Microbiological Status and Antifungal Properties of Irradiated Spices

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The bacterial counts of commercially available species were found to be in the range of 10^2–10^7/g, whereas the fungal counts varied between 10^2 and 10^3/g. Among the five spices studied, pepper, cardamom, and nutmeg mace had a high microbial load compared to cinnamon and clove. Exposure to γ-irradiation in the dose range of 7.5–10 kGy was adequate to sterilize all the spices. The essential oil of clove and cinnamon exhibited inhibitory properties against aflatoxin-producing aspergilli. γ-Irradiation did not affect fungal inhibitory principles present in clove, though marginal reduction was observed in that of cinnamon.

India is among the leading suppliers of spices, and the export trade is often faced with the problems of insect infestation and microbial contamination. The efficacy of γ-irradiation for the decontamination of certain spices has been reported (Bachman and Gieszczynska, 1973; Vajdi and Pereira, 1973; Inal et al., 1975; Farkas, 1983). The present experiments were undertaken to assess the microbial load in some of the common Indian spices to arrive at an effective dosage of radiation for decontamination. Some of the spices are known to have antifungal properties (Bullerman et al., 1977; Hitokoto et al., 1978). Therefore, the influence of radiation treatment on the fungal inhibitory principles in spices was also ascertained. The presence of aflatoxin, produced by *Aspergillus flavus/parasiticus* (Goldblatt, 1969), was observed in some spices (Seenappa and Kempton, 1980). The experiments presented in this report are concerned mainly with this aspect.

MATERIALS AND METHODS

Spices. The following spices were obtained from the local market: clove (*Syzygium aromaticum*), cinnamon (*Cinnamomum zeylanicum*), black pepper (*Piper nigrum*), cardamom (*Elettaria cardamomum*), and nutmeg (*Myristica fragrans*).

Irradiation Treatment. For irradiation, the spices were packed in polyethylene bags (gauge 400) in 25-g quantities. The irradiation of polyethylene bags at the average absorbed doses of 5, 7.5, and 10 kGy was carried out in a Gamma Cell-220 (Atomic Energy of Canada, Ltd.) with a maximum dose rate of 0.025 kGy/min.

Enumeration of Microbial Load. A 1-g sample in triplicate of each of the spice (whole) from three different bags was added to a test tube containing 25 mL of sterile distilled water. A 1-mL aliquot from this was withdrawn to be added to blank sterile distilled water for the purpose of dilution. The plating was done by the standard plate count method. For the total bacterial count nutrient agar (Difco) and for the enumeration of fungi potato–dextrose agar (Difco) were used. Bacterial and fungal counts were done after irradiation to different doses, as well as prior to irradiation.

Preparation of Spice Extracts. *Chloroform Extracts.* A 20-g sample of each spice was ground in an omnimixer and a slurry was prepared by adding 100 mL of water. The slurry was extracted with 100 mL of $CHCl_3$ 3 times. The extracts were pooled and vacuum evaporated. The residue was used for estimation of aflatoxin and also for preliminary screening of antifungal activity.

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Steam-Distilled Oil. This fraction was obtained by steam distilling 20 g quantity of a spice. Aqueous condensate so obtained was extracted with peroxide-free diethyl ether. Ether was removed by flash evaporation and the yield of oil was determined by weighing (Guenther, 1973; Pruthi, 1980).

Preparation of the Spore Suspension: Organism. *Aspergillus parasiticus* NRRL 3145 was obtained from Northern Regional Research Laboratory, Peoria, IL. The organism was subcultured on potato–dextrose agar (Difco Laboratories, Detroit, MI) from the stock cultures. The slants were incubated for 7 days at ambient temperature. The spore material from the slants was suspended in 10 mL of sterile water and transferred for surface culture to Roux bottles containing potato–dextrose agar (200 mL, 4%), which were subsequently incubated for 10 days at ambient temperature. The harvesting of the spores from Roux bottles and preparation of the spore suspension (ca. 10^6 spores/mL) was carried out according to the method described earlier (Padwal-Desai et al., 1976).

Assay of Antifungal Activity. The antifungal activity of the spice extracts was assayed by using two techniques as follows.

Standard Cup Assay (Booth, 1972). One milliliter of the spore suspension was used to seed the assay plates, prepared by pouring 20 mL of potato–dextrose agar. After the agar was allowed to solidify, cups were prepared by using a sterilized glass tube and a propipet. Various extractives were then added to the cups in appropriate quantities in triplicate. In each case, a separate cup with pure solvent alone was employed as a control. The diameter of the zone of inhibition was measured after incubation for 48 h at ambient temperature (28–30 °C).

Assay in Flask Cultures. The spore suspension containing ca. 5×10^3 spores/mL of *A. parasiticus* (NRRL 3145) was inoculated in 100-mL conical flasks containing 50 mL of glucose–salt medium (pH 6.5) of Shih and Marth (1974). On the basis of the preliminary information obtained from the cup assay, appropriate quantities of various extractives were added in triplicate to the flasks containing spores and the medium. Flasks not containing spice extracts served as a control. Flasks were incubated at ambient temperature for 15 days. Thereafter, the mycelium was separated from the culture broth, washed with water, dried at 85 °C for 24 h, and weighed. The flasks that did not show any sign of visual growth were further observed up to a period of 35 days.

Estimation of Aflatoxin. The culture filtrate after filtering out the mycelium was extracted with 50-mL chloroform 3 times. The three extracts were pooled and dried on a boiling water bath. The residue was diluted with an appropriate volume of the solvent (chloroform) and

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Table I. Effect of γ-Irradiation on the Total Viable Bacterial and Fungal Population in Spices

	total bacterial count/g				total fungal count/g				
spice	0	5 kGy	7.5 kGy	10 kGy	0	1 kGy	5 kGy	7.5 kGy	10 kGy
pepper	1.4×10^7	1.2×10^3	4.2×10^2	0	8.7×10^2	1.2×10	0	0	0
nutmeg mace	3.6×10^4	5.5×10^2	0	0	8.1×10^3	7.5×10	0	0	0
cinnamon	2.1×10^3	1.2×10^2	0	0	3.1×10^2	0	0	0	0
cardamom	1.3×10^4	3.0×10^2	2.5×10	0	1.3×10^3	5.0×10	0	0	0
clove	8.7×10^2	3.7×10	0	0	9.3×10^2	3.7×10	0	0	0

Table II. Diameter of Zone of Inhibition Produced by Chloroform Extracts of Various Spices against *A. parasiticus* NRRL 3145 in the Standard Cup Assay[a]

	zone of inhibition, mm, resulting from the aliquots		
treatment	0.01 mL	0.02 mL	0.03 mL
control (chloroform)	0	0	0
clove	21 ± 1	23 ± 1	28 ± 4
cinnamon	18 ± 3	21 ± 1	28 ± 4
cardamom	0	0	0
nutmeg	0	0	0
pepper	0	0	0

[a] Spices (20 g of each) were extracted with chloroform as described in the text, and the residue was dissolved in 2 mL of chloroform from which 0.01-, 0.02-, and 0.03-mL aliquots were added to the cup in agar.

aflatoxin was estimated in a direct-reading Velasco fluorotoxinmeter (Neotec Instruments, Inc., Silver Spring, MD) by using a minicolumn (Velasco, 1975).

Thin-Layer Chromatography of Aflatoxin. An aliquot from the residue obtained after extracting the culture with chloroform was spotted on silica gel plates (0.25 mm, 20 × 20 cm). The plates were initially developed in diethyl ether and then in a chloroform and acetone (9:1 v/v) solvent system. The plates were viewed under ultraviolet light to detect the fluorescent aflatoxins after drying the plates in air.

Gas–Liquid Chromatography. The analysis of the steam-distilled oil of unirradiated and irradiated spices was carried out in a BARC model gas chromatograph equipped with a flame ionization detector. A glass column (6 ft × 0.25 in. o.d.) packed with 10% Carbowax 20 M on Chromosorb W, 60–80 mesh, was used. The column temperature was maintained at 130 °C with a carrier gas (nitrogen) flow rate of 30 mL/min.

RESULTS

Determination of Radiation Doses. Table I shows the bacterial and fungal counts per gram of each spice. It was observed that the bacterial counts were low in clove and cinnamon, whereas the counts were higher in nutmeg mace and cardamom followed by pepper, clove, and cinnamon. Data show that for peper, cardamom, and nutmeg mace, a dose of 10 kGy and for cinnamon and clove a dose of 7.5 kGy was sufficient for sterilization. A dose of 5 kGy was adequate to eliminate the fungal spores in all the spices.

Growth Inhibition of *A. Parasiticus* by Spice Extracts. In an antibiotic-type assay of the chloroform extracts of various spices, it was found that the extracts from clove and cinnamon showed pronounced zones of inhibition, indicating the presence of inhibitory compounds against aflatoxin-producing fungi (Table II). However, these inhibitory substances seemed to be absent in pepper, cardamom, and nutmeg as no zones of inhibition could be observed. This was further observed in the flask assay of inhibitory activity.

Table III shows the growth and aflatoxin production by the cultures of aflatoxin-producing fungi in the presence of the spice extracts. Whereas no visual growth was observed in case of flasks containing extracts of cinnamon and clove, even after prolonged incubation up to 1 month, the growth and aflatoxin biogenesis were observed in all other flasks.

Table III. Growth and Aflatoxin Production in the Presence of Various Spice Extracts (Flask Assay, Incubation Time 7 Days)[a]

	growth g dry wt	total aflatoxin, mg
control	1.23 ± 0.05	0.34 ± 0.03
cinnamon	0	0
clove	0	0
cardamom	1.39 ± 0.01 (1.38 ± 0.01)	0.94 ± 0.3 (2.02 ± 0.7)
nutmeg	1.22 ● 0.01 (1.22 ± 0.10)	1.10 ± 0.1 (1.70 ± 0.1)
pepper	1.03 ± 0.25 (0.89 ± 0.10)	0.60 ± 0.1 (3.5 ± 0.1)

[a] 20 g of each spice extracted with chloroform (described in the text). The residue was dissolved in 2 mL of chloroform from which 0.1-mL aliquots were added to the flask. In parentheses are the values obtained with 0.2 mL of the extracts.

Table IV. Diameter of the Zone of Inhibition Produced by Steam-Distilled Oil of Cinnamon and Clove against *A. parasiticus* NRRL 3145 in the Standard Cup Assay[a]

	zone of inhibition, mm, resulting from adding the aliquots		
	0.01 mL	0.02 mL	0.03 mL
cinnamon oil	26 ± 1	35 ± 1	43 ± 0.3
clove oil	27 ± 1	38 ± 3	43 ± 0.3

[a] Aliquots of the steam distillate of each spice were drawn from 20% pure cinnamon oil and 70% pure clove oil diluted with chloroform.

Table V. Diameter of the Zone of Inhibition Produced by Steam-Distilled Oil of Irradiated and Unirradiated Cinnamon and Clove against *A. parasiticus* NRRL 3145 in the Standard Cup Assay

	zone of inhibition, mm	
treatment	unirradiated	irradiated (10 kGy)
cinnamon oil (0.06 mg)	44 ± 1	33 ± 2
clove oil (0.07 mg)	24 ± 1	24 ± 1
cinnamon oil + clove oil	47 ± 7	30 ± 1

Table IV depicts the diameter of the zone of inhibition obtained with steam-distilled oil of clove and cinnamon in a cup assay. The inhibition was observed to be concentration dependent. The inhibitory activity of steam-distilled oil also appeared to be higher than that of chloroform extracts (see Table II). The residues left after steam distillation, on the other hand, did not have the inhibitory activity.

Effect of γ-Irradiation on the Inhibitory Activity. Table V shows the comparison of inhibitory activity of oils from unirradiated and irradiated (10 kGy) spices. The inhibitory activity of the steam-distilled oil from the irradiated cloves remained unchanged, whereas in the case of irradiated cinnamon it was reduced when compared with the unirradiated controls. Figure 1 shows the comparison of GLC profile of the oils of unirradiated and irradiated (10 kGy) cinnamon, which indicates a reduction in the cinnamaldehyde peak. The two compounds, cinnamaldehyde and eugenol were detected in GLC with the help

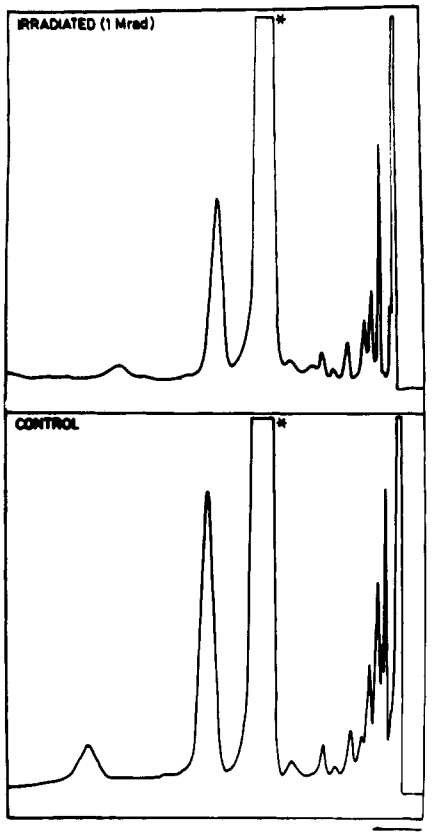

Figure 1. Gas–liquid chromatographic profiles of steam-distilled oil from irradiated (10 kGy) and unirradiated cinnamon. (*) Cinnamaldehyde peak.

of authentic samples on the basis of their retention time.

DISCUSSION

The foregoing results indicate the relatively high microbial load present in some of the common Indian spices. Most of the earlier reports deal with the bacterial load in ground spices with little attention given to fungal contamination (Farkas, 1983; Vajdi and Pereira, 1973; Bachman and Gieszczynska, 1973; Inal et al., 1972). It is apparent that the microbial load varies with each spice variety. It is possible that practices employed for harvesting, drying, and storage could influence the incidence of microbial population. In the present studies black pepper was found to be the most contaminated spice. The lower microbial counts in cinnamon and clove could be attributed to the inhibitory substances present in these spices.

Exposure to γ-irradiation was effective in decontaminating these spices. The dose required was related to the initial bacterial count. Thus, a dose of 10 kGy was necessary to sterilize black pepper, which showed a high incidence of bacterial population. The other spices could be sterilized at 7.5 kGy. The effective dose for the elimination of the fungi was only 5 kGy. An assessment of the fungal population indicated that cinnamon and clove had the lowest fungal load. This may be due to the antifungal principles present in these spices.

On a commercial scale fumigation with methyl bromide is being used for insect disinfestation and ethylene oxide or propylene oxide for sterilization. Exposure of workers to the noxious chemicals is the main cause of concern with the gas treatment. Ethylene oxide treatment of spices has been shown to affect the oil content as well as the flavor and color qualities of spices (Vajdi and Pereira, 1973). Besides, the possible retention of toxic residues in chemically treated products is currently causing concern. The radiation treatment has distinct advantages over the current practices using chemicals. Irradiation is effective in prepacked spices, and there is no risk of recontamination. It is not known to produce harmful residues. It can completely rid the spices of their microflora without affecting their essential attributes, and since the spices are highly valued commodities, the cost of irradiation per unit weight would be comparatively low. Consequently, the irradiation treatment is gaining increasing acceptance (*W. H. O. Tech. Rep.*, 1981; *Fed. Regist.*, 1983).

The antifungal activity in some of the spices under investigation could be attributed to volatile compounds. The major components causing growth inhibition of *A. Parasiticus* were found to be cinnamaldehyde in cinnamon and eugenol in clove (Bullerman et al., 1977). The zone of inhibition in the case of irradiated clove was not affected. It was observed that eugenol levels were not reduced as a result of radiation exposure (10 kGy). From the GLC profiles, the eugenol peak was found to be 52.2% against 48.2% of the total volatile content in the unirradiated controls. On the other hand, the cinnamaldehyde content in cinnamon was reduced from 61.8% to 45.5% in the irradiated samples. The aldehyde group in this compound is presumably sensitive to radiation (Diehl et al., 1978).

In the present experiments aflatoxin was not detected in any of the spices examined. The incidence of this toxic substance was reported in black pepper (Seenappa and Kempton, 1980). This could be attributed to differences in moisture content and other factors between the two studies. Since black pepper, cardamom, and nutmeg do not contain growth inhibitors of *A. parasiticus*, they are susceptible to incidence of this fungus and formation of aflatoxin.

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