Cytotoxicity of Extracts of Spices to Cultured Cells

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Abstract

The cytotoxicity of the extracts from eight different spices used in the Indian diet was determined using Dalton's lymphoma ascites tumor cells and human lymphocytes in vitro *and Chinese Hamster Ovary cells and Vero cells in tissue culture. Alcoholic extracts of the spices were found to be more cytotoxic to these cells than their aqueous extracts. Alcoholic extracts of several spices inhibited cell growth at concentrations of 0.2–1 mg/ml* in vitro *and 0.12–0.3 mg/ml in tissue culture. Ginger, pippali (native to India; also called dried catkins), pepper, and garlic showed the highest activity followed by asafetida, mustard, and horse-gram (native to India). These extracts also inhibited the thymidine uptake into DNA.*

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Introduction

Spices are part of Indian culinary art. But some of them have been implicated in carcinogenesis. Ingredients like capsaicin, which is present in the red pepper and piperine derivatives in black pepper, have been reported to be mutagenic in *in vitro* assay methods (1,2), although their effect on carcinogenesis has not been proven experimentally. Similarly, 4-alkyl-1,2-methylene-dioxy benzene, which is present in safrole, has been found to be mutagenic and carcinogenic in experimental systems (3).

According to the traditional Indian medicines, many of the spices are used medically, including for the treatment of cancer (4). This may be partially true because the extracts of many spices are rich in ingredients that could be of value in cancer prevention. Some of the ingredients are listed in Table 1. Many of these ingredients whose chemical structures are well defined are phenolic acid derivatives, which reduce substances, and isothiocyanates, which can theoretically scavenge the free radicals in the body or conjugate with chemical carcinogens. This can prevent carcinogen activation and prolong the expression of tumors in the body (5–7). As has been calculated, a delay of nearly 10 years in the expression of tumors in the general population can be of high value in cancer treatment strategies (8).

Recently, we determined the cytotoxic and tumor-reducing properties of turmeric and its

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Table 1. Common and Botanical Names of Spices Used, Their Reported Ingredients, and Postulated Properties

Spice Name	Reported Ingredients	Postulated Properties
Asafetida *(Ferula assofoetida)*	Umbelliferone allyl sulfides	Reducing agent, free radical scavenging
Black pepper *(Piper nigrum)*	Piperine, piper longumin	Cytotoxic, tumor reduction, mutagenic
Garlic *(Allium sativan)*	Allicin, allithiamine, disulfides, and sulfides	Reducing cytotoxic, free radical scavenging
Ginger *(Zingiber officinale)*	Shogal, gingerone, gingerol	Cytotoxic, free radical scavenging
Horse-gram *(Dolichos biflorus)*	Lectins	Cytotoxic, tumor reduction
Mustard *(Brassica computris)*	*P*-Hydroxyphenyl isothiocyanate	Chemoprotective
Pippali *(Piper longum)*	Piperine, piper longumin	Cytotoxic, tumor reduction, mutagenic
Sesame *(Sesamum indicum)*	Sesamin, sesamolin	Cytotoxic, tumor reduction

active ingredient curcumin (9). Turmeric was found to be cytotoxic *in vitro* at concentrations of nearly 400 μg/ml and curcumin at 8 μg/ml. Both of them reduced the ascites tumor induced in mice by Dalton's lymphoma ascites tumor cells.

The effect of extracts of various spices on mammalian cells are largely unknown. Therefore, the present work reports on the cytotoxic properties of eight different spices as assayed by different methods.

Materials and Methods

All the spices used in this study were bought from a local (Indian) market, where the storage time was approximately two to three months. After the spices were brought to the laboratory, they were kept in the refrigerator. Table 1 indicates the spices used in this study in addition to the botanical names.

Except ginger and garlic, all the other spices were directly powdered (using a mechanical grinder) just before use. The skin was peeled from the ginger and garlic, then, both ginger and garlic were dried in the oven at 50°C and powdered. The powdered drugs were extracted with either hot water or ethanol as described below.

Extraction

One gram of the dry powder was extracted with 100 mls of 90% ethanol overnight and was stirred at room temperature (30°C). After the extraction, the extract was evaporated in vacuum and was suspended in 10 mls of ethanol. This concentrated extract was diluted 10 times in saline so that the final preparation had extract from 10 milligrams of the spice per milliliter. There was no visible precipitate when the ethanolic extract was diluted with saline. Aliquots (200–10 μl) of this extract were used for further analysis. Aliquots were selected in such a manner that the alcohol content did not interfere in the assay procedure (1.8–0.09%).

Aqueous extraction was done by boiling 1 g of the powder in 100 mls of water for one hour. This extract was filtered and made up to 100 mls, and the aliquot was used for assay.

Assessment of In Vitro Cytotoxicity to Tumor Cells

In vitro cytotoxicity was measured using Dalton's lymphoma ascites tumor cells (originally grown from a spontaneously grown tumor of mouse thymus) that were propagated in female

Swiss albino mice (weighing 16–20 g) by injection of 1 million cells in the peritoneal cavity. Visible tumors appeared within 16–20 days. Cells were aspirated and were washed in phosphate-buffered saline (PBS). At 37°C, 1 million cells were incubated with various concentrations (minimum 5 concentrations) of drug for three hours. At this time, the percentage of live cells was determined using the trypan blue exclusion method. Using various concentrations of the drug (in triplicate), the amount needed to produce 50% cytotoxicity was calculated from a graph by plotting drug concentration against percentage of dead cells after assay. The percentage of dead cells after a three-hour incubation during the assay with and without the ethanol (1.8%) remained the same (approx 3–5%).

Determination of Cytotoxicity in Cultured Cells

Vero cells (NIV, Pune, India) and Chinese Hamster Ovary (CHO) cells were used to determine the cytotoxicity in cultured cells. Nearly 0.5 million cells (Vero) were incubated in tissue culture bottles and were fed with Eagle's Minimum Essential Medium containing 10% goat serum. After 24 hours, aliquots of the drug were added. After seven days, the cells were removed by trypsinization and stained with trypan blue and live cells were counted using a hemocytometer.

Clonogenic assay was used to determine the cytotoxicity to CHO cells. Cells (10^3) were plated on tissue culture bottles containing growth medium as described above. After 24 hours, various concentrations of the extract were added and incubation was continued for seven days. The medium was further removed, cells were washed in PBS, and colonies were stained with crystal violet and counted. Colonies with more than 50 cells were counted.

Cytotoxicity to Isolated Lymphocyte

The Ficoll-Hypaque procedure was used for the isolation of lymphocytes from the blood of leukemia patients. These cells, after washing with PBS, were treated with extracts at various concentration and incubated for three hours at 37°C. After the incubation, the viability of lumphocytes was determined by trypan blue exclusion method.

Determination of Inhibition of DNA Synthesis

Incorporation of tritiated thymidine (Bhabha Atomic Research Centre, Bombay, India) into DNA was done by incubating Dalton's lymphoma ascites tumor cells (5×10^6) with 4 μCi of [^{3}H]thymidine in the presence of various concentrations of the drug. In the actual experiment, the cells were pretreated with drug for three hours; thereafter, [^{3}H]thymidine was added and incubated for another six hours. After the incubation, DNA was precipitated with 0.8 M perchloric acid at 4°C and redissolved in 0.5 N NaOH and counted in a liquid scintillation counter, after the addition of dioxan-based coctail.

Results

We used the extracts from eight different spices that are commonly used in Indian food to determine their cytotoxic activity to various cells.

Cytotoxicity of Spices in In Vitro Cultures

All the spice extracts were tested for *in vitro* cytotoxicity using Dalton's lymphoma ascites cells. Both aqueous and alcoholic extracts were used. Ethanolic extracts showed a much higher activity than did aqueous extracts (Table 2). Among the ethanol extracts, ginger showed maximum activity. The amount needed to produce 50% cell death for ginger was found to be 200 μg/ml. Pippali (native to India; also called dried catkins) showed the next

Table 2. *In Vitro* Cytotoxicity of Spice Extracts on Dalton's Lymphoma Ascites Tumor Cells[a, b]

	Amount of Spices Needed for 50% Cytotoxicity, mg/ml	
	Alcoholic extract	Aqueous extract
Ginger	0.2 (0.170–0.235)	4.2 (4.180–4.220)
Pippali	0.3 (0.283–0.310)	1.5 (1.493–1.520)
Pepper	0.45 (0.44–0.454)	1.6 (1.580–1.625)
Asafetida	0.60 (0.590–0.608)	2.7 (2.670–2.715)
Garlic	0.50 (0.497–0.511)	10.0 (9.5–10.5)
Mustard	0.50 (0.494–0.503)	0.8 (0.775–0.810)
Sesame	1.20 (1.16–1.215)	1.50 (1.485–1.520)
Sesame (stored sample)	0.15 (0.147–0.156)	ND[c]
Horse-gram	0.70 (0.688–0.720)	10.0 (9.40–11.0)

a: Values are expressed as amount of spices needed as alcoholic or aqueous extracts in milligrams to produce 50% cytotoxicity in 3 hours at 37°C. Values are means of 3 separate determinations.

b: Amount of spices needed for 50% cytotoxicity was determined from group by plotting drug concentration against percent of dead cells. Standard deviation from other batches of spices was <10% unless otherwise stated, as in the case of sesame. Solvent used by itself did not produce any more cell death than control (<5%).

c: ND, not determined.

highest activity (300 μg/ml), and mustard. Sesame and horse-gram (native to India) were toxic only at higher concentrations (0.7–1.2 mg/ml). Except mustard (800 μg/ml), none of the aqueous extracts of spices showed any appreciable cytotoxicity to Dalton's lymphoma ascites tumor cells (1.5–4.2 mg), and that for garlic and horse-gram cell death could be seen only at concentrations of more than 10 mg. There was no considerable difference (<10%) in the activity using different batches of spices. Moreover there was no change in the activity (except for that of sesame powder) when the powdered material was stored up to one month at room temperature.

Effect of Extract of Spices in Tissue Culture Cells

Alcoholic extract of spices decreased the cell growth of Vero cells in tissue culture. All the extracts showed higher cytotoxicity in cultured cells than by *in vitro* methods. The concentrations needed for 50% reduction in cell number is given in Table 3. The extract of ginger showed the highest activity (120 μg/ml), followed by pippali, black pepper, asafetida, garlic, etc. The effect of the extract on cell death on long-term cultures in Vero cells was comparable to that of the *in vitro* experiment. However, the concentrations needed to produce 50% cytotoxicity were found to be lower in the case of cultured Vero cells.

When clonogenic assays were conducted using CHO cells to assess the cytotoxicity, we found that the amount needed for the 50% cell death was almost the same as that needed for Dalton's lymphoma cells (Table 3). Pippali and ginger had the highest activity (0.15–0.3 mg) followed by garlic, black pepper, asafetida, etc. The concentration needed for 50% cytotoxicity was found to be higher than that needed for Vero cells. Thus, depending on the cell line used, the drug extracts produced varying effects. The reason for the higher amount needed for cytotoxicity to CHO cells and Dalton's lymphoma cells than for Vero cells has not been investigated at present.

Effect of Extracts on Lymphocytes

Lymphocytes prepared by the Ficoll-Hypaque method were found to be sensitive to the extract of spices. It was seen that some extracts are more toxic to lymphocytes than in other

Table 3. Effect of Spice Extracts on Growth Inhibition of Vero cells and Chinese Hamster Ovary Cells [a, b]

	Amount of Spices Needed for 50% Reduction in Cell Growth, mg/ml	
	Vero cells	Chinese hamster ovary cells
Ginger	0.120 (0.115–0.125)	0.245 (0.240–0.247)
Pippali	0.130 (0.128–0.133)	0.145 (0.142–0.150)
Pepper	0.140 (0.139–0.142)	0.290 (0.284–0.295)
Asafetida	0.150 (0.147–0.154)	0.575 (0.560–0.585)
Garlic	0.155 (0.153–0.156)	0.275 (0.270–0.283)
Mustard	0.400 (0.395–0.410)	0.490 (0.485–0.495)
Sesame	0.360 (0.358–0.363)	0.440 (0.438–0.450)
Horse-gram	0.180 (0.178–0.185)	1.00 (0.800–1.110)

a: Values represent amount of spices as alcoholic extract needed for 50% reduction in cell growth (Vero cells) or colony formation (Chinese Hamster Ovary cells). Each experiment is a mean of 3 separate determinations.

b: Amount of spices needed for 50% cytotoxicity was determined by plotting percentage of live cells or colonies formed against drug concentration. Standard deviation from other batches of spices was < 10%. Ethanol used to dissolve extract by itself did not have any effect on cell growth at concentrations used here.

Table 4. Cytotoxicity of Spice Extracts on Human Lymphocytes *In Vitro* [a]

	Live Cells After Incubation with Various Drug Concentrations, %			
	1 mg/ml	0.5 mg/ml	0.25 mg/ml	0.1 mg/ml
Ginger	12±6	17±6.1	80± 7.8	100±0
Pippali	0±0	0±5.2	18± 6.1	74±3.6
Pepper	0±0	37±6.1	78± 4.0	100±4.2
Asafetida	0±0	0±1.0	22±10.2	89±3.8
Garlic	18±5.7	29±7.2	100± 5.0	100±3.2
Mustard	0±3.5	34±4.0	100± 3.5	100±3.2
Sesame	0±0	0±4.5	41± 3.1	67±5.5
Horse-gram	0±3.5	87±6.0	100± 2.3	100±4.2

a: Ethanol used to dissolve extract did not produce any cytotoxicity at concentrations studied. Values are means of 3 separate experiments.

cell lines (Table 4). Asafetida and sesame were found to have more activity on lymphocytes than on other cells, and there was a slight reduction on the activity of ginger. The activity of all other spices was almost the same as that for other cell lines.

Effect of Extract of Spices on Thymidine Incorporation Into Cellular DNA

The effect of the drug on DNA synthesis was studied by incorporating tritiated thymidine into Dalton's lymphoma tumor cell DNA. [^{3}H]Thymide (4 μCi) was incubated into cells along with two different concentrations of the drugs. The results show a concentration-dependent decrease of thymidine incorporation in all the spice extracts (Table 5). Ginger extract showed the maximum effect on thymidine incorporation into DNA. In this case, the effective concentration needed for 50% reduction in incorporation was 1 mg/ml of the extract. Black pepper and garlic also showed similar results. Other spice extracts showed moderately higher values (1.5–2 mg/ml), whereas the extract of horse-gram did not produce any inhibition of thymidine incorporation into DNA. Concentration of extracts needed for

Table 5. Effect of Spice Extracts on [^{3}H] Thymidine Incorporation Into Cellular DNA[a]

	Inhibition of DNA Synthesis, %	
	0.25 mg/ml	1 mg/ml
Ginger	24±4.6	52± 3.60
Pippali	35±6.0	44±13.00
Pepper	10±0.71	55± 7.50
Asafetida	17±1.52	28± 2.31
Garlic	22±2.10	44± 1.20
Mustard	22±5.00	40± 5.00
Sesame	34±1.50	45± 1.20
Horse-gram	6±5.70	21± 2.50

a: Values are expressed as percentage of inhibition of DNA synthesis produced during incubation with [^{3}H] thymidine with 0.25 mg/ml and 1 mg/ml of spice extract. Values are means of 3 separate determinations and were compared with untreated cells to which same value of ethanol was added.

the inhibition of thymidine incorporation was higher than the cytotoxic concentration. This indicates that the inhibition of DNA synthesis may not be the only mechanism of action of spices on cells.

Discussion

Spices are regular ingredients of traditional Indian food. The structure of chemical compounds present in some of the spices show that they may have some antimutagenic and/or anticancer properties. We selected eight spices (ginger, pepper, sesame, pippali, garlic, mustard, asafetida, and horse-gram) for the primary screening experiments. The screening was based on the cytotoxic activity of the spice extracts using trypan blue as the marker and *in vivo* tissue culture methods.

There are only very few reports that indicate the possible antitumor activity of spices. Goodpasture and Arrighi (10) did show that several spices in tissue culture produced chromosomal changes, including changes in chromosomal condensation and disintegration. Our own studies (9) on turmeric and its active principle, curcumin, showed that they produce cellular disintegration *in vitro*. However, neutralized curcumin, at physiological pH, does not produce these changes. Similarly, combined with cellular proteins, its antitumor property is reduced considerably (11). Recently, we found that curcumin reduced the induction of 7,12-dimethylbenz[*a*]anthracene-induced tumors in mice (M.C. Unnikrishnan and R. Kuttan, unpublished observations).

The present experiments indicate that the aqueous extracts of most of the spices are not cytotoxic at the levels we studied. However, the alcoholic extracts of all spices were cytotoxic to tumor cells, CHO cells, lymphocytes, and Vero cell lines. Ginger, pippali, and black pepper were the most cytotoxic and exerted their action between 120 and 300 μg to various cell lines. Asafetida, mustard, garlic, sesame, and horse-gram were much less cytotoxic. Sesame was found to produce a highly cytotoxic component when stored, the nature of which has not been studied at present (see Table 2).

Results of thymidine incorporation assay shows that DNA synthesis was inhibited by the extracts of the spices, which is reflective of the cytotoxic potential. Because the concentration needed for the inhibition of DNA synthesis was higher than what was needed for cytotoxicity, it seems very possible that the inhibition of DNA synthesis may not be the only mechanism of action.

Whether the cytotoxicity of spices causes cancer or reduces tumors is not known at present. The mutagenicity of several spice extracts have been tested by the *Salmonella* testing

method (12). However, none of the spices tested in the report (except pepper) produced any appreciable mutagencity or teratogenicity (13,14). Pepper contained the irritants such as piperine and capsaisin, both of which have been reported to be irritants to rat intestinal epithelium (15).

Some of the extracts used in this study were reported to have anticancer activities. Compounds isolated from garlic extract have also been shown to produce regression of Sarcoma 180 (16). Similarly, horse-gram lectin was found to be cytotoxic, and F^9 embryonal carcinoma and leukemia cells were found to have receptors for this lectin (17). Many isothiocyanate (18) derivatives present in plants are highly bactericidal, and spices in the ginger family contain many different cytotoxic principles (19). The role of these cytotoxic components in tumor reduction needs further evaluation.

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