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In-vitro comparative studies : Three techniques for comparing the mutagenicity, genotoxicity of irradiated and processed food

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ABSTRACT

The present investigation was performed by three techniques for comparing toxicity in treated food resulted after irradiation with food processing. The extract of the inhibited sprouts potatoes (EISP) was used, the first was Ames test using Salmonella/reversion assay in two strains of *S. typhimurium* namely TA98 and TA100, second was chromosomal aberrations (CA)techniques. Besides using samples of EISP after irradiation at low dose (0.01kGy)-the applied recommended dose on commercial scale-through boiling or freezing. Whereas,another samples of food as coffee beans were used for genotoxicity test in the last one after irradiation (5.0 and 10.0kGy) or roasting (200 ^{0}C) for short (10 min.)or long time (20 min). The obtained results of Ames test showed slight mutagenic agent, for all the tested irradiate samples. Only ethyl alcohol extract (EAE) showed high significant values, whereas, whole sprouts or residue has less values approximately. Using EAE recorded high significant values as 93,115,120 ig/plate by using 0.01,1.0 and 10.0 kGy at TA 98 respectively. Whereas, using TA 100 recorded 92,115.0,124.0 ìg/plate by using 0.01,1.0 and 10.0 kGy respectively. A significant linear dose-response relationship was resulted with correlation coefficient R^2 were resulted after using different doses which use for decontamination in food. These values were in proportion to irradiation dose and fortunately CA, whereby check (EISP) at low dose (0.01 kGy),with different concentration of EAE (1.0, 0.5,0.05 and 0.005%), different types of CA. All these types showed percent of CA which proportion with EAE (%)of irradiated samples at 0.01 kGy. These toxic compounds were more affected markedly by storage freezing or genotoxicity test results markedly higher values in roasted samples at 200 ^{0}C either at short time 10 min.(light coffee) or long time 20 min.(dark coffee), recorded high values of genotoxicity (BN/MN). Whereas, irradiation caused less values.

Finally, there are toxic compounds resulted after irradiation process in the inhibited cells even at low doses but the mutagenicity were less at the applied dose and less than resulted from processed foods. Furthermore, safety food studies are needed.

KEYWORDS

Safety food;
Ames test;
Chromosomes aberrations;
Genotoxicity;
Irradiated food.

Regular Paper

INTRODUCTION

Cooking of food is a process unique to humans. It enhances the taste and the digestibility of food so much so that its beneficial nature is taken for granted; however, it induces profound changes in all types of food. It has been well established that these changes may be of concern to human health[1,2]. General cooking procedures such as broiling, frying, barbecuing, heat processing and pyrolysis of protein rich foods like beef, chicken and fish induce the formation of potent mutagenic and carcinogenic compounds called heterocyclic amines[3-7,10]. These are potent mutagens and carcinogens in rodents, inducing tumors of several organs[5]. In Egypt, processed ready meals studies showed high values of genotoxicity[6].(TABLE 1). The authors showed that different food processing produced some food reactions due to high temperature during processing as Millard reaction, Carmalization[3-7,10]. However, these compounds also occur more widely as environmental pollutants through emission from a wide range of combustion sources including vehicle exhausts, furnaces, etc. and may also enter the human food chain through deposition on the surface of food crops[11].

TABLE 1 : The genotoxicity of ready meals as collected from local markets in Egypt.

Samples	Average ± SD	Samples	Average ± SD
Blank samples	2.0±0.707		
Roasted bread spots	14.25±3.4	Corn-rice crisps (Sandose)	5.75±2.5
Potato chips additives free	44.75±5.76	Poultry (roasted)	30.5±8.29
		Fish (grilled with wheat bran)	18.5±6.1
cheese tasty	12.63±2.2	Sweet candy (caramel candy)	12.5±3.28

Food irradiation considered as safety treatments as alternative pesticides. To date, health and safety authorities in over 60 countries worldwide have approved the application of food irradiation. Whereas, more 40 countries used food irradiation on commercial scale for sterilize spices, herbs, chicken besides others near 120 product.

In spite of successful of using food irradiation on commercial scale but another groups like Germany and other countries have apprehensive for using irradiation. Mutagenicity studies either *in vitro-vivo* showed that famous mutagenic compound is 2-alkylcyclobutanones (2-ACBs) which can cause colon tumor as resulted from fatty food after irradiation. That a compound found exclusively in irradiated dietary fats may promote colon carcinogenesis in animals treated with a chemical carcinogen[12]. But WHO (Joint FAO/WHO, 2002)[13] announced after long –term feeding studies, 2-DCB and 2-alkylcyclobutanones in general do not appear to pose a health risk to consumers of irradiated fatty diets. Same compound showed weak positively effect of mutagenicity *in-vitro* in rats. Also, feeding Irradiated potatoes or different diets at low doses caused changes behavior, abnormal growth and fertility of different stages of insects[14-17].

To day, still now some questions about the reasons of presence toxicity of irradiated diets either using in extract form or dry. Therefore, our task in the present work to cover the knowledge lack around safety of damaged cells after inhibition sprouts by irradiation besides determination the toxicity resulted after irradiation separately or combined with food processing.

MATERIAL AND METHODS

Ames test

Preparing samples

The inhibition of sprout by irradiation depend on damage of DNA in meristimatic cells. Therefore, in the present work, we used meristimatic cells of potatoe sprouts. These parts were removed from sprouted potatoes areas then irradiated to different doses (0.0,0.01,1.0 and 10.0 kGy). Assay of the mutagenicity for Ames test was done with sprouts of potatoes, in three forms, fresh irradiated and non irradiated sprouts alcohol extracts (IPSAE), residue after extract and powder of whole sprouted.

Amest test

Mutagenic effects of treated samples were assayed according to the Ames test using *S. typhimurium* strains TA98 and TA100[18]. The Ames mutation assay was performed with modification samples (500µl),filtered by using 0.45µm HAWP 01300 Millipore filter for sterilization and for termination of the microsomal activating reaction. More than 90% of the mutagenicity was recovered in the filtrate by using this filtration procedure. The mixture (10 µL). was poured onto a 400 µL of 100

Regular Paper

mM sodium phosphate (pH7.4) plus 100 µL of the bacterial suspension. The mixture was incubated at 37^{0}C for 10 min to ensure the contact of the mutagen,2ml of molten soft agar was poured gently onto minimal glucose agar plates containg 0.1 µmol of L histidine and 0.1 µmol of biotin. The histidine revertants (His+ revertant colonies) were counted after incubation at 37^{0}C for 48h. The number of spontaneous revertants (18-25rev./plate) was susbtracted from each revertants count. The data represented are the means of four replicates. All steps of that techniques according[18].

Chromosomal aberrations

Aqueous extract of treated or untreated samples were added to culture at different concentrations (1.0%, 0.5%,0.05% and0.005%)being dissolved in media 199. The culture media was prepared according to recommended methods[19,20]. Each culture 5ml of media 199(Gibco)contained 5 µg reconstituted phytohaemoglutinin and 20%foetal calf serum (Gico). Blood human lymphocytes obtained from four donors were added to each culture tube. Inculation was done at 37^{0}C,duration of culture extended to 48 hrs., colchicines was added at time three hrs before end the period (48 hrs.) with concent.10 µg/ml, then cells was harvested then fixed with Gremsa.(ALEX). One hundred metaphases were counted per each concentration, where the frequency of dicentrics rings,acentrics, gaps chromated and chromosome breaks were recorded.

GENOTOXICITY EXPERIMENT

Genotoxicity tests using human to measure the toxicity of some processed ready meal and some irradiated samples.

Preparing samples

Coffee green beans were collected from local markets to carry the following experiments. Coffee beans irradiated to 5.0 and 10.0 kGy. Besides roasted coffee beans at 200 ^{0}C for two periods,as10 min. and 20 min. After roasting, beans were cooled to 25 ^{0}C while the third considered as positive control. All These samples were checked for genotoxicity test.

Genotoxicity test

Preparation of blood samples

Heparinzed blood was obtained from six healthy, non-smoker volunteers who had no recent diagnostic or occupational exposure to ionizing radiation, laser, or chemicals and had not had any experience to recent allergic responses or drug administration. Buffy coats were separated and concentrated in plasma at a cell density of 2 x 10^5 cells /100ml. Aliquots of cells were distributed in 96 well tissue culture plates. Every treatment of six individuals was investigated in duplicate.

Cell culture

Immediately, cells were transferred into 15-ml sterile tubes containing only media 199 (Sigma, Saint Loius MO, USA). Cells were incubated for 72 hours, adding cytochalasin B 48 hours before harvesting[21].

Harvesting of cells

Forty eight hours after the addition of cytochalasin B, cells were collected and treated with 0.8 % sodium citrate for 3-5 minutes and then fixed in 5:1 methanol: acetic acid. Fixed cells were dropped gently onto clean microscope slides, air-dried and stained with 4% Giemsa (Sigma, Sigma, Saint Loius MO, USA) using standard procedures.

Scoring under the microscope

Slides were scored at 1000X magnification using a Leica Biomed microscope (Leica Lasertechnik GmbH, Heidelberg, Germany). Identification of cytokinesis blocked binucleatid cells and the frequencies of micronuclei in such cells were estimated according to the criteria stated. Binucleatid cells were selected on the basis of having a well-preserved cytoplasm with two distinct nuclei of approximately equal size, which may be attached by a fine nucleoplasmic bridge or alternatively be overlapped. The micronuclei scored were therefore located within the cytoplasm and were not refractile nor linked to the main nuclei via nucleoplasmic bridge. From each culture the ratio of binucleotide (BN) to mononucleotide cells (MN) was determined by counting the number of BN per 2000 MN. Additionally 500 binucleated cells were scored for micronuclei.

RESULTS

Ames Test

The results of Ames test using *S. typhimurium* TA98 and TA100 (as shown in TABLES 2–3 and Figures 1-

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4) clearly indicate that used extracts is a slight mutagenic agent, that could effectively mutagenicity all the tested irradiate samples. Only ethyl alcohol extract (EAE) showed high significant values of mutagenicity, whereas, whole sprouts and residue has less values approximately. Using EAE recorded high significant values as 93,115,120 ìg/plate by using 0.01,1.0 and 10.0 kGy after tested by TA 98 respectively. Whereas, using TA 100 recorded 92,115.0,124.0 ìg/plate by using 0.01,1.0 and 10.0 kGy respectively.

TABLE 2 : Effect of different extracts of irradiated sprouts potatoes on mutagenicity (revealants/plate) of TA 98,TA100 strain of *Salmonella typhimurium* ,*Significant, ** High significant., all values are expressed as mean ± S.D. (n = 6)

TA 98			
Whole(powder)	Residual	Ethyl Extract	Dose (kGy)
21±1.3	21±1.6	18±1.0	Blank
19.0±2.2	28.5±1.3	70.2±10.3	Control
30.0±2.6**	25.8±1.1	93.3±4.3*	0.1
39.7±1.9**	50.0±4.1**	115.8±5.1**	1.0
41.2±2.5**	65.7±2.8**	120.0±12.9**	10.0
4.89	4.121	17.68	LSD 5%
6.07	5.92	25.42	LSD 1%
Y=6.1x+11.9	Y=11.3x+4.8	Y=23.9x+8.5	Linear equation
0.8801	0.8505	0.9003	R^2
TA 100			
21±1.4	21±1.5	18±1.0	Blank
18.5±2.1	28.3±1.3	65.5±3.2	Control
30.8±1.5**	26.0±1.2	92.0±2.1**	0.1
39.8±2.7**	51.5±2.3**	115.0±3.1**	1.0
41.8±0.5**	64.8±2.5**	124.0±4.2**	10.0
1.73	2.44	4.98	LSD 5%
2.49	3.50	7.09	LSD 1%
Y=6.3x+11.7	Y=11.2x+4.8	Y=26.1x+4.7	Linear equation
0.8915	0.8608	0.9332	R^2

A linear dose-response relationship was observed in TABLE 1, a significant values of correlation coefficient R^2 were resulted after using different doses. These values were in proportion to irradiation dose and fortunately, the low values were recorded like the applied low dose as 0.01 kGy. The low doses were recorded values near control treatment. A logartimatic values- in relative to control - a relationship was resulted as shown in (Figures 3, 4).

TABLE 3 : The cytogenic analysis of the frequency of chromosomal aberrations effects of aqueous extract from irradiated samples(0.01kGy) at different doses after irradiation and storage (120days).

Typed of Chromosomal aberrations					Abnormal cells (count)	Normal cells (count)	Treatments (concent %)
Gs	Dic.	Acen.	Chs b	Cd b			
							Un irradiated
1	0	1	0	2	4	96	1
1	0	1	0	1	4	96	0.5
2	0	0	0	0	2	98	0.05
1	0	0	0	0	1	99	0.005
							Irradiated (0.01 kGy)
5	6	12	7	15	45	55	1.0
3	4	10	4	12	33	67	0.5
2	2	11	--	10	25	75	0.05
4	0	1	--	2	7	93	0.005
							After storage 120 days:
							Un irradiated
1	0	1	0	2	4	96	1.0
2	0	1	0	1	4	96	0.5
2	0	0	0	0	2	98	0.05
1	0	0	0	0	1	99	0.005
							Irradiated (0.01 kGy)
1	1	1	1	11	15	85	1.0
2	-	-	-	10	12	88	0.5
1	-	-	-	-	1	99	0.05
-	-	-	-	-	-	100	0.005

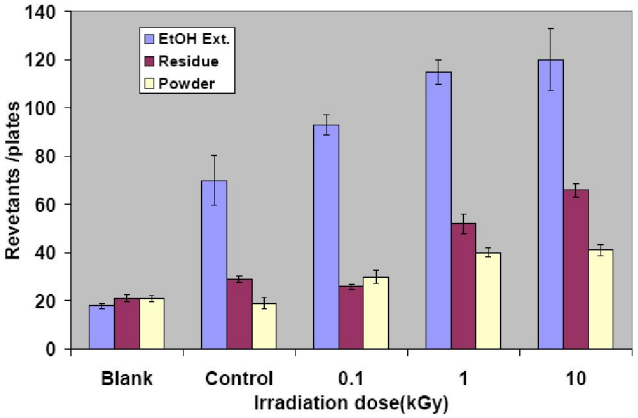

Figure 1 : Effect of different extracts of irradiated sprouts potatoes on mutagenicity (revealants/plate) of TA 98, strain of *Salmonella typhimurium*. (all values are expressed as mean ± S.D.,n = 6).

Chromosome aberrations (CA)

As shown in (TABLES 3-4),low concentrations of alcohol extract (1.0, 0.5,0.05 and 0.005%), used

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to follow the chromosome aberrations. Different types were involved for abnormal chromosomes as chromated breaks, chromosome break, centric, di-centrics, gaps, absence of metaphase besides cell death. All these types showed different figures of abnormality types. Generally,the percent of (CA) was proportion with concentration (%) of ethyl alcohol extract.(EAE) of irradiated samples. High percent of CA was higher markedly after irradiation directly then decreased gradually even after four months of storage samples of potatoes. In the same time, after storage period (120 days). high dose (1.0 kGy) recorded high values of CA than low dose significantly. In the same time these percent of CA was higher after food processing as boiling or freezing either using different concentration of EAE (TABLES 3-4) and (Figure 5).

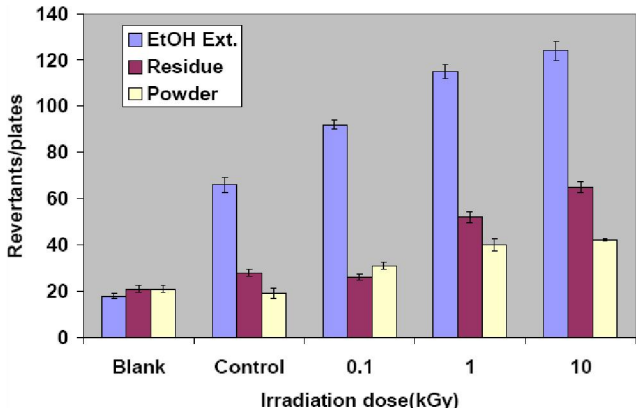

Figure 2 : Effect of different extracts of irradiated sprouts potatoes on mutagenicity (revealants/plate) of TA 100, strain of *Salmonella typhimurium*. (all values are expressed as mean ± S.D., n = 6).

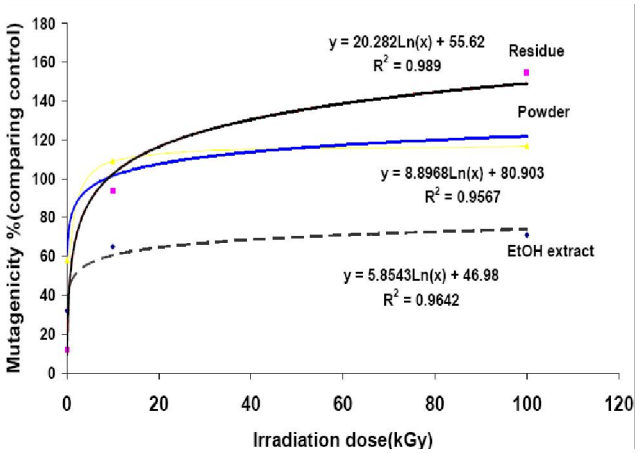

Figure 3 : Linear regression analysis (log values) between different concentrations extract of irradiated sprouts potatoes and mutagenicity (revealants/plate) of TA 98, strain of *Salmonella typhimurium*. (all values are expressed as mean ± S.D., n = 6).

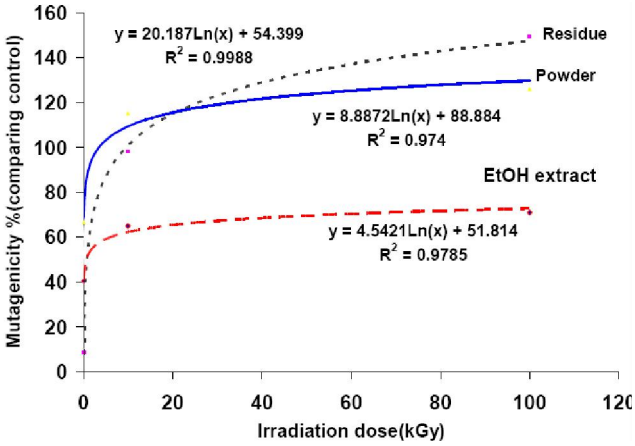

Figure 4 : Linear regression analysis (log values) between different concentrations extract of irradiated sprouts potatoes and mutagenicity (revealants/plate) of TA 100, strain of *Salmonella typhimurium*. (all values are expressed as mean ± S.D., n = 6).

TABLE 4 : Effect of food processing on cytogynic effects of aqueous extract from irradiated samples (Cd b=Chromated breaks, Chs b=Chromosome break, Ace=a centric, Dic=dicentrics, Gs=gaps and no rings was recorded. -No metaphase , *=cell death).

Typed of chromosomal aberrations					Abnormal cells (count)	Normal cells (count)	Treatments (concent. %)
Gs	Dic.	Acen.	chs b	Cd b			
							Freezing after irradiation:
8	8	10	7	28	61	39	1.0
5	4	7	5	21	42	58	0.5
4	2	8	2	11	27	73	0.05
2	0	4	1	5	12	88	0.005
							Boiling after irradiation :
6	10	12	8	32	68	32	1.0
7	6	9	5	21	48	52	0.5
5	4	5	0	12	26	74	0.05
2	1	2	2	4	11	89	0.005

Genotoxicity

The third techniques was performed for testing the potentiality of producing genotoxicity substances either by irradiation or processing food for coffee green beans. The results of treated coffee are shown in (TABLE 5). The roasted samples at 200 ^{0}C either at short time 10 min.(light coffee) or long time 20 min.(dark coffee), recorded high values of genotoxicity (BN/MN). Whereas, irradiation caused less values. The genotoxicity values

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were significantly difference than blank value, these values were (35.2 ± 5.2), (44.75±5.7) for roasted coffee at 10,20 min. Whereas, less values of genotoxicity were clear significantly in irradiated samples as (19.7±3.3),(17.5±2.3) for 5.0 and 10.0kGy in respectively. A linear relationship between roasting time, irradiation doses and the ratio of BN/MN of genotoxicity (Figure 5). These results were significant with high values of correlation coefficient (R^2).

TABLE 5 : Ratio of binucleotide (BN) to mononucleotide cells (MN) as influenced by irradiation doses and roasting process for coffee beans.

Average ± SD	Treatments	
6.3±3.3	Blank	
	--Coffee beans	
22.0 ±6.2	-control-	
19.7±3.3	-Irradiation	5.0 kGy
17.5±2.3	-	10.0 kGy
	-Roasting 200C:	
35.2 ± 5.2	-	10 minutes
44.75±5.76	-	20 minutes

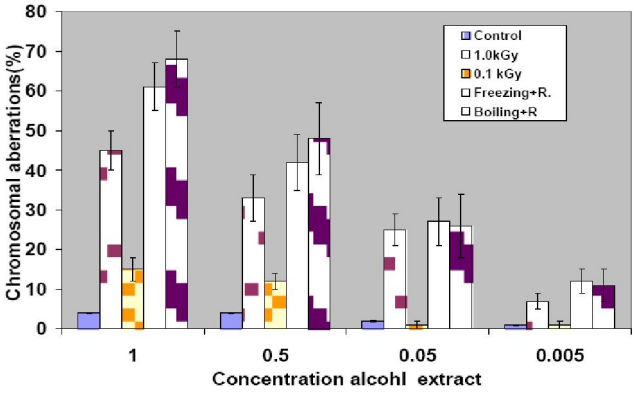

Figure 5 : The frequency of abnormal chromosomal aberrations after irradiation (0.01kGy), storage period (120days) besides

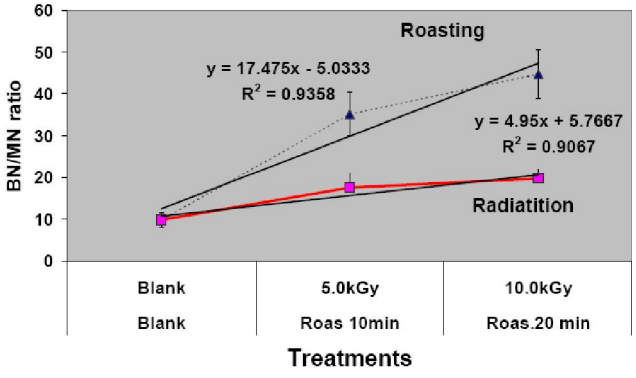

Figure 6 : The linear relationship between mutagenicty ratio of binucleotide (BN) / mononucleotide cells (MN) and treatment methods.

DISCUSSION

Food safety is a global issue with paramount environmental and public health consequences of inadequately maintained. Therefore, a variety of procedures have been developed and used to reduce food-borne contamination Since the late 1980's, different clearance announced by the World Health Organization and the US Food and Drug Administration have approved the irradiation of food by ionizing radiation at the beginning of the food supply chain as an inexpensive and effective procedure.[20-23]. The energy from the irradiation breaks chemical bonds and produces toxic ions and free radicals that react with cellular constituents in food to form altered products (often classified as radiolytic products). In spite of more than50 countries have given approval for over 60 foodstuffs to be irradiated for local consumption and/or for export, and approximately40 different countries are using the food irradiation technology on commercial scale but another groups like Germany, UK and other countries have apprehensive for using irradiation[17,24-25].

Irradiation cause breaking the bonds in a microorganism's DNA structure and prohibiting its replication that food irradiation prevents spoiling and foodborn illness. The main role in sprouting inhibition depend on damaging DNA, leading to its fragmentation either by single- or double-strand breaks. In addition, denaturation of the DNA helix, crosslinking and base modifications occurs. These radiation induced changes in DNA could be a basis for detection of irradiation treatment in a number of foods call Comet assay. No, studies concerning safety of DNA-fragmentation or DNA –damaged[26,27].

Therefore, in present work, we used three techniques to compare the values of toxicity due to using irradiation with food processing. In our work before, we used the same test of genotoxicity for determination values in ready meals, as proved before that fast food contains double or more values than the obtained data heir in irradiated samples with the same genotocicity test.

Whereas, Ames test techniques concerning first one, inhibition sprouts by irradiation cause death of meristematic cells consequently prevent sprouting or rooting of bulbs or tubers. Same function of irradiation can inhibits sprouting and rooting of garlic, onion, ginger and

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shallot[28]. These cells change to nerotic cells due to damaged of DNA, these parts can consume fresh as in garlic or onion or processed as in potatoes. The mutagenicity of injured cells of these parts mainly sprouted parts-were neglected in past studies, no reports focus before in details in spite of presence some reports from IAEA which introduced same results but unfortunately, these results were encrypted soon. Inspite of same reports introduced same trends before[29-33].

In our results of Ames test showed that ethyl extract contains high levels of mutagenic compounds in irradiated samples which increased gradually with increasing doses. The mutagenic compounds concentrated in Alcohol extract but less amount are present in residue or whole dry parts. The published data by WHO[35], showed to presence of mutagenic effects of ethyl potato extract after irradiation but same effects decreased after 40 days[34]. Our data are parallel with obtained earlier by studies researchers from Soviet Union which reported the formation of radiotoxins in irradiated potatoes, showed cytotoxic and mutagenic effects in mice[25,26]. It was claimed that alcoholic extracts of freshly irradiated potatoes containing these radiotoxins induced dominant lethal mutations in mice[27-29]. Different repots for positive trend –in-*vivo* of mutagenicity were reported early[30-35].

In the second test using (CA),-only used with applied dose (0.01 kGy)-using different concentrations of alcohol extract of sprouts caused slight increase of abnormal chromosomes.(AC). Boiling or freezing same irradiated sprouts caused increasing the CA- percentage (TABLES 3, 4).

The first and second test proved presence toxic compounds in injured cells which can eat freshly as common food as garlic, onion or processed as potatoes. Fortunately, the results of Ames test or CA of applied dose (0.01 kGy) near control results but more doses increased the toxicity as showed in our results. The inhibited parts can eat freshly as habitats or processed, therefore, the consumers can eat the toxic compounds directly.

Finally, the third techniques using genotoxicity, proved that roasting at 200 ^{0}C of coffee beans either at short time (10min.) or long time (20 min.) increased the genotoxicity values than irradiation treatments. In the same time using same techniques by same author proved high values of genotoxicity of processed ready meals (TABLE 1) as coffee than low values in irradiated coffee beans.

Our results *in-vivo* showed presence of toxic compounds due to using irradiation even at low dose or after roasting process. The famous mutagenic compound is 2-alkylcyclobutanones (2-ACBs) which can cause colon tumor which resulted from fatty food after irradiation. The toxicological potential of radiolytic derivatives of triglycerides found exclusively in irradiated food, is scarce. That a compound found exclusively in irradiated dietary fats may promote colon carcinogenesis in animals treated with a chemical carcinogen[31]. But WHO (Joint FAO/WHO[13] announced after long –term feeding studies, 2-DCB and 2-alkylcyclobutanones in general do not appear to pose a health risk to consumers of irradiated fatty diets. Therefore,the workers still in doubt for the the mutagenicity or toxicity of 2-DCB and 2-alkylcyclobutanones,some authors concluded that this compound may be regarded as a possible risk factor for the initiation and progression processes in colon carcinogenesis. The cytotoxic and genotoxic potentials of various highly pure synthetic 2-alkylcyclobutanones were investigated in bacteria and human cell lines[35].

CONCLUSION

Generally, using ã-irradiation either at low dose for inhibition sprouting (0.01 kGy) or high dose for treating coffee beans produced mutagenicity or genotocicty but less than processing methods food. Using the recommended dose must use at low dose not more. Moreover, additional in vitro and in vivo tests with regards to the tumor-promoting activities of unique radiolytic products should be conducted.

Lack of sufficient data on the effect of long-term consumption of irradiated foods on human health and on long-term health effects of eating a diet based on irradiated foods is still considered to be a problem, and thus, precautionary principles should be applied until such data are available. In this regard, WHO continues to encourage further research to be conducted in accordance with scientifically accepted protocols. for assessing food safety to help resolve any remaining uncertainties about the toxicity or carcinogenicity of 2-alkylcyclobutanones. WHO reiterates its previously stated[13] (Joint FAO/WHO Food Standards 2002)willingness to reopen the risk assessment of irradiated foods

if new evidence indicates a potential public health risk.

Foods are irradiated to provide the same benefits as when they are processed by heat, refrigeration, freezing or treated with chemicals. Far from sterilizing the food, which still requires proper handling and cooking, irradiation destroys disease-carrying bacteria and reduces the incidence of food borne illnesses making it possible to keep food longer, while at the same time ensuring a higher level of safety and quality.

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