JOURNAL OF PURE AND APPLIED MICROBIOLOGY, April 2010. Vol. 4(1), p. 69-77

Antimicrobial Activity and HPTLC Profiling of *Piper longum* Linn. after Microbial Decontamination by Gamma Irradiation

Prakash Chandra Gupta[1], Neelam Garg[2]*, Devki Pant[1], Prakash Joshi[1] and D.R. Lohar[1]

[1]Microbiology Laboratory, Homoeopathic Pharmacopeia Laboratory, Ghaziabad - 201 002, India.
[2]Department of Microbiology, Kurukshetra University, Kurukshetra - 132 119, India.

(Received: 30 August 2009; accepted: 20 October 2009)

In the present study, the effects of gamma irradiation of *P. longum* root was carried out at dose levels of 0, 2.5, 5.0, 10.0, 20.0, and 25.0 kGy. The dose of 2.5 kGy reduced microbial load by 2 log cycles. However, complete sterilization was achieved at 10.0 kGy. The un-irradiated and irradiated powdered *P. longum* root were extracted by hexane, chloroform, acetone and methanol. The results showed that the extraction yields increased with an increase in irradiation dose for all the test solvents. The antimicrobial activity was performed by using agar well diffusion assays against *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus, Bacillus cereus, Enterococcus faecalis, Candida albicans* and *Saccharomyces cerevisiae*. Of the various extracts the methanol extract showed the prominent activity against microorganisms used in this study. The minimum inhibitory concentration (MIC) values were determined by tube dilution method. No significant change was observed in the antimicrobial activity and minimum inhibitory concentration after gamma irradiation at different doses. The alteration in the phytoconstituents after gamma irradiation was analyzed by HPTLC. The results showed that the gamma irradiation can be considered as an available and useful sterilization technique without any undesirable effect on phytoconstituents of *P. longum* root.

Key words: Gamma irradiation, *Piper longum*, Microbial decontamination, Antimicrobial activity.

Medicinal plants are the most important source of life saving drugs for the majority of world's population, for thousands of years. Even today, WHO estimates that upto 80 percent of people still rely mainly on traditional remedies such as herbs for their medicines[1]. The global market of medicinal plants has been growing at a fast pace of 7% annually, capitalizing on the growing awareness of herbal and aromatic plants worldwide. Two of the largest producers of raw materials of herbal medicines are China and India. In India, herbs are generally collected from the wild sources. Only in few cases, medicinal plants are being cultivated. These medicinal plants are often contaminated with high level of

* To whom all correspondence should be addressed.
E-mail: nlmgarg@yahoo.com

bacteria, molds and yeast. Most often the micro-organisms, which are present on the surface of the plant, are a miniature of epiphytic microflora growing on specific varieties of plants and the microflora originating from the plant environment, namely soil, water and air. Pathogenic micro-organisms may grow on some medicinal plants not only on the surface but also inside the plant tissue, if untreated they can result in serious illnesses[2]. It is very essential that medicinal plants be decontaminated before using as raw material to get good quality of the herbal products. The conventional methods of decontamination were fumigation with gaseous ethylene oxide or methyl bromide, which are now prohibited or being increasingly restricted in most advance countries for health, environmental and occupational safety reasons[3]. Gamma Irradiation results in much more effective microbial decontamination and is often the only treatment efficient enough to meet standards set by processors operating under Hazard Analysis Critical Control Points (HACCP) or International Standards Organization (ISO) standards[4-9].The maximum dose allowed for herb treatment in USA is 30 kGy[10]. However, it is utmost important to optimize the gamma irradiation doses for medicinal plants used for herbal products.

Piper longum Linn., (Piperaceae) sometimes called Indian Long Pepper is one of the herbs mentioned in all ancient scriptures of Ayurveda. It is a close relative of the black pepper plant, and has a similar, though generally hotter taste. The roots and fruits of *P. longum* are used in palsy, gout and lumbago. Since ancient times the dried fruits and roots of the plant *P. longum* have been used as thermogenic, stomachic, anti-asthmatic, antiseptic and also active against bacterial diseases[11-12].

The objective of this work was to evaluate the effects of different doses of gamma irradiation on the microbial decontamination, extraction yield, antimicrobial activity and phytoconstituents present in *P. longum* root.

MATERIAL AND METHODS

Plant material

Piper longum roots were purchased from the local market in Ghaziabad, Uttar Pradesh and identified by botanists. The collected plant materials were air- dried for 5-7 days under shade at room temperature, powdered with a grinder and packed in polythene bags.

Gamma irradiation

The polythene packed powdered root samples (50 g) of *P. longum* were irradiated using gamma rays from a cobalt-60 radiation source at room temperature. Samples were irradiated in air-packed cardboard boxes and the dosimeters were placed at different positions in the boxes to ascertain uniform dose of gamma irradiation. Different doses of gamma irradiation (2.5, 5.0, 10.0, 20.0 and 25.0 kGy) were applied. Un-irradiated sample was used as a control.

Microbiological analysis

Total aerobic microbial count and sterility testing were done following the method described by Indian Pharmacopoeia[13].

Extraction of phytoconstituents

The irradiated and control samples (50 g each) were separately extracted in hexane, chloroform, acetone, and methanol using a soxhlet extractor. All the extracts were filtered through Whatman No. 1 filter paper and concentrated under vacuum at 45° C. The dry extract obtained with each solvent was weighed and extraction yields were calculated. The extracts were stored at 4° C until further processing.

Screening for antibacterial activity

The antimicrobial activity of the hexane, chloroform, acetone and methanol extracts of irradiated and non- irradiated samples were performed by agar-well diffusion method[14] against *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus, Bacillus cereus, Enterococcus faecalis, Candida albicans and Saccharomyces cerevisiae*. An inoculum size of 10^8 cfu mL^{-1} of microorganisms, compared with 0.5 MacFarland turbidity standard was used. About 100µL of the extracts (stock 500 mg mL^{-1}) were added carefully in wells of 8 mm diameter in Muller Hinton agar plate.

The minimum inhibitory concentration (MIC) values were evaluated by the tube dilution method. The MIC was tested in the concentrations range 0.97 – 250.0 mg mL^{-1} Antibacterial assay plates and tubes were incubated at 37 ± 1ºC for 24 h, whereas antifungal assay plates were incubated at 25 ± 1ºC for 48 h in an incubator. Zone of inhibition surrounding the wells were measured to evaluate the antimicrobial activity. Tube showing no turbidity was recorded as the MIC value[15]. Antibiotics such as gentamicin (20 µg mL^{-1}) for bacteria, nystatin (10 µg mL^{-1}) for fungus were used as positive control and 100% dimethyl sulfoxide (DMSO), a dissolving solvent was used as a negative control.

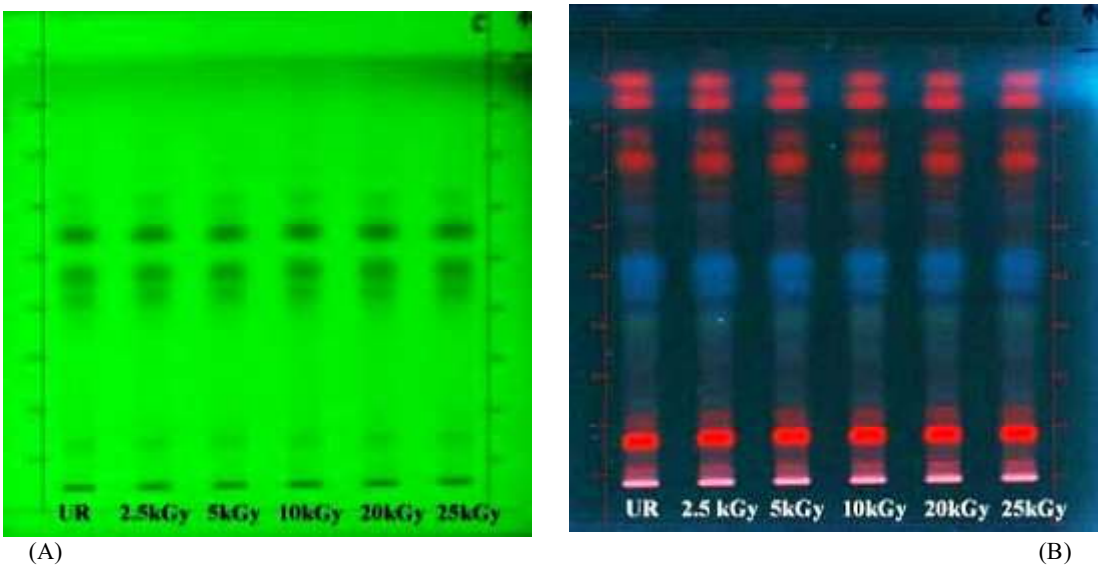
High performance thin layer chromatography profiling

The HPTLC analysis of chloroform extract was performed on aluminum sheet pre-coated with silica gel 60 F_{254} (E-Merck grade). Before use, plates were washed with methanol, and dried in an oven at 105°C for 1 hour. The extracts (5 µL) were applied on the plates as bands of 7-mm width with the help of a linomat-5 sample applicator. The plates were developed in a Camag twin-trough chamber previously equilibrated with a mobile phase for 20 minutes. The solvent system containing toluene: ethyl acetate (7:3) was used as a mobile phase. After development, plates were analyzed with TLC scanner 3 and Win-CATS software under UV at 254 nm and 366 nm.

RESULTS AND DISCUSSION

Total aerobic microbial count in the un-irradiated sample *of P. longum* root was found to be about 3 x 10^5 cfu g^{-1}. Gamma irradiation at a dose of 2.5 kGy resulted in 2 log reduction in the total microbial count (1.6 x 10^3 cfu g^{-1}) and at a dose of 5.0 kGy not even a single microbial cell was recorded in the samples. However, in the sterility test, the sample treated with 5.0 kGy gamma radiation showed bacterial growth in fluid thioglycollate medium (Hi-media) on 5th day after incubation and no fungal growth was observed in soyabean casein digest medium. Gamma irradiation at 10 kGy resulted in complete sterilization and the sterility was maintained for 14 days. Our results are in agreement with those who reported that a minimum dose of 10 kGy was necessary to obtain a product of good microbiological quality[16-18].

The extraction yields of *P. longum* root in hexane, chloroform, acetone and methanol were determined and are shown in Table 1.Solvents extraction significantly affected the dry weight yield in control samples of the plant. The non-irradiated methanol extracts showed highest



(A) (B)

Fig. 1. HPTLC profiling of un-irradiated and gamma irradiated *P. longum* root at UV - 254 nm (A) and UV - 366 nm (B)

extraction yield (7.79 %). However, control sample extracted with hexane, chloroform and acetone gave extraction yield of 1.86%, 1.64% and 1.25 % respectively. Radiation treatment resulted in significant increase in the extraction yields for all the test solvents. The methanol extract showed a linear increase in the dry weight with increase in the dose of gamma irradiation.

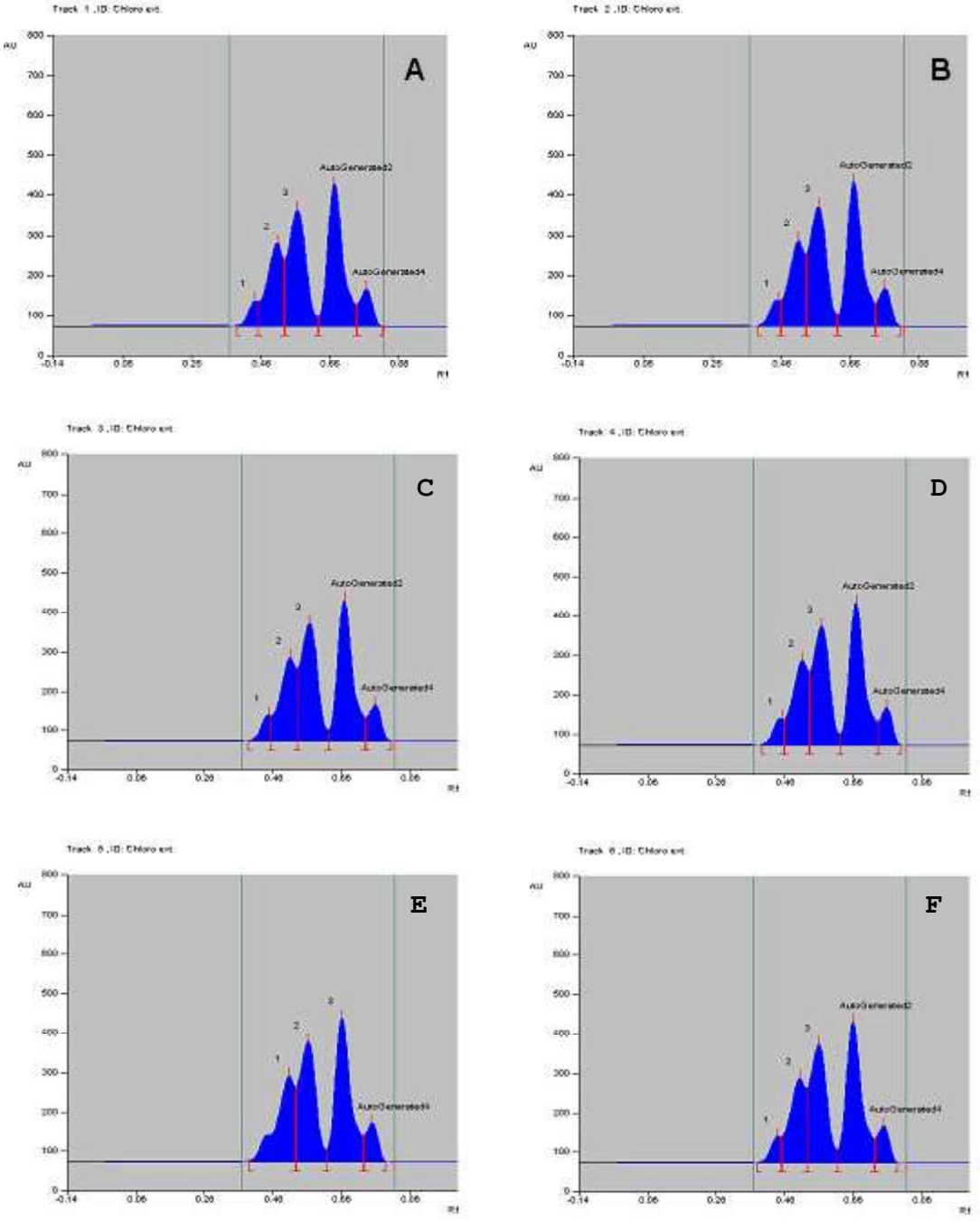


Fig. 2. Densitometric chromatogram of *P. longum* root at 254 nm treated with different doses of gamma irradiation. Un-irradiated (A), irradiated with 2.5 (B), 5.0 (C), 10.0 (D), 20.0, (E) and 25.0 kGy (F).

Similarly, the extraction yield in acetone extract was increased from 1.25 % to 1.51 % at 25 kGy gamma irradiation. The dry weight in the hexane extract increased from 1.86 % to 2.08 %. However, the extraction yield of chloroform extract was increased by 16.4 %. The increase in the dry weights of extracts following irradiation might be due to degradation of some high molecular weight components, and changing these components from non-soluble to soluble ones in

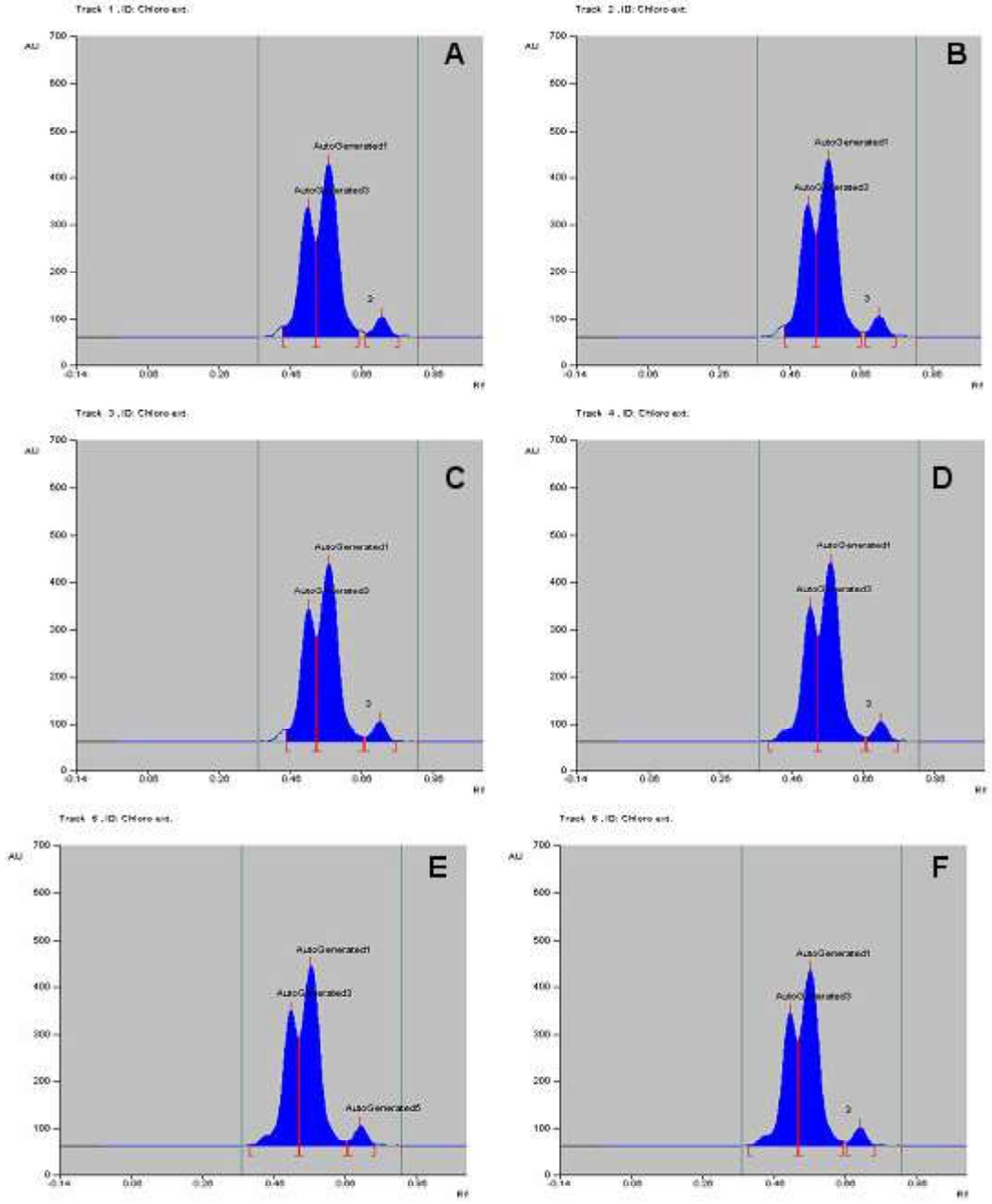


Fig. 3. Densitometric chromatogram of *P. longum* root at 366 nm *treated with different doses of gamma irradiation*. Un-irradiated (A), irradiated with 2.5 (B), 5.0 (C), 10.0 (D), 20.0, (E) and 25.0 kGy (F)

the test solvents. Overall, the results show that gamma irradiation up to 25 kGy is an effective method for enhancing extraction yields in *P. longum* root. The increase in the extraction yield with radiation treatment has also been reported earlier[19].

The changes observed in the extraction yield of *P. longum* root necessitated the test for the antimicrobial activity of the extracts after exposure to different doses of gamma irradiation.

The effect of gamma irradiation on antimicrobial activity was tested at different radiation doses in a wide range between low (2.5 kGy) up to high radiation dose (25.0 kGy) Table 2. The DMSO negative control showed no inhibitory effect. The positive control (gentamicin) showed inhibition zone ranging from 16 mm to 23 mm against bacteria. The inhibition zone of nystatin was 18mm and 20 mm against *C. albicans* and *S. cerevisiae* respectively. The methanol extract showed activity against all the microorganisms tested and maximum activity was observed against *S. aureus* (22 mm). As can be seen in Table 2, it seems that the antimicrobial activity against Gram – positive bacteria was more pronounced than against Gram – negative bacteria. These differences may be attributed to the fact that the cell wall in Gram –positive bacteria consist of single layer, whereas the Gram – negative cell wall is a multilayer structure and quite complex[20-21].The zone of inhibition being prominent in the methanol extract, therefore the minimum inhibitory concentration (MIC) study of methanol extract was carried out. The results showed that The MIC value for the *S. aureus* was 62.5 mg mL^{-1}, whereas, *B.cereus* required 125 mg mL^{-1}, of the methanol extract for inhibition (Table 3). No significant change has been found in antimicrobial activity and minimum inhibitory concentration after gamma irradiation at different doses.

In order to further verify the change in phytoconstitutents present in *P. longum* root HPTLC profiling was performed. The method as described in the present work, utilizes pre coated silica gel 60 $_{F254}$ plate with toluene: ethyl acetate (7:3), as mobile phase resulted good separation in the chloroform extract. The HPTLC profiling of the chloroform extract of un-irradiated and irradiated *P. longum* root showed in (Fig. 1). The HPTLC of the chloroform extracts showed five compounds with R_f value 0.44, 0.51, 0.57, 0.67, and 0.77 at UV 254 nm and three compounds with R_f values 0.51, 0.57, and 0.72 at UV-366 nm. The *densitometric chromatogram* evaluation at UV 254 nm (Fig. 2 and Table 4) and 366 nm (Fig. 3 & table 5) showed that the phytoconstituents of *P. longum* root did not change significantly with irradiation up to 25.0 kGy.

With attention of these results it is concluded that gamma irradiation effectively reduced microbial count. The antimicrobial activities were not changed although the extraction yield increased by 11.8 % to 32.6% after gamma irradiation. However, no changes in the phytoconstitutents have been observed up to 25 kGy. These results further support the notion that gamma irradiation process is chemically inert and could be used effectively and efficiently for the sterilization of medicinal herbs.

Table 1. Effect of gamma irradiation on the extraction yield of *Piper longum* root.

Radiation dose	Extraction yield (% w/w)			
(kGy)	Hexane	Chloroform	Acetone	Methanol
0	1.86	1.64	1.25	7.79
2.5	1.93	1.68	1.35	7.86
5.0	1.95	1.73	1.38	8.07
10.0	1.98	1.80	1.42	9.22
20.0	2.04	1.86	1.47	9.86
25.0	2.08	1.91	1.51	10. 33

The value in each column is mean of two independent experiments with four replicates each.
The maximum variation from mean value was less than 5%.

Table 2. Effect of gamma irradiation on Antimicrobial activity of *Piper longum* root.

Solvent	Irradiation Dose (kGy)	Inhibition zone diameter (mm)							
		E.coli	*P aeruginosa*	*S.typhi*	*E. faecalis*	*S. aureus*	*B.cereus*	*C. albicans*	*S. cervicae*
Hexane	0	11	18	10	NZ	13	14	NZ	NZ
	2.5	11	18	11	NZ	12	14	NZ	NZ
	5.0	12	18	10	NZ	12	14	NZ	NZ
	10.0	12	17	10	NZ	13	15	NZ	NZ
	20.0	11	18	10	NZ	12	14	NZ	NZ
	25.0	12	18	10	NZ	13	14	NZ	NZ
Chloroform	0	10	12	12	16	13	15	NZ	NZ
	2.5	12	12	12	15	13	15	NZ	NZ
	5.0	11	12	12	14	13	16	NZ	NZ
	10.0	12	12	12	16	13	16	NZ	NZ
	20.0	10	12	12	16	13	15	NZ	NZ
	25.0	10	12	12	16	13	15	NZ	NZ
Acetone	0	12	12	NZ	12	10	15	NZ	NZ
	2.5	13	12	NZ	12	10	16	NZ	NZ
	5.0	12	12	NZ	12	10	16	NZ	NZ
	10.0	12	12	NZ	12	10	16	NZ	NZ
	20.0	12	12	NZ	12	10	15	NZ	NZ
	25.0	12	12	NZ	12	10	15	NZ	NZ
Methanol	0	16	14	15	20	22	18	14	13
	2.5	16	15	16	20	22	18	14	13
	5.0	17	15	15	20	23	17	14	13
	10.0	16	14	15	20	22	18	14	13
	20.0	16	14	16	20	22	17	14	13
	25.0	16	15	16	20	22	18	14	13
Gentamicin		21	21	16	19	23	20	-	-
Nystatin		-	-	-	-	-	-	18	20

NZ – No inhibition zone

The value in each column is mean of two independent experiments with four replicates each. The maximum variation from mean value was less than 5%.

Table 3. Determination of Effect of gamma irradiation doses on MIC (Minimum Inhibitory Concentration) of the methanol extract of *Piper longum* root.

S. No.	Organism	MIC (mg/mL) Irradiation dose (kGy)					
		0	2.5	5.0	10.0	20.0	25.0
1.	*S. aureus*	62.5	62.5	62.5	62.5	62.5	62.5
2.	*B.cereus*	125	125	125	125	125	125

The value in each column is mean of two independent experiments with four replicates each.
The maximum variation from mean value was less than 5%.

Table 4. HPTLC profiling of chloroform extracts of *Piper longum* root treated with various doses of gamma radiation at UV 254 nm.

Irradiation dose (kGy)											
0		2.5		5.0		10.0		20.0		25.0	
Rf	Area (%)	Rf	Area (%)	Rf	Area (%)	Rf	Area (%)	Rf	Area (%)	Rf	Area (%)
0.44	3.84	0.45	4.51	045	4.12	0.45	4.65	0.44	4.50	0.44	4.41
0.51	21.14	0.51	20.84	0.51	21.26	0.51	21.11	0.51	22.56	0.50	21.48
0.57	32.12	0.57	31.71	0.57	31.83	0.57	31.57	0.56	31.86	0.56	31.35
0.67	35.36	0.67	35.41	0.67	35.28	0.67	35.47	0.66	35.64	0.66	35.37
0.77	7.53	0.77	7.53	0.76	7.51	0.76	7.19	0.75	7.52	0.75	7.38

The value in each column is mean of two independent experiments with four replicates each.
The maximum variation from mean value was less than 5%.

Table 5. HPTLC profiling of chloroform extracts of *Piper longum* root treated with various doses of gamma radiation at UV 366 nm.

Irradiation dose (kGy)											
0		2.5		5.0		10.0		20.0		25.0	
Rf	Area (%)	Rf	Area (%)	Rf	Area (%)	Rf	Area (%)	Rf	Area (%)	Rf	Area (%)
0.51	35.66	0.51	35.41	0.51	35.31	0.51	36.67	0.51	37.07	0.51	38.50
0.57	58.98	0.57	59.14	0.57	59.68	0.57	58.37	0.56	58.01	0.58	59.62
0.72	5.36	0.71	5.45	0.71	5.01	0.71	4.69	0.70	4.92	0.71	5.63

The value in each column is mean of two independent experiments with four replicates each.
The maximum variation from mean value was less than 5%.

REFERENCES

1. Abdel-Hady, M.S., Okasha, E.M., Soliman, S.S.A., Talaat, M. Effect of Gamma Radiation and Gibberellic Acid on Germination and Alkaloid Production in *Atropa belladonna* l. *Aust. J. of Basic and Applied Sci*., 2008; **2**(3): 401-405.
2. Andrzej, G., Chmielewski, Wojciech M. Radiation decontamination of herbs and spices. *NUKLEONIKA*., 2005; **50**(4): 179-184.
3. Uijl, C.H. Heat treatment of spices. Beating the bugs! *Inter. Food Ingredients,*1992; **3**: 9-11.
4. Mossel, D.A.A. Adequate protection of the public against food-transmitted diseases of microbial aetiology: Achievements and challenges, half a century after the introduction of the Prescott- Meyer-Wilson strategy of active intervention. *Int. J. Food Microbiol*., 1989; **9**: 271-294.
5. Radomyski, T., Murano, E.A., Olson, D.G., Murano, P.S. Elimination of pathogens of significance in food by low-dose irradiation: A review. *J. Food Prot*., 1994; **57**: 73-86.
6. Harding, R.B., Pinteric, F.J.A. Validation of gamma irradiator controls for quality and regulatory compliance. *Rad. Phys. Chem*., 1995; **46**: 1335-1341.
7. Farrar IV, H. Twenty new ISO standards on dosimetry for radiation processing. *Rad. Phys. Chem*., 2000; **57**: 717-720.
8. Panisell. P.J., Rooney, R., Quantick, P.C., Stanwell, S. R. Application of foodborne disease outbreak data in the development and maintenance of HACCP systems. *Int. J. Food Microbiol*., 2000; **59**: 221-234.
9. Molins, R.A., Motarjemi, Y., Kaferstein, F.K. Irradiation: a critical control point in ensuring the microbiological safety of raw foods. *Food Control*, 2001; **12**: 347-356.
10. Food and Drug Administration. Irradiation the production, processing and handling of foods; Final rule (21CFR Part. 179), Federal Register.,1986; **55**(75): 13376-13399.
11. kirtikar, K.R., Basu., B.D. (ed): *Piper longum* Linn, Indian Medicinal Plants; *Perdodical expert Book Agency*, New delhi, India, 1984.
12. Warrier, P.K. , Nambiar, V.P.K., Raman, K.C. *Piper longum* Linn; Indian medicinal plants; orient Longman Ltd. Madaras, India, 1995.
13. Anonymous. (ed): *Indian Pharmacopeia,* vol I. Indian pharmacopeia commission, Ghaziabad, 2007.
14. Perez, C., Pauli, M., Bazerque, P. An antibiotic assay by the agar-well diffusion method. *Acta Biol Med Exp*.,1990; **15**: 113-115.
15. NCCLS (National Committee for Clinical Laboratory Standards), Approved standard M7-A5, Standard methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically. Villanova PA, 1990.
16. Kim, M.J., Yook, H.S., Byun, M.W. Effects of gamma irradiation on microbial contamination and extraction yields of Korean medicinal herbs. *Rad Phys Chem*., 2000; **57**(1): 55-58.
17. Van, D.H., Bosch, E.H., Zwaving, J.H., Elema, E.T.Gamma irradiation of *Sennaae folium* microbiological and phytochemical studies. *Pharm Weekly Sci*., 1988 ; **10**(5): 217-220.
18. Gupta P. C., Bajpai V., Mishra V., Saxena R. K., Singh S. Effect of gamma irradiation on microbial load, aflatoxins and phytochemicals present in *Trigonella foenum-graecum*.*World J. of Micro. and Biotec*. 2009; Published online: 2 August 2009.
19. Huang,S.J., Mau, J.L. Antioxident properties of methanolic extracts from *Antrodia camphorate* with various doses of γ-radiation. *Food Chem.,* 2007; **105:** 1702-1710.
20. Essawi, T., Srour, M. Screening of some Palestinian medicinal plants for antibacterial activity. *J Ethnopharmacol.*, 2000; **70**: 343-349.
21. Kaushik, P., Garima, Chauhan A., Goyal P. Screening of *Lyngbya majuscula* for potential antibacterial activity and HPTLC analysis of active ethanolic extract. *J. of Pure and Applied Microbiol.*, 2009; **3**(1): 169-174.