

Effects of Limonene on the PAHs Mutagenicity Risk in Roasted Fish Skin

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Abstract

Traditional edible barbecue products use with lemon juice not only makes the barbecue more delicious but also reduces the risk of PAHs in the barbecue products. One of the major economics crops in Taiwan, the waste from citrus fruits was very tremendous mass. However, the peelings of citrus fruits are rich in essential oil, especially, the limonene is the major. Whether the anti-carcinogenesis activities of terpene, such as limonene, in citrus fruits essential oil extraction. This study to demonstrate the PAHs content in fish skin increased markedly after being roasted at 210°C for 20 minutes and greater Mutagenicity Risk of roasted fish skin was observed by Ame's test. The reduction of Mutagenicity Risk of roasted fish skin, which the anti-mutagenic abilities of substances in descending order were limonene>cold pressure oil>lemon>grapefruit. The anti-mutagenicity rate and ability of the three extracts were limonene: 18%-23%; cold-pressed lemon oil: 18%-22%; and steam distilled lemon essential oil: 8%-16%. The obvious anti-mutagenicity effects against the PAHs mutagenicity of roasted fish skins can be found in citrus fruits essential oil extraction.

Keywords: Roasted fish skin; PAHs Mutagenicity risk; Limonene

Introduction

Citrus is one of the most abundant agriculture crops worldwide. With a global production of 4,200,000 metric tonnes, lemon (*Citrus limon* (L.)) is the third most important cultivated citrus species after orange and mandarin. Countries with the most important lemon productions for processing are Argentina (1,000,000 tonnes), Italy (304,000 tonnes), Spain (300,000 tonnes), USA (230,000 tonnes), and Mexico (296,000 tonnes). Of the 2,130,000 tonnes of lemons processed globally in 2007, peel waste made up 57% (~1,200,000 tonnes) [1]. Citrus peels are rich in essential oils, which in various plants exhibit antioxidant properties that can extend the shelf life in foods by lowering lipid oxidation [2]. High-purity limonene, a major component of lemon essential oil found in the peels, can be used as fragrance, flavour, insecticide, or renewable solvent for coating or replacing aromatic and mineral oils [3]. Researchers worldwide are investigating this substance as a method to protect food from bacterial growth without the need for harmful chemicals [4].

Polycyclic aromatic hydrocarbons (PAHs) are organic toxins consisting of two or more aromatic rings fused in linear, angular, or clustered arrangements. These compounds do not undergo self-degradation easily in nature owing to their stable chemical structures [5]. Some PAHs such as acenaphthene anthracene, phenanthrene, fluorene, fluoranthene, pyrene, benzo[a]pyrene are included in the list of priority pollutants by the United States Environmental Protection Agency [5]. In 1976, there are at least 30 PAHs that are recognised internationally to be carcinogens, which is the largest group of known carcinogens in the world currently.

PAHs cause severe pollution in the biosphere due to its environmental dissemination and bioaccumulation characteristics. PAHs are mainly produced from incomplete combustion of carbon-containing compounds or use of fossil fuels. Examples include the combustion of plants that releases and spreads PAHs into nature; spillage of crude oil in the petrochemical industry [5]; roasted meats, smoked fishes, and cured meats. The use of baking, roasting, and other cooking techniques will generate more PAHs and increase PAH content in foods [6-9]. Consumption of fish and roasted and smoked meats by humans allow PAHs to enter the human body. In this study, tilapia

skins were soaked in limonene and lemon essential oil to examine their ability to prevent PAH-induced mutagenicity induced by roasting.

Materials and Methods

Materials

Lemons (*C. limon*) purchased from a supermarket in Kaohsiung city were washed and wiped dry. Lemon skins were collected without disrupting the oil sacs and were directly used for subsequent experiments.

Limonene, glucose-6-phosphate, β-Nicotine adenine dinucleotide phosphate, and sodium ammonium hydrogen phosphate tetrahydrate were purchased from Fluka Chemical Corp, US. The fragrance company provided cold oil presses for lemon skins. Benzo [a]pyrene was purchased from Supelco Inc., US. 4-Nitroquinoline-N-oxide L-histidine, Sodium chloride and D-biotin were purchased from Sigma Inc., US. Nutrient agar, nutrient broth, tryptic soy agar, tryptic soy broth, and Bacto agar were purchased from Difco Inc., US. Magnesium sulfate heptahydrate, citric acid, H₂O, dipotassium hydrogen phosphate, magnesium chloride anhydrous, potassium chloride, sodium dihydrogenphosphate anhydrous, disodium hydrogenphosphate, and glucose were purchased from Showa Inc., JP.

Methods

Extraction and analysis of essential oil from citrus peels

Steam distillation: 700 g of distilled water was added to 150 g of citrus peels for blending before being placed in a three-neck round-

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bottom flask for steam distillation. After the distillate separated into layers, the upper layer containing distilled essential oil was collected. This oil was stored in the dark at -20°C before gas chromatography-mass spectrometry (GC/MS) was used to analyse the types and levels of compounds [10,11].

Cold pressing of lemon essential oils: The method of Ou [12] was used in which lemons were cut in half, the flesh was removed, and the peels were pressed. Afterward, distilled water was used to flush the pressed juice. The mixture was then centrifuged at 3000 rpm for 10 min. Cold pressed oil was then collected from the upper layer.

Analysis of citrus peel essential oil: The Agilent 6890 (Agilent technologies Inc., US) GC system used in this was connected in tandem to an Agilent 5973N quadrupole very selective detector (MSD). The temperature of the ion source was 230°C, and the quadrupole temperature was 150°C. A DB-1 separating column with a length of 60 m and inner diameter of 0.25 mm was used; the injection port temperature was 150°C. The initial temperature of the column was 40°C for 0 min, which increased to 75°C at 35°C/min before holding for 2 min. This was followed by an increase of 5°C/min to 120°C and holding for 0 min. Finally, the temperature was increased at 15°C/min to 210°C and was held at 2 min. The carrier gas used was helium, and the flow rate was 1 mL/min. The obtained MS data were aligned with the Wiley7n Mass Spectral Library.

Toxicity, mutagenic, and anti-mutagenic effects of limonene and citrus essential oil on *Salmonella typhimurium* TA100

***S. typhimurium* TA100 experiment strains:** Biotechnology was used to modify this genetically defective strain from that growing in a histidine-deficient environment to a strain that requires histidine for growth. Samples containing mutagenic substances will revert to a histidine-independent form. This experiment strain was obtained from Chung Shan Medical University.

Toxicity testing: This test examines whether the experimental samples exhibit toxicity towards bacteria in the presence or absence of metabolism by liver enzymes. Toxicity is present if the colony count of the experimental group is 80% of that in the control group. The experimental samples of limonene and citrus essential oils were diluted by 1% Tween into different concentrations. The control group consisted of 1% Tween. Hereafter, these samples were referred to as “experimental Tween samples” and “Tween control group,” respectively.

First, 0.1 mL of the Tween sample was added into 0.1 mL of phosphate buffer (pH 7.4) before 0.1 mL of overnight bacterial culture was added. For the group without S9 mixture, 0.5 mL of the phosphate buffer was added. For that with S9, this mixture was added in place of the phosphate buffer. After mixing, the mixtures were precultured for 20 min at 37°C. They were then diluted to 10²-10³ CFU/mL, and 1 mL of diluate was mixed with a suitable volume of melted nutrient agar. The plates were grown in an inverted position at 37°C for 48 h before the colony counts were enumerated.

Mutagenicity experiments: The method used to perform the mutagenicity test (Ames test) involved the pre-incubation method as described by Maron and Ames [13]. This test examined whether the experimental samples exhibited mutagenic effects towards bacteria in the presence or absence of metabolism by liver enzymes. If the data of the experimental group were more than twice that of the control group, the sample was mutagenic.

First, 0.1 mL of the experimental Tween sample was added to 0.1 mL of the phosphate buffer before 0.1 mL of overnight bacterial culture

was added. For the group without S9 mixture, 0.5 mL of the phosphate buffer was added. For that with S9, this mixture was added in place of the phosphate buffer. After mixing, the mixtures were precultured for 20 min at 37°C. Then, 2 mL melted top agar containing 40% glucose and 0.5 mM histidine/biotin solution was added. The plates were grown in an inverted position at 37°C for 48 h before the colony counts were enumerated [14].

Antimutagenicity experiments: This experiment examines whether the experimental samples have antimutagenic ability following the addition of standard mutagens that do or do not require liver enzyme metabolism. The mutagen selected for the group without S9 mixture added was 4-Nitroquinoline-N-oxide, which does not require metabolism by liver enzymes. Benzo(a)pyrene, which requires liver enzyme metabolism, was used for the group with S9 mixture added. In both cases, 0.1 mL of overnight bacterial culture was added.

First, 0.1 mL of the experimental Tween sample was added into 0.1 mL of commercial mutagens. For the group without S9 mixture, 0.5 mL the phosphate buffer was added. For that with S9, this mixture was added in place of the phosphate buffer. After mixing, the mixtures were pre-cultured for 20 minutes at 37°C before 2 mL of melted top agar containing 40% glucose and 0.5 mM histidine/biotin solution was added. The plates were grown in an inverted position at 37°C for 48 hours before the colony counts were enumerated [15].

Antimutagenicity was calculated according to the following formula:

Inhibition (%)=[1-(number of spontaneous revertants sample group/control group)] × 100

Antimutagenicity of limonene and citrus essential oil on roasted fish skins

Extraction of roasted fish skins: 70 g of descaled tilapia skins 20 cm long × 8 cm wide was weighed and washed before being wiping dry (treated samples). Afterwards, the skins were placed in an oven for 20 min of roasting at 210°C. After the skins were crushed, three volumes of dichloromethane were added for 1 h of extraction. The dichloromethane layer was collected and concentrated under reduced pressure. The concentrate obtained was stored in the dark at -20°C.

Toxicity of extracts from roasted fish skins: This test examines whether the extracts from roasted fish skins exhibit toxicity towards bacteria in the presence or absence of metabolism by liver enzymes. Toxicity is present if the colony count of the experimental group is 80% of that in the control group. Extracts from roasted fish skins were dissolved in dimethyl sulfoxide (DMSO). The control group contained DMSO alone; these are referred to as “experimental DMSO samples” and “DMSO control group,” respectively. First, 0.1 mL of experimental DMSO samples was added to 0.1 mL of phosphate buffer (pH 7.4) and 0.1 mL of overnight culture. The group without S9 mixture had 0.5 mL of the phosphate buffer added, whereas the group with S9 had the S9 mixture added in place of the aforementioned 0.5 mL phosphate buffer. After mixing, the mixtures were pre-cultured for 20 min at 37°C. The mixture was then diluted to 10²-10³ CFU/mL, and 1 mL of diluate was mixed with a suitable volume of melted nutrient agar. The plates were grown in an inverted position at 37°C for 48 h before the colony counts were enumerated [13-15].

Mutagenicity of extracts from roasted fish skins: This test examines whether the experimental samples exhibit mutagenic effects towards bacteria in the presence or absence of metabolism by liver

enzymes. If the data of the experimental group is more than twice that of the control group, the sample is mutagenic. First, 0.1 mL of DMSO experimental samples was added to 0.1 mL of phosphate buffer (pH 7.4) before 0.1 mL of overnight bacterial culture was added. The group without S9 mixture had 0.5 mL of the phosphate buffer added, whereas the group with S9 had the S9 mixture added in place of the aforementioned phosphate buffer. After mixing, the mixtures were pre-cultured for 20 min at 37°C before adding 2 mL of melted TOP agar containing 40% glucose and 0.5 mM histidine/biotin solution. The plates were grown in an inverted position at 37°C for 48 h before the colony counts were enumerated [14].

Antimutagenicity of limonene and citrus essential oil on extracts from roasted fish skins: This experiment examines whether limonene and lemon essential oil, including cold pressed oil and that from steam distillation, have antimutagenic ability when extracts are added from roasted fish skin in the presence or absence of liver enzyme metabolism. First, 0.1 mL of experimental Tween samples was added to 0.1 mL of extracts from roasted fish skins; the extracts were dissolved in DMSO as 50 µg/plate and 200 µg/plate. The group without S9 mixture had 0.1 mL of overnight bacterial culture added before the addition of 0.5 mL of phosphate buffer (pH 7.4). For the group with S9, S9 mixture was added in place of the aforementioned phosphate buffer. After mixing, the mixtures were precultured for 20 min at 37°C before adding 2 mL of melted TOP agar containing 40% glucose and 0.5 mM histidine/biotin solution. The plates were grown in an inverted position at 37°C for 48 h before the colony counts were enumerated [13-15].

Evaluation of toxicity and mutagenicity of fish skin extracts regarding *S. typhimurium* TA 100: 70 g of descaled tilapia skins 20 cm long × 8 cm wide was weighed and washed before being wiped dry. Afterwards, the skins were soaked in 15 mL of 10% limonene and lemon essential oil, including cold pressed oil and that extracted from steam distillation, until the oils were completely absorbed, about 10-15 min. The skins were then placed in an oven for 20 min of roasting at 210°C. Afterwards, the skins were crushed, and three volumes of dichloromethane were added for 1 h of extraction. The dichloromethane layer was collected and concentrated under reduced pressure. The concentrate obtained was used for toxicity and mutagenicity evaluation following the procedures in described in sections 2.6.2 and 2.6.3.

Evaluation of toxicity and mutagenicity of fish skin extracts regarding *S. typhimurium* TA 100: 70 g of descaled Tilapia skins 20 cm long × 8 cm wide was weighed and washed before being wiped dry. Afterwards, the skins were soaked in 15 mL of 10% limonene and lemon essential oil, including cold pressed oil and that extracted from steam distillation, until the oils were completely absorbed, about 10-15 min. The skins were then placed in an oven for 20 min of roasting at 210°C. Afterwards, the skins were crushed and, three volumes of dichloromethane were added for 1 h of extraction. The dichloromethane layer was collected and concentrated under reduced pressure. The concentrate obtained was used for toxicity and mutagenicity evaluation, following the procedures described in sections 2.6.2 and 2.6.3.

Statistical analysis

Statistical analysis was conducted with one-way analysis of variance (ANOVA) with an LSD post hoc test for multiple comparisons. Data are reported as the mean ± standard error of mean (SEM) with a p value less than 0.05 being considered statistically significant. Analyses were performed using the Statistical Package for the Social Sciences (SPSS) software (Chicago, IL, USA).

Results and Discussion

Identification of components in citrus essential oils

In total, 26 aromatic compounds were identified from the citrus essential oil obtained by different extraction methods (Table 1). Terpenes had the highest levels, accounting for 20 of the 26 aromatic compounds; the remaining compounds consisted of 4 types of aldehydes and 2 types of esters. The limonene content in the four types of citrus essential oils was 70%-90%, showing that limonene content in citrus essential oils is extremely high. In addition, the lemon essential oils all contained β-Pipene and γ-Terpinene regardless of whether cold pressing or steam distillation was used. Studies have shown that these two compounds have antimicrobial and anti-cancer properties [16-20].

Toxicity, mutagenicity, and antimutagenicity tests

The method used to perform the mutagenicity test (Ames test) involved the pre-incubation method as described by Maron and Ames [13]. In the Ames test, toxicity testing is an extremely important step. The test sample must first undergo toxicity testing so that the sample concentration used in subsequent experiments will not have toxic effects on the experimental bacterial strains. This is because if toxicity occurs when the sample concentration is not high, error in colony counts can occur in subsequent experiments [14]. Therefore, it is necessary to determine the concentration with no toxicity effects when conducting the Ames test [15]. While determining whether the sample concentrations have toxicity effects, we can monitor whether the colony counts of the test sample group are lower than 80% of the control group. If so, concentration is toxic to the experimental bacterial strain and is not suitable for subsequent experiments.

Table 2, shows that as the sample concentration increased, the

Retention time (min)	Compound	Cold pressed oil (%)		Steam distilled oil (%)	
		Lemon	Lemon	Valencia	Grapefruit
4.95	α- Thujene	-	0.50	-	-
5.10	α- Pipene	2.96	2.04	1.06	0.87
5.91	Sabinene	1.17	1.30	0.52	0.75
6.01	β- Pipene	13.32	6.54	-	-
6.22	Myrcene	1.51	2.30	3.23	2.94
6.50	Octyl aldehyde	-	-	0.25	0.78
6.74	δ- Carene	-	-	0.21	-
6.87	α- Terpinene	-	0.23	-	-
7.19	Limonene	72.68	74.66	92.96	92.39
7.90	γ- Terpinene	5.34	7.48	-	-
8.67	α- Terpinolene	-	0.41	0.52	-
11.76	Decanal	-	-	0.32	0.44
12.80	Citral	-	0.78	-	-
13.57	Geranial	0.55	0.96	-	-
14.92	Neryl acetate	-	0.39	-	-
15.11	α- Copaene	-	-	-	0.32
15.12	Geranyl acetate	-	0.21	-	-
15.25	β- Cubebene	-	0.22	-	0.15
15.56	β- Caryophyllene	-	0.40	-	0.39
15.84	Cycloundecatriene	-	-	-	0.07
16.04	Germacrene	-	-	-	0.25
16.13	Valencene	-	-	0.33	-
16.16	Calarene	-	-	-	0.08
16.19	β- Bisabolene	0.22	0.57	-	-
16.32	δ- Cadinene	-	-	-	0.29

Table 1: Volatile compound compositions of citrus essential oil extracted by different methods.

bacterial counts exhibited a gradual decreasing trend regardless of whether liver enzymes were added. Toxicity occurs when the concentration of limonene is greater than 100 µg/plate and when the concentration of limonene, cold pressed oil, lemon essential oil, and the extract from roasted fish skins are greater than 200 µg/plate. Therefore, this value was used as the maximum concentration for the four samples in subsequent experiments. However, within this concentration range, the colony counts exhibited a significant increasing trend as the concentration of roasted fish skin extracts increased. This shows that animal tissues undergoing high-temperature roasting contain large amounts of toxic substances that could cause DNA mutations, which is similar to the document released by the World Health Organization [5].

After identifying the concentration not causing toxicity, mutagenicity experiments were conducted to confirm that the test samples are safe and not mutagenic in order to avoid errors in colony counts and doubts about safety. Therefore, this test must be conducted if the sample is determined to be antimutagenic or edible so that antimutagenicity and safety can be accurately calculated in subsequent antimutagenicity experiments [21].

When determining whether the test sample is mutagenic, the colony counts of the test sample and control groups are compared. If the colony counts of the sample group are more than twice that of the control group, safety issues and mutagenicity are present for that concentration, which makes it unsuitable for subsequent experiments [22,23]. Table 2 shows that the colony count trends of the four test samples at various concentrations were comparable with the control group, regardless of whether liver enzymes were added. This shows that the concentrations of these samples are not mutagenic towards the bacterial strain used. The results showed that when 100 and 200 µg/plate concentrations of limonene and various citrus essential oils were

used, respectively, no mutagenicity was present, and the safety of the samples was confirmed.

Anti-mutagenicity analysis was conducted according to the confirmed sample concentrations in the two aforementioned experiments. The aims of this experiment were to examine whether these four samples can decrease mutagenicity caused by interaction with mutagens and to calculate the anti-mutagenicity rate. Table 3 shows that as the concentrations of these four samples increased, the anti-mutagenicity rate against two standard mutagens, 4-Nitroquinoline-N-oxide and benzo(a)pyrene, both increased regardless of whether liver enzymes were added. The antimutagenicity rate and ability of these samples at their highest concentrations were limonene: 49%-52%; cold pressed lemon oil: 45%; steam distilled lemon essential oil: 342%; and extract from roasted fish skins: 286%-551% Overall, the three citrus essential oils all have antimutagenic effects, which were strongest in limonene and lemon essential oil. Therefore, subsequent studies were conducted using limonene and lemon essential oil for toxicity, mutagenicity, and anti-mutagenicity evaluation on extracts from roasted fish skins.

Anti-mutagenicity of limonene and citrus essential oils on extracts from roasted fish skins

Anti-mutagenicity tests using the three citrus essential oils were conducted using a high mutagenicity concentration. In this experiment, limonene, cold pressed lemon oil, and steam distilled extracted lemon essential oil were used as experimental samples. Table 3 shows the results. Under the maximum concentrations of these three samples, the anti-mutagenicity rate and ability of these samples towards extracts from roasted fish skin (200 µg/plate) were limonene: 18-23%; cold pressed lemon oil: 18-22%; and steam distilled extracted lemon

Amount (µg/plate)	Colony number				Number of His ⁺ revert ant colonies			
	Limonene	Cold pressed oil	Lemon essential oils	Extract from roasted fish skin	Limonene	Cold pressed oil	Lemon essential oils	Grilled fish skin
Without S9 mixture								
Control	250 ± 12	246 ± 9	210 ± 16	237 ± 21	162 ± 31	206 ± 10	139 ± 16	152 ± 36
100	200 ± 45	201 ± 20 [#]	188 ± 23	207 ± 14	155 ± 16	153 ± 28 [#]	133 ± 6	588 ± 38 [#]
200	NT [#]	197 ± 16 [#]	173 ± 16	195 ± 27	NT [#]	172 ± 16	131 ± 16	990 ± 70 [#]
With S9 mixture								
Control	321 ± 13	300 ± 22	248 ± 15	254 ± 10	211 ± 17	250 ± 13	189 ± 15	197 ± 23
100	262 ± 18 [#]	250 ± 15 [#]	210 ± 14	243 ± 5	199 ± 14	238 ± 16	165 ± 21	640 ± 50 [#]
200	NT [#]	239 ± 19 [#]	201 ± 15	206 ± 10 [#]	NT [#]	244 ± 27	168 ± 14	1231 ± 56 [#]

[#]NT: Not tested
[#]p<0.05 versus control (N=3/Group)

Table 2: Toxicity and mutagenicity of essential oil from citrus and the extract from grilled fish skin regarding *S. typhimurium* TA100 with and without S9 mixture.

Amount (µg/plate)	Number of His ⁺ revertant colonies			Amount (µg/plate)	Colony number			
	Without S9 mixture				Without S9 mixture			
	Limonene	Cold pressed oil	Lemon essential oils		Tween 20	Limonene	Cold pressed oil	Lemon essential oils
Control	776 ± 30	899 ± 41	997 ± 83	Control	174 ± 33 [#]	128 ± 14	154 ± 34	120 ± 23
100	672 ± 34 [#]	784 ± 30 [#]	949 ± 97	100	146 ± 24	106 ± 13	130 ± 15	101 ± 15
200	NT [#]	760 ± 14 [#]	907 ± 36	150	142 ± 16	101 ± 11 [#]	122 ± 22	97 ± 16
With S9 mixture								
Control	944 ± 49	1013 ± 44	1296 ± 32	Control	150 ± 13	166 ± 20	222 ± 16	165 ± 27
100	831 ± 77 [#]	931 ± 49	1317 ± 24	100	205 ± 19 [#]	140 ± 16	184 ± 22	134 ± 19
200	NT [#]	888 ± 66 [#]	1224 ± 24	150	193 ± 17 [#]	135 ± 15	175 ± 19 [#]	132 ± 13

[#]NT: Not tested
[#]p<0.05 versus control

Table 3: Antimutagenic effects and toxicity of limonene and lemon essential oil mixed with the extract from grilled fish skin (200 µg/plate) regarding *S. typhimurium* TA100 (N=3/Group).

Concentration (150 µg/plate)	Number of His ⁺ revertant colonies	
	Different samples	
	Without S9 mixture	With S9 mixture
Control	185 ± 34 [†]	231 ± 41 [†]
Untreated	997 ± 30 [#]	985 ± 58 [#]
Tween 20	909 ± 56 [#]	961 ± 40 [#]
Limonene	949 ± 57 [#]	1040 ± 41 [#]
Cold pressed oil	1015 ± 77 [#]	1119 ± 68 ^{#†}
Lemon essential oils	1083 ± 121 ^{#†}	1157 ± 48 ^{#†}

[†]NT: Not tested
[#]p<0.05 versus control
[†]p<0.05 versus untreated

Table 4: Mutagenicity of the same extract from grilled fish skin after immersion in limonene and lemon essential oil regarding *S. typhimurium* TA100 (N=3/Group).

essential oil: 8-16%. The anti-mutagenicity rate exhibited significant increases only under high doses of samples, which is likely attributed to the overly high concentration of the fish extracts. Therefore, a lower concentration of mutagenic fish skin extract (50 µg/plate) was used for subsequent identical experiments. The anti-mutagenicity rate and ability of these samples towards extracts from roasted fish skins (50 µg/plate) were limonene: 24%-35%; cold pressed lemon oil: 14%-24%; and steam distilled extracted lemon essential oil: 15%-20%. When compared with high concentrations of fish skin extracts (200 µg/plate), the anti-mutagenicity rates of the three samples increased by 12%, 2%, and 4% for limonene, cold pressed lemon oil, and steam distilled extracted lemon essential oil, respectively (data not shown).

Toxicity and mutagenicity tests before roasting

Table 2 shows the toxicity of extracts from fish skins after pre-treatment with citrus essential oils and roasting. As the concentration the extract increased, the colony counts showed a decreasing trend. No toxicity occurred when a dose of 150 µg/plate was used regardless of whether liver enzymes were added.

The most suitable concentration for toxicity testing, 150 µg/plate, was used for anti-mutagenic ability comparison for various groups. The results are shown in Table 4. Limonene pre-treatment before roasting showed only a slight reduction in mutagenicity when liver enzymes were added. However, similar results were not obtained after liver enzymes were added. We speculate that limonene pre-treatment followed by roasting may not have effectively inhibited mutagenic substances activated by liver metabolism. In addition, neither cold pressed lemon oil nor steam distilled essential oil was able to reduce the mutagenicity in the roasted fish skins.

Toxicity and mutagenicity tests after roasting

Table 4 shows the toxicity of extracts from fish skins that were treated with citrus essential oils after roasting. As the concentration of the extract increased, the colony counts showed a decreasing trend. Regardless of whether liver enzymes were added, no toxicity occurred when a dose of 150 µg/plate was used.

The most suitable concentration for toxicity testing, 150 µg/plate, was used for anti-mutagenic ability comparison for various groups. The results are shown in Table 4. Limonene pre-treatment before roasting showed only a slight reduction in mutagenicity regardless of whether liver enzymes were added. Because limonene and other citrus essential oils are volatile substances, high-temperature roasting may dramatically reduce their effective levels, thereby decreasing their anti-mutagenic abilities. However, treatment with limonene and other volatile

substances after roasting does not require high-temperature roasting and thus limonene may be present in the roasted material. This greatly increases the anti-mutagenic effects of limonene. We hypothesize that the effective inhibition of mutagenicity by roasting before limonene treatment, with or without addition of liver enzymes, may attribute the loss of volatile substances during high-temperature roasting, although it doesn't reduce mutagenicity after various treatments, at the same results demonstrate addition of limonene and cold pressure oil will not cause mutagenic risk (Table 4), but lemon essential oil groups (with or without S9 mixture) versus untreated had significantly difference, may be caused by the evaporation of effective substances during the roasted.

Conclusion

This study demonstrated that among the four types of citrus essential oils extracted from lemons using different methods, limonene had the highest content of 70% to more than 90% [24-28]. This shows that the limonene content in citrus essential oils is extremely high. When standard mutagens were used in anti-mutagenicity experiments, the anti-mutagenic abilities of substances in descending order were limonene>cold pressure oil>lemon>grapefruit [29-33]. When extracts from roasted fish skins were used as a mutagen at a concentration of 200 µg/plate, the anti-mutagenicity rate and ability of the three extracts were limonene: 18%-23%; cold pressed lemon oil: 18%-22%; and steam distilled lemon essential oil: 8%-16%. When a concentration of 50 µg/plate of extracts from roasted fish skins was used, the anti-mutagenicity rate and ability of the three extracts were limonene: 24%-35%; cold pressed lemon oil: 14%-24%; and steam distilled lemon essential oil: 15%-20%. In experiments simulating roasting of fish skins, limonene was found to decrease the mutagenicity caused by roasted substances when the three samples were used for pre-soaking before and after roasting.

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