Kr	0	7.3	7.3
papaya - 100Kr	0	0	0
papaya - 50Kr	0	0	0
Total	8.9	17.9	26.8

$$\text{Correction factor (CF)} = \frac{(\text{Grand Total})^2}{50}$$

$$= \frac{(26.8)^2}{50} = 14.36$$

$$SS_{Total} = \sum\sum x^2 - CF = 49.06 - 14.36 = 34.70$$

Hypotheses:

Ho#1 = There is no significant difference between the zones of inhibition produced by the different extracts.

Ho#1' = There is a significant difference in the zones of inhibition produced by the different extracts.

Ho#2 = There is no significant difference between the zones of inhibition produced in the two strains of Bacillus subtilis.

Ho#2' = There is a significant difference between the zones of inhibition produced in the two strains of Bacillus subtilis.

Ho#3 = The interaction between the strains of Bacillus subtilis and the extracts is not significant.

Ho#3' = The interaction between the strains of Bacillus subtilis and the extracts is significant.

$$SS_{extract} = \frac{(19.5)^2 + (7.3)^2}{10} - CF$$

$$= \frac{433.54}{10} - 14.36$$

$$= 28.99$$

$$SS_{organism} = \frac{(17.9)^2 + (8.9)^2}{25} - CF$$

$$= \frac{399.62}{25} - 14.36$$

$$= 1.62$$

$$SS_{interaction} = SS_{total} - (SS_{organism} + SS_{extract})$$

$$= 34.70 - (1.62 + 28.99)$$

$$= 4.09$$

$$SS_{treatment} = \frac{0^2+0^2+10.6^2+ 8.9^2+7.3^2+0^2+0^2+0^2+0^2+0^2}{5} - CF$$

$$= 34.6$$

$$SS_{error} = SS_{total} - SS_{treatment}$$

$$= 34.70 - 34.60$$

$$= 0.1$$

summary table:

SV	SS	df	Ms	F comp.	F tabular $\alpha = .05$
extract	28.99	4	7.25	2,900	2.61
organism	1.62	1	1.62	648	4.08
interaction	4.09	4	7.72	409	2.61
error	0.1	40	0.67		

Conclusions:

$SS_{extract}$ - Reject Ho#1
Accept Ho#1' - There is a significant difference in the zones of inhibition produced by the different extracts.

$SS_{organism}$ - Reject Ho#2
Accept Ho#2' - There is a significant difference between the zones of inhibition produced in the two strains of Bacillus subtilis.

$SS_{interaction}$ - Reject Ho#3
Accept Ho#3' - The interaction between the strains of Bacillus subtilis and the extract is significant

Appendix C: Statistical Analysis for Micronucleus Test Using Complete Randomized Design (CRD)

Summary Table:

Extract	Total of five trials
distilled water	35
tetracycline·HCl	162
papaya - 150Kr	151
papaya - 100Kr	40
papaya - 50Kr	37
	425

Ho#1 = uH_2O = utetracycline·HCl = upapaya 150Kr = upapaya 100Kr = upapaya 50Kr

Ho#1'= not all u's are equal

α = 0.05

Statistical Test: $F = \frac{\text{MS Treatment}}{\text{MS error}}$

$SS_{total} = \sum x^2 - CF \qquad CF = \frac{(GT)^2}{N} = \frac{(425)^2}{25} = 7225$

= 10735 - 7225

= 3510

$$SS_{treatment} = \frac{(\text{Treatment total})^2}{\text{treatment sample size}} - CF$$

$$= \frac{(35)^2 + (162)^2 + (151)^2 + (40)^2 + (37)^2}{5} - CF$$

$$= \frac{53239}{5} - 7225$$

$$= 3422.8$$

$$SS_{error} = SS_{total} - SS_{treatment}$$

$$= 3510 - 3422.8$$

$$= 87.2$$

Summary Table:

Source of variance	sum of square	df	mean of square	F ratio
treatment	3422.8	4	855.7	196.26
error	87.2	20	4.36	

$F_{tabular}$ (0.05, 4, 21) = 2.84

Conclusion:

Reject Ho#1

Accept Ho#1' - not all u's are equal