Mutagenicity of Four Natural Flavors: Clove, Cinnamon, Thyme and *Zataria multiflora* Boiss.

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Abstract

Background: Natural flavors are widely used in various foods, cosmetic and pharmaceutical products. These kinds of additives are applied as colors, preservatives, aroma and tasting agents.

Objective: The large-scale use of certain food flavors requires accumulation of toxicological data on these substances, particularly in cases where structural similarities with other known substances showing genotoxic or carcinogenic properties indicate that some restrictions on human consumption or exposure should be implemented the case of the flavors.

Methods: In this study, Concentrations of 50, 100, 200, 300, 500, 1000 and 2000 µg/ml of four essential oils, *Eugenia caryophyllata* (Clove), *Cinnamum zeylanicum* (Cinnamon), *Thymus vulgaris* (Thyme) and *Zataria multiflora*, were tested in *Salmonella typhymurium* strains TA100 with and without rat liver S9 using Ames Salmonella reversion assay.

Results: Without S9 fraction, increase in mutant colonies per plate was not observed in all used concentrations. Also with S9 fraction all of samples had no significant increase in mutant colonies per plate except Clove in 500 µg/ml, and higher concentrations.

Conclusion: Based on obtained results, Clove oil may have mutagenic effect in 500 µg/ml and higher concentration.

Keywords: *Eugenia caryophyllata* (Clove), *Cinnamum zeylanicum* (Cinnamon), *Thymus vulgaris* (Thyme), *Zataria multiflora*, Mutagenicity

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Introduction

One of the most important objectives in human's life is food safety. Foods and beverages have a wide variety, so inevitably many different synthetic and natural compounds (additives) used for the various purposes such as stability, shape and consistency, preservation, spoilage prevention, increase of consumer tendency, nutritional value and increase of shelf life of products; [5, 6, 7, 8, 9] however safety of these products should be considered.

Because of incremental use of food additives, evaluation of their harmful side effects should be considered as an important topic in food safety. Harmful effects of theses compounds include allergic reaction, gastrointestinal disorders, cardiovascular diseases, respiratory or renal failures and in severe cases carcinogenicity, mutagenicity and teratogenicity; may be presented in short or long time, and in the permanent and/ or irreversible patterns. Animal, technical and safety studies are performed for confirming food additives prior using, but some specific factors like sex, ethnic, age groups, genetic, geographical conditions, intake doses, route of consumption, intake with other compounds, storage condition of products may convert them to toxic agents or increase their side effects.

Food additives could be classified based on their origin, synthetic, semi-synthetic and natural. The two first could be controlled due to their constant physicochemical characteristic, usage and intake level and purity, but in natural ones like essential oils, extracts, spices, powders, and oleoresins differs. This type of additives derivates from natural origins and doesn't have any identified contents. They are a complex of known and unknown compounds; so in their usage should be paid more attention. The unknown compounds may intrinsically be harmful for consumers and also can be converted to toxic and harmful compounds due to interfering with product ingredients, processing effects, long time storage, changing in GI tracts and so on. Therefore risk assessment of unknown and complex compounds is so critical and essential. Aromatic plants are used in various forms such as essential oils, powders, oleoresins, and dried, in food, cosmetic and pharmaceutical products. They are used because of their sweet-smelling and flavor, therapeutic, anti-microbial [5, 6, 7, 8, 9], anti-septic, analgesic, spasmolytic, anti-diarrhea, anti-emetic and expectorant effects. The active ingredients of these plants are usually tannin, essential oils, terpens, mucilage, hydrocarbons, saponin, minerals, fatty acids and phenols.

In the present study four common used plants: Clove, Cinnamon, Thyme and Zataria multiflora were selected. Their known active ingredients include Caryophyllene, Eugenol, Humulene in Clove, Cymene, Cineole, Terpinolene, Thymol, Carvacrol in Thyme, Phenol, Carvacrol, p-Cymene, Oleanolic acid, Linalool in Zataria multiflora and Cinnamaldehyde, Cinnamic acid, Eugenol, Limonen, Cinnamyl alcohol in Cinnamon.

These four plants were selected for evaluation of mutagenicity effects. Dose-dependent inhibitory effect of them, their genotoxicity and mutagenicity in the Ames Salmonella reversion assay were studied in 2 microbial test systems [4] with and without S9 fraction (microsomal mutagenesis assay) for 7 dilutions of each.

The large-scale use of certain food flavors requires accumulation of toxicological data on these substances [11], particularly in cases where structural similarities with other known

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substances showing genotoxic or carcinogenic properties indicate that some restrictions on human consumption or exposure should be implemented the case of the flavors.

The average maximum used levels of some essential oils in food products, such as beverages, ice cream, baked goods, gelatins and puddings, and chewing gums, range from 1.4 to 500 µg/ml.

Materials and Methods

Bacterial strains

The test strain used for the Ames test was *Salmonella typhymurium* strains TA100 (hisG46/rfa/Δ uvrB/pKM101), developed by Dr B.N.Ames of the university of California, Berkeley.

S9 fraction

Induction of liver enzymes

For enzyme induction at least 6 male Sprague-Dawley rats of body mass about 200 g to 300 g intra-peritoneal treated with Phenobarbital at doses of 30 mg/kg/day (96 h before killing) and 60 mg/kg/day (24, 48 and 72 h before killing) and with Β- naphtoflavone at a dose of 80 mg/kg/day (48 h before killing).

In case of Phenobarbital /Β- naphtoflavone induction, the rats received 80 mg/kg body mass Phenobarbital intraperitoneally and 80 mg/kg body mass Β- naphtoflavone orally on three consecutive days simultaneously, in an appropriate vehicle. The rat livers were analyzed 24 h after the last treatment.

Animals were kept in special adequately marked cages which were only used for this purpose. Husbandry was standardized, with 12 h of electrical lighting daily, 20°C to 23°C room temperature and about 60% mean relative humidity, on a bedding of softwood granules. Air change was about 10 times per h. The animals received an appropriate standard diet and water [2].

Preparation of S9 fractions

Liver were removed under sterile conditions immediately after termination and kept at 4°C until all animals had been prepared, all the remaining steps were carried out under sterile conditions at 4°C.

The livers were washed with cold (4°C) 0.15 mol/l KCl solutions (approximately 1 ml KCl per 1 g liver) and homogenized in fresh, cold (4°C) 0.15 mol/l KCl (approximately 3 ml KCl per 1 g liver). Then the homogenate was centrifuged in a cooling centrifuge at 4°C and 9000 g for 10 min. The supernatant (the S9 fraction) was stored below -70°C in small portions. On the day of the test, was thawed, divided in appropriate portions and stored at room temperature until used.

Plant material and chemicals

Plant Material

Aerial parts of the plants were collected at the flowering stage from Tokestan, 11th Km. Gorgan-Mashhad Road,Gorgan, Iran. Thymol (99.5 %) was purchased from DBH, and Carvacrol, γ-Terpinene and *p*-Cymene (all 99.5 %) from Fluka.

Isolation of Essential Oil

The air dried and powdered aerial parts of the plants for Zataria and thymus, flowers for Eugenia and bark parts for Cinnamon were subjected to hydro distillation for 4h using a Clevenger type apparatus according to the procedure described in the European Pharmacopoeia. The obtained essential oil was dried with anhydrous sodium sulphate and stored at +4°C before using and analyzed by GC and GC-MS. Gas chromatography (GC) and GC–mass spectrometry (MS) analysis of

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essential oils were performed. The oil of clove showed high contents of Eugenol, B-caryophyllene and Eugenyl acetate. The oil of thyme showed high contents of carvacrol and thymol. The oil of thyme showed high contents of *p*-cymene, carvacrol and thymol. Cinnamon sticks were finely chopped before being boiled and analyzed by GC and GC-MS. [5, 6, 7, 8, 9].

Identification and Quantification of the Oil Components

Analytical gas chromatography was carried out using a HP-6890 GC with capillary column DB-1 (30 m × 0.25 mm i.d., 0.25 µm f.t.); carrier gas, He; flow rate 1.5 ml/min; split ratio, 1:25 and using a flame ionization detector. The column temperature was programmed at 50 °C for 1 min and then heated to 265 °C at a rate of 2.5 °C/min, then kept constant at 265 °C for 20 min; injector temperature, 265 °C; detector temperature, 300 °C; H_2 flow, 35 ml/min; air flow, 400 ml/min.

Gas Chromatography – Mass Spectrometry

GC-MS was performed on a Thermo quest 2000 with a quadrupole detector, on capillary column DB-1. The MS operated at 70 eV ionization energy. Retention indexes were calculated using retention times of n-alkenes that were injected after the oil at the same chromatographic conditions. The compounds were identified by comparison of retention indexes (RI, DB-1) with those reported in the literature and by comparison of their mass spectra with the Wiley library. Essential oil was also analyzed by Hewlett Packard GC –MS (model 5890 series II) with mass selective detector (model 5971A). Two columns of different polarity were used: an HP-101 column (Methyl silicone fluid, Hewlett Packard; 25 m × 0.2 mm i.d., film thickness 0.2 μm) and an HP-20 M column (Carbowax 20 M, Hewlett Packard; 50 m × 0.2 mm i.d., film thickness 0.2 μm). Oven temperature was programmed as follows: isothermal at 70°C for 4 min, then increased to 180°C, at a rate of 4°C min^{-1} and subsequently held isothermal for 15 min (for HP-20M column); isothermal at 70°C for 2 min, then increased to 200°C, at a rate of 3°C min^{-1} and held isothermal for 15 min (for HP-101 column). Carrier gas was helium, flow rate: 1 ml min^{-1}; injector temperature: 250°C; volume injected: 1 μl; split ratio: 1: 50. MS conditions: ionization voltage: 70 eV; ion source temperature: 280°C; mass range: 30 – 300 mass units.

Qualitative and quantitative determination

The individual peaks were identified by comparison of their retention indices to those of authentic samples, as well as by comparing their mass spectra with the Wiley 6·0 library (Wiley, New York, NY, USA) and NIST98 (National Institute of Standards and Technology, Gaithersburg, MD, USA) mass spectral database and literature.

The percentage composition of the samples was computed from the GC peak areas by using the normalization method (without correction factors). Quantitative results are mean of data derived from duplicate GC-FID analyses. [5, 6, 7, 8, 9].

Mutagenicity assay

The Ames test (pre- incubation method at 37°C for 20 min) was conducted to examine the mutagenicity of the concentrations of 50, 100, 200, 300, 500, 1000 and 2000 µg/ml of four essential oils, *Eugenia caryophyllata* (Clove), *Cinnamum zeylanicum* (Cinnamon), *Thymus vulgaris* (Thyme) and *Zataria multiflora* [1], using *Salmonella typhymurium* strains TA100 with and without rat liver S9. A mixture containing each test component in 0.1 ml of dimethyl sulfoxide (DMSO), 0.1 ml of

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the test strain cell culture in the early stationary phase and 0.5 ml of S9 mix was incubated at 37 °C for 20 min in each test tube with a shaking frequency of 120 strokes per minute. After incubation, 2 ml of 0.05 mM L-histidine/0.05 mM biotin molten top agar were added to each test tube, mixed and poured onto the surface of minimal glucose agar medium. The plate was incubated for 48 h at 37°C and the number of reverent colonies was counted. The Ames test using the S9 fraction and without S9 mix (phosphate buffer in place of the S9 mix) for all the test compounds was performed on the same occasion.

The S9 mix (0.5 ml) contained 0.05 ml of the S9 fraction and 0.45 ml of a cofactor solution (ISO 16240,. 2005.04.01).The S9 mix composed of 8 mM MgCl2, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADPH, 4mM NADH, and 100 mM sodium phosphate (pH 7.4). The protein amount of each S9 fraction that was used was 1 mg/plate.

To validate the assay, positive controls (benzo [α] pyrene at 27 µg/plate, nitrofurantoin 0.2 µg/plate and Sodium azaide 1.5 mg/plate) for TA100) were used and the sterility of each S9 fraction was checked. The dose-finding and main assays were conducted using one plate and two plates, respectively. DMSO was used to dissolve the test and positive control articles and was also used as negative (solvent) control.

Evaluation of mutagenicity

The Induction rate is difference between the mean value of mutant colonies counted on the plates treated with a dose of the test sample or with a positive control and the mean value of the corresponding plates treated with the negative control using the same strain under identical activation conditions. For TA100, this induction rates 80.

A test sample is evaluated as being mutagenic if an increase in mutant colonies per plate above the defined induction rate defined per strain is achieved in relation to dose in at least one strain under at least one activation condition.

Mutagenicity was evaluated according to the so-called "2-fold" rule. Thus, the test samples were judged to be mutagenic if the following criteria were satisfied: (a) the maximum number of revertants was 2-fold or more relative to the negative control, (b) a dose dependent increase in the number of revertants was observed and (c) the dose-finding and main assays produced reproducible results.

Validity criteria

By using TA100, the mean values of negative controls were within the range of 80-180 mutant colonies per plate, the mean values of positive controls showed at least the induction rate of +100 colonies.

Results

All 4 essential oils in all 7 tested dilutions were negative in the Ames Salmonella reversion assay without S9 (microsomal mutagenesis assay), induction rates were from 0 to 13.

However all 3 essential oils in each 7 tested dilution except for Clove oil (*Eugenia caryophyl*) were negative in the Ames Salmonella reversion assay with S9 (microsomal mutagenesis assay), induction rates were from 0 to 15.

Clove oil has dose related response, and showed a positive result in 500 µg/ml in the Ames Salmonella reversion assay with S9

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(microsomal mutagenesis assay), induction rates were 127, >207 and >207 for 500, 1000 and 2000 µg/ml concentration respectively. (Tables 1, 2, 3, 4, 5).

Table 1: Mutagenicity test results of Clove

Conc. (µg/ml)	with S9			without S9		
	Mean	SD	IR	Mean	SD	IR
50	93	1.73	0	82	1.73	2
100	94	1.00	1	84	2.65	4
200	103	3.61	10	84	2.00	4
300	130	4.36	37	90	2.65	10
400	135	6.00	42	91	5.29	11
500	220	5.57	127	91	3.46	11
1000	>300		>207	92	4.58	12
2000	>300		>207	95	3.00	15

IR: Induction Rate
Mean: Average of colonies in triplicate plates

Table 2: Mutagenicity test results of Cinnamon

Conc. (µg/ml)	with S9			without S9		
	Mean	SD	IR	Mean	SD	IR
50	93	3.00	0	80	0.00	0
100	94	2.00	1	81	1.00	1
200	102	4.35	9	81	1.73	1
300	103	8.18	10	80	1.00	0
400	107	2.00	14	81	3.60	1
500	107	3.46	14	81	5.13	1
1000	107	2.64	14	81	3.00	1
2000	108	4.35	15	83	1.00	3

IR: Induction Rate
Mean: Average of colonies in triplicate plates

Table 3: Mutagenicity test results of Thyme

Conc. (µg/ml)	with S9			without S9		
	Mean	SD	IR	Mean	SD	IR
50	92	2.64	-1	81	4	1
100	93	2.00	0	81	1.73	1
200	93	1.00	0	81.3	6.65	1.3
300	100	5.56	7	82	2.64	2
400	102	1.00	9	83	4.35	3
500	105	3.60	12	82	3.00	2
1000	105	5.56	12	83	3.60	3
2000	105	5.19	12	85	2.64	5

IR: Induction Rate
Mean: Average of colonies in triplicate plates

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Table 4: Mutagenicity test results of Zataria multiflora

Conc. (µg/ml)	with S9			without S9		
	Mean	SD	IR	Mean	SD	IR
50	92	0.00	0	82	4.58	2
100	92	1.00	0	82	5.56	2
200	92	2.64	0	84	2.00	4
300	93	2.64	7	85	2.64	5
400	93	2.00	9	86	0.00	6
500	95	3.60	12	85	2.64	5
1000	97	2.00	12	87	2.00	7
2000	97	4.58	12	87	4.35	7

IR: Induction Rate
Mean: Average of colonies in triplicate plates

Table 5: Mutagenicity test results of positive and negative controls

Concentration	with S9	without S9
	Mean	Mean
1.5(µg/ml) Sodium azaide	>300	>300
0.2(µg/ml) Nitrofurantoin	>300	>300
benzo [µ] pyrene at 27 (µg/ml)	>300	>300
Negative control	93	80

Mean: Average of colonies in triplicate plates

Discussion

Safrole and eugenol at high level are known carcinogens in animals and methyleugenol is a suspected carcinogen compound [3]. These phenylpropenes and some of carcinogens are not detectable by the Ames assay without S9.Zataria multiflora was reported mutagen in the Ames assay with S9 [5, 6, 7, 8, 9]. In contrast with improving the method which can screen for microsomal enzymes or also for intra and inter chromosomal recombination in logarithmic phase cultures, some negative compounds in the Ames assay without S9, can give positive results [10]. We also used the Ames test with S9 for promising.

Suggestion

These results confirm the promutagen character of clove oil (*Eugenia caryophyl*) in 500 µg/ml Due to its widely use as a flavoring agent in various foodstuffs and also due to its application in cosmetics and perfumes too, so its risk assessment for carcinogenicity and

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mutagenicity should be noticed in further studies. This would be required by evaluating this effect in proper cell lines and in vivo tests.

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