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Evaluation of Mutagenic Activity of Turmeric Extract Containing Curcumin, Before and After Activation with Mammalian Cecal Microbial Extract of Liver Microsomal Fraction, in the Ames *Salmonella* Test

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Turmeric, a ground powder of the rhizomes of the plant Curcuma longa and its alcoholic extract are commonly used for spicing of foods in many countries. Curcumin (diferuloyl methane) the active colour principle of turmeric, has increasing use as a food colorant (Timberlake and Henry, 1986). The joint FAO/WHO Expert Committee on food additives has allocated a temporary Acceptable Daily Intake (ADI) for turmeric and curcumin of 2.5 and 0.1 mg/kg body weight respectively (Anon, 1982). However, they have indicated the need for additional studies on the mutagenicity and teratogenicity of these substances.

Mutagenic activation or inactivation of the ingested compounds is known to occur through various metabolic processes in the body (Hoensch and Schwenk, 1984). In such transformations of compounds, liver microsomal enzymes and intestinal microflora play a major role (Lu et al., 1972; Prins, 1978). Bonin and Baker (1980) and Jensen (1982) have reported nonmutagenicity of turmeric oleoresin and pure curcumin, in the presence and in the absence of rat liver microsomal activation systems in the Ames assay with S.typhimurium tester strains TA 100 and TA 98. However, as far as we are aware, no study has addressed whether turmeric and curcumin can be mutagenic after activation by cecal microorganisms.

The work reported here examines the mutagenicity of alcohol soluble turmeric extract containing curcumin, before and after activation with a cecal cell free extract or a liver microsomal fraction of rat, in the Ames assay with S.typhimurium tester strains TA 100, TA 98 and TA 97a. The recently developed strain TA 97a is reported to be more sensitive than strain TA 98 (Levin et al., 1982).

MATERIALS AND METHODS

Pure curcumin (99%) was the generous gift from Bombay Oil Industries, Bombay, India; Nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate (G-6-P), histidine-HCl, biotin, rutin, benzo(a)pyrene, sodium azide, 4-Nitroquinoline-N-oxide, ICR-191 were purchased from Sigma Chemical Company,

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St. Louis, Mo, U.S.A.; Aroclor-1254 from Monsanto, St. Louis, U.S.A.; S.typhimurium strains TA 100, TA 98 and TA 97a were kindly provided by Prof. B.N. Ames, Univ. California, Berkeley, California, U.S.A.

An ethanol soluble extract from turmeric was prepared essentially according to the procedure of Goodpasture and Arrighi (1976). Turmeric, a ground powder (100 gms, 100 mesh) of dry rhizome of the plant Curcuma longa was refluxed with a 400 ml mixture of double distilled ethanol and water (70 : 30 v/v) at 84°C ± 2° for 1 hr, kept overnight at room temperature and the insoluble portion was separated by filtration. To the filtrate (about 100 ml) cold distilled water (about 200 ml at 5°C) was added, the precipitate was collected by centrifugation (12,000 x g at 5°C) and dried at 37° for 24 hr. The precipitate thus obtained is termed turmeric extract. The yield of turmeric extract from turmeric powder generally ranged from 4.5 to 5.0%. The concentration of curcumin in the extract was determined by the method recommended by Joint WHO/FAO Expert Committee on Food Additives (Anon, 1971) using pure curcumin as a standard. The curcumin content of the turmeric extract was usually in the range of 33 to 35% (w/w).

The turmeric extract was dissolved in double distilled ethanol at three different concentrations 5, 10 and 20 mg/ml. Mutagenic activity of turmeric extract was determined at three concentrations (50, 100 and 200 µg/plate, curcumin concentrations, 17.5, 35 and 70 µg/plate respectively) by adding 0.01 ml of the above stock solutions to the agar layer in the Ames assay. The solubility limit of the turmeric extract in the agar layer of 2 ml was found to be 200 µg. The concentration of ethanol in the assay was maintained at 0.5% (v/v).

Aroclor 1254 was dissolved (200 mg/ml) in dimethyl sulphoxide (DMSO) and administered intraperitoneally (500 mg/kg body wt.) to male rats (Wistar strain) and the liver S9 factor was prepared according to Ames et al. (1975). The S9 mix was freshly prepared and contained per ml, S9 factor, 0.1 ml; NADP, 4 mM; G-6-P, 5 mM; Na_2HPO_4, 100 mM; $MgCl_2$, 8 mM and KCl, 33 mM. The metabolic activity of the S9 mix was examined before use by assaying the mutagenicity of benzo(a)pyrene with tester strain TA 100. The compound showed mutagenicity after metabolic activation (around 900 revertants/µg/plate) (Ames et al., 1975).

A cell free extract of microorganisms located in the rat cecum was prepared by the procedure of Brown and Dietrich (1979). The procedure involved removal of rat cecal contents (about 4.5 gms) in Krebs-Ringer-0.25 M phosphate buffer (pH 7.4) containing 0.25 mg/ml of Clelands reagent, sonication for disruption of cells in a cell disruptor (Model W-220F, Heat Systems - Ultrasonics, Inc., N.Y., U.S.A.; 5 burts of 30 sec with 60 sec gaps), centrifugation (13,000 x g for 20 min at 5°C) and collection of the supernatant termed as cecal cell free extract (CCE). Freshly prepared CCE was filter sterilised and used in the mutagenicity assay as described below. That CCE is metabolically active was ensured by assaying the mutagenicity of rutin, with and without CCE activation, with TA 98. Mutagenic activity of rutin (around 200 revertants/100 µg/

Table 1. Assessment of reversion properties of S.typhimurium strains TA 100, TA 98 and TA 97a with standard mutagens by spot tests.

Mutagen	Concentration µg/disk	Tester strain TA 100	TA 98	TA 97a
Sodium azide	1.5	+ + + +	-	+
4-Nitroquinoline-N-oxide (NQNO)	10.0	+ + +	+ +	—
ICR-191	1.0	+ +	-	+ + +

ICR-191 and NQNO were dissolved in DMSO and sodium azide in water.

Symbols for the number of revertants/plate (spontaneous substracted): - = <25; + = 25 - 100; + + = 100 - 200; + + + = 200 - 500; + + + + = >500.

plate) was discernible exclusively after CCE activation (Brown and Dietrich, 1979; Pamukcu et al., 1980; Tamura et al., 1980).

The mutagenicity assay using Salmonella typhimurium tester strain TA 100, TA 98 and TA 97a was conducted according to Ames et al. (1975). The tester strains were examined for the stability of their genetic properties before use (Ames et al., 1975).

Cells were grown in nutrient broth for 18-20 hr and the culture (0.1 ml, 10^9 cells/ml), turmeric extract in ethanol (0.01 ml) or ethanol (0.01 ml), liver S9 mix (0.1 ml) or CCE (0.4 ml) were added to 2 ml of molten agar (40-42°C) containing agar, 0.6%; NaCl, 0.5%; histidine, 0.05 mM; biotin, 0.05 mM; glucose, 4% for TA 100, TA 98 and 0.4% for TA 97a. This agar was spread in plates having a preset agar layer (20 ml) of modified M9-medium (per litre, Na_2HPO_4, 7.0 gm; K_2HPO_4, 3.0 gm; NaCl, 0.5 gm; NH_4Cl, 1.0 gm; $MgSO_4$, 2 gm; $CaCl_2$, 20 mg and agar 15.0 gm). The plates were incubated at 37°C for 48 hr and histidine revertant colonies were counted. As positive controls, concurrent spot-tests were carried out with mutagens sodium azide (1.5 µg/plate), 4-nitro-quinoline-N-oxide (10 µg/plate), ICR-191 (1.5 µg/plate) for tester strains TA 100, TA 98, TA 97a, respectively. The responses of the strains to the mutagens (Table 1) were similar to those reported by Maron and Ames (1983). Ethanol (0.01 ml/plate), a solvent used for dissolving turmeric extract, was included as a negative control. Each dosage of turmeric extract was tested in 5 parallel plates and all the tests were performed in quadruple.

RESULTS AND DISCUSSION

It is now recognised that microflora from the intestine can metabolise or transform a large number of exogenous and endogenous compounds by enzymic hydrolysis, reduction, degradation and various other reactions (Prins, 1978). Mutagenic activation of the ingested compounds can occur through such reactions eg., rutin, a flavonol present in edible plants, exhibits mutagenicity after its activation with intestinal microflora (Brown and Dietrich, 1979).

Ravindranath and Chandrasekhara (1980) reported that about two-thirds (60 to 65%) of the given dose of curcumin (a major pigment of turmeric, doses 0.05, 0.4 and 2.0 gm/kg body wt., given by gavage) is absorbed in the rat and the remaining portion of 35-40% of the administered dose is eliminated mainly through feaces with very little through the urine. As reported by Wahlstrom and Blennow (1978), they also observed low levels of curcumin in blood, liver and kidney though the proportion of the given dose absorbed was quite high (Ravindranath and Chandrasekhara, 1980). In vitro studies with (^{3}H)curcumin and averted rat intestinal sacs indicated transformation of the pigment during its absorption from intestine and also the movement of the transformed products towards the serosal side (Ravindranath and Chandrasekhara, 1981). Additionally, it has been reported (Holder et al., 1978; Ravindranath and Chandrasekhara, 1982) that around 99% of the administered radioactivity is excreted in the feces of the rat over 72 hrs after the administration of (^{3}H)curcumin and that about one third

Table 2. Results of mutagenicity tests using S.typhimurium strains TA 100, TA 98 and TA 97a on curcumin containing turmeric extract, before and after activation with rat caecal cell free extract (CCE).

Concentration of Turmeric extract (µg/plate)	TA 100		TA 98		TA 97a	
	- CCE	+ CCE	- CCE	+ CCE	- CCE	+ CCE
	(Number of revertants/plate)					
0 (SR)	87.2 ± 4.1	108.7 ± 3.5	16.6 ± 1.9	19.1 ± 2.2	66.4 ± 1.7	69.6 ± 2.5
*Control	81.6 ± 6.1	67.9 ± 4.6	13.3 ± 1.0	16.0 ± 1.0	64.6 ± 2.0	69.4 ± 2.5
50 (17.5)	69.8 ± 2.7	66.6 ± 4.9	17.3 ± 3.1	21.5 ± 2.6	60.9 ± 2.1	64.4 ± 1.5
100 (35)	74.8 ± 3.8	75.0 ± 7.0	15.0 ± 2.0	18.6 ± 1.9	60.1 ± 1.5	67.7 ± 2.5
200 (70)	77.0 ± 4.2	73.9 ± 4.6	21.0 ± 2.2	21.1 ± 1.6	59.4 ± 1.1	69.0 ± 2.0

(SR), spontaneous revertants. *Control, 0.01 ml ethanol per plate.

In each experiment, as positive controls, concurrent spot-tests were performed with the mutagens indicated in Table 1. The genetic properties of the tester strains and the metabolic activity of CCE were examined as described in 'Materials and methods'.

Values are means of ± S.E. for 5 replicates of 4 independent experiments.

Figures in parenthesis indicate concentrations of curcumin.

Table 3. Results of mutagenicity tests using S.typhimurium strains TA 100, TA 98 and TA 97a on curcumin containing turmeric extract, before and after activation with rat liver microsomal fraction (S9).

Concentration of turmeric extract (µg/plate)	TA 100		TA 98		TA 97a	
	- S9	+ S9	- S9	+ S9	- S9	+ S9
	(Number of revertants/plate)					
0 (SR)	94.6 ± 2.6	94.5 ± 2.6	16.5 ± 1.8	21.0 ± 3.2	66.4 ± 1.7	63.1 ± 2.3
*Control	107.1 ± 5.5	104.2 ± 3.9	25.1 ± 3.8	24.2 ± 2.4	64.6 ± 2.0	68.7 ± 2.1
50 (17.5)	99.0 ± 4.3	97.1 ± 3.8	13.8 ± 2.6	18.6 ± 2.3	60.9 ± 2.1	68.0 ± 2.0
100 (35)	97.8 ± 2.4	94.0 ± 2.6	20.1 ± 2.4	18.0 ± 2.5	60.1 ± 1.5	65.0 ± 2.1
200 (70)	106.8 ± 6.0	108.5 ± 3.6	21.1 ± 4.0	20.8 ± 3.9	59.4 ± 1.1	69.4 ± 2.0

(SR), spontaneous revertants. *Control, 0.01 ml ethanol per plate.

In each experiment, as positive controls, concurrent spot-tests were performed with the mutagens indicated in Table 1. The genetic properties of the tester strains and the metabolic activity of S9 mix were examined as described in 'Materials and methods'.

Values are mean ± S.E. for 5 replicates of 4 independent experiments.

Figures in parenthesis indicate concentrations of curcumin.

of the excreted radioactivity is present as curcumin. This suggested that the remaining portion of the radioactivity present in the feces (60-65%) would be due to metabolites of the pigment. Thus, these reports point out absorption, metabolism and excretion of curcumin in animals, the level of metabolism in liver and that the elimination through urine is lower.

Results from Table 2 show that turmeric extract, at three different doses (50, 100, 200 µg/plate; curcumin concentrations 17.5, 35, 70 µg/plate; respectively) does not exhibit mutagenicity, in the presence and in the absence of activation by CCE in Ames Salmonella assay system with tester strains TA 100, TA 98 and TA 97a. Similarly, the extract does not display mutagenicity after activation with rat liver S9 mixture (Table 3) as has been reported by others (Bonin and Baker, 1980; Jensen, 1982) with tester strains TA 100 and TA 98. The results (Table 2 and 3) also demonstrate that the solvent, ethanol, at the concentration (0.5, v/v) used in the experiments does not affect the growth of the strains and their respective pattern of spontaneous revertants.

Clastogenicity of turmeric has been shown in root tip cells of Allium cepa (Abraham et al., 1976) and in mammalian cells under in vitro conditions (Goodpasture and Arrighi, 1976). In contrast to these studies, investigations in vivo, of both turmeric and curcumin by Vijayalaxmi (1980) did not exhibit genotoxic effect in the micronucleus test in mice, the bone marrow chromosome analysis test in mice and rats, and the dominant lethal test in mice. Abraham and Keshavan (1984) observed negative results for turmeric in the Drosophila test for sex chromosome loss and recessive lethals. Jensen (1982) reported an absence of a mutagenic effect for turmeric oleoresin and curcumin in the Salmonella/mammalian microsome test. In addition, the present investigations reveal nonmutagenicity of turmeric extract containing curcumin after activation with mammalian cecal microflora in the Ames Salmonella assay system.

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