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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 make the barbecue more delicious but also reduce 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

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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℃ 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 antimutagenic abilities of substances in descending order were limonene > cold pressure oil > lemon >grapefruit. The antimutagenicity 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

1. 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

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55 tonnes). Of the 2,130,000 tonnes of lemons processed globally in 2007, peel waste made
56 up 57% (~1,200,000 tonnes) [1]. Citrus peels are rich in essential oils, which in various
57 plants exhibit antioxidant properties that can extend the shelf life in foods by lowering
58 lipid oxidation [2]. High-purity limonene, a major component of lemon essential oil found
59 in the peels, can be used as fragrance, flavour, insecticide, or renewable solvent for
60 coating or replacing aromatic and mineral oils [3]. Researchers worldwide are
61 investigating this substance as a method to protect food from bacterial growth without the
62 need for harmful chemicals [4].
63
64 Polycyclic aromatic hydrocarbons (PAHs) are organic toxins consisting of two or
65 more aromatic rings fused in linear, angular, or clustered arrangements. These compounds
66 do not undergo self-degradation easily in nature owing to their stable chemical structures
67 [5]. Some PAHs such as acenaphthene anthracene, phenanthrene, fluorene, fluoranthene,
68 pyrene, benzo[a]pyrene are included in the list of priority pollutants by the United States
69 Environmental Protection Agency [5]. In 1976, there are at least 30 PAHs that are
70 recognised internationally to be carcinogens, which is the largest group of known
71 carcinogens in the world currently.
72 PAHs cause severe pollution in the biosphere due to its environmental dissemination

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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.

2. Materials and methods

2.1 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, ß-Nicotine adenine dinucleotide phosphate, and sodium ammonium hydrogen phosphate tetrahydrate were purchased from Fluka Chemical Corp, US. Cold oil presses for lemon skins were provided by the fragrance

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company. Benzo [a]pyrenene 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, H_2O, dipotassium hydrogen phosphate, magnesium chloride anhydrous, potassium chloride, sodium dihydrogenphosphate anhydrous, disodium hydrogenphosphate, and glucose were purchased from Showa Inc., JP.

2.4. Methods

2.4 Extraction and analysis of essential oil from citrus peels

2.4.1.1 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-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℃ before gas chromatography-mass spectrometry (GC/MS) was used to analyse the types and levels of compounds [10], [11]

2.4.1.2 Cold pressing of lemon essential oils

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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.

2.4.2 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 mass selective detector (MSD). The temperature of the ion source was 230℃, and the quadrupole temperature was 150℃. 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℃. The initial temperature of the column was 40℃ for 0 min, which increased to 75℃ at 35℃/min before holding for 2 min. This was followed by an increase of 5℃/min to 120℃ and holding for 0 min. Finally, the temperature was increased at 15℃/min to 210℃ 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.

2.5 Toxicity, mutagenic, and anti-mutagenic effects of limonene and citrus essential oil

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on *Salmonella typhimurium* TA100

2.5.1 *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 back to a histidine-independent form. This experiment strain was obtained from Chung Shan Medical University.

2.5.2 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 oil were diluted by 1% Tween into different concentrations. The control group consisted of 1% Tween. Hereafter, these samples are 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

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146 mixture, 0.5 mL of the phosphate buffer was added. For that with S9, this mixture was
147 added in place of the phosphate buffer. After mixing, the mixtures were precultured for
148 20 min at 37℃. They were then diluted to 10^2–10^3 CFU/mL, and 1 mL of diluate was
149 mixed with a suitable volume of melted nutrient agar. The plates were grown in an
150 inverted position at 37℃ for 48 h before the colony counts were enumerated.
151
152 2.5.3 Mutagenicity experiments
153 The method used to perform the mutagenicity test (Ames test) involved the pre-
154 incubation method as described by Maron and Ames [13]. This test examined whether
155 the experimental samples exhibited mutagenic effects towards bacteria in the presence or
156 absence of metabolism by liver enzymes. If the data of the experimental group were more
157 than twice that of the control group, the sample was mutagenic.
158 First, 0.1 mL of the experimental Tween sample was added to 0.1 mL of the
159 phosphate buffer before 0.1 mL of overnight bacterial culture was added. For the group
160 without S9 mixture, 0.5 mL of the phosphate buffer was added. For that with S9, this
161 mixture was added place of the phosphate buffer. After mixing, the mixtures were
162 precultured for 20 min at 37℃. Then, 2 mL melted top agar containing 40% glucose and
163 0.5 mM histidine/biotin solution was added. The plates were grown in an inverted position

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164 at 37℃ for 48 h before the colony counts were enumerated [14].

165

166 2.5.4 Antimutagenicity experiments

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

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

180 Antimutagenicity was calculated according to the following formula:

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

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100

2.6 Antimutagenicity of limonene and citrus essential oil on roasted fish skins

2.6.1 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℃. 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℃.

2.6.2 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

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200 samples was added to 0.1 mL of phosphate buffer (pH 7.4) and 0.1 mL of overnight
201 culture. The group without S9 mixture had 0.5 mL of the phosphate buffer added, whereas
202 the group with S9 had the S9 mixture added in place of the aforementioned 0.5 mL
203 phosphate buffer. After mixing, the mixtures were precultured for 20 min at 37℃. The
204 mixture was diluted to 10^2–10^3 CFU/mL, and 1 mL of diluate was mixed with a suitable
205 volume of melted nutrient agar. The plates were grown in an inverted position at 37℃ for
206 48 h before the colony counts were enumerated [13-15].
207
208 2.6.3 Mutagenicity of extracts from roasted fish skins
209 This test examines whether the experimental samples exhibit mutagenic effects
210 towards bacteria in the presence or absence of metabolism by liver enzymes. If the data
211 of the experimental group is more than twice that of the control group, the sample is
212 mutagenic. First, 0.1 mL of DMSO experimental samples was added to 0.1 mL of
213 phosphate buffer (pH 7.4) before 0.1 mL of overnight bacterial culture was added. The
214 group without S9 mixture had 0.5 mL of the phosphate buffer added, whereas the group
215 with S9 had the S9 mixture added in place of the aforementioned phosphate buffer. After
216 mixing, the mixtures were precultured for 20 min at 37℃ before adding 2 mL of melted
217 TOP agar containing 40% glucose and 0.5 mM histidine/biotin solution. The plates were

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grown in an inverted position at 37℃ for 48 h before the colony counts were enumerated [14]..

2.6.4 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 ℃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℃ for 48 h before the colony counts were enumerated [13-15]..

2.6.5 Evaluation of toxicity and mutagenicity of fish skin extracts regarding *S.*

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236 *typhimurium* TA 100

237 70 g of descaled tilapia skins 20 cm long × 8 cm wide was weighed and washed
238 before being wiped dry. Afterwards, the skins were soaked in 15 mL of 10% limonene
239 and lemon essential oil, including cold pressed oil and that extracted from steam
240 distillation, until the oils were completely absorbed, about 10–15 min. The skins were
241 then placed in an oven for 20 min of roasting at 210℃. Afterwards, the skins were crushed,
242 and three volumes of dichloromethane were added for 1 h of extraction. The
243 dichloromethane layer was collected and concentrated under reduced pressure. The
244 concentrate obtained was used for toxicity and mutagenicity evaluation following the
245 procedures in described in sections 2.6.2 and 2.6.3.

246

247 2.6.6 Evaluation of toxicity and mutagenicity of fish skin extracts regarding *S.*
248 typhimurium TA 100

249 70 g of descaled Tilapia skins 20 cm long × 8 cm wide was weighed and washed
250 before being wiped dry. Afterwards, the skins were soaked in 15 mL of 10% limonene
251 and lemon essential oil, including cold pressed oil and that extracted from steam
252 distillation, until the oils were completely absorbed, about 10–15 min. The skins were
253 then placed in an oven for 20 min of roasting at 210℃. Afterwards, the skins were crushed

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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.

2.7 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).

3. Results and discussion

3.1 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

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of whether cold pressing or steam distillation was used. Studies have shown that these two compounds have antimicrobial and anti-cancer properties [16-20].

3.2. 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 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

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295 undergoing high-temperature roasting contain large amounts of toxic substances that
296 could cause DNA mutations, which is similar to the document released by the World
297 Health Organization [5].

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

304 When determining whether the test sample is mutagenic, the colony counts of the
305 test sample and control groups are compared. If the colony counts of the sample group
306 are more than twice that of the control group, safety issues and mutagenicity are present
307 for that concentration, which makes it unsuitable for subsequent experiments [22-23].
308 Table 2 shows that the colony count trends of the four test samples at various
309 concentrations were comparable with the control group, regardless of whether liver
310 enzymes were added. This shows that the concentrations of these samples are not
311 mutagenic towards the bacterial strain used. The results showed that when 100 and 200
312 μg/plate concentrations of limonene and various citrus essential oils were used,
313 respectively, no mutagenicity was present, and the safety of the samples was confirmed.

314 Antimutagenicity analysis was conducted according to the confirmed sample
315 concentrations in the two aforementioned experiments. The aims of this experiment were
316 to examine whether these four samples can decrease mutagenicity caused by interaction
317 with mutagens and to calculate the antimutagenicity rate. Table 3 shows that as the
318 concentrations of these four samples increased, the antimutagenicity rate against two

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319 standard mutagens, 4-Nitroquinoline-N-oxide and benzo[a]pyrene, both increased
320 regardless of whether liver enzymes were added. The antimutagenicity rate and ability of
321 these samples at their highest concentrations were limonene: 49–52%; cold pressed lemon
322 oil: 45%; steam distilled lemon essential oil: 342%; and extract from roasted fish skins:
323 286–551% Overall, the three citrus essential oils all have antimutagenic effects, which
324 were strongest in limonene and lemon essential oil. Therefore, subsequent studies were
325 conducted using limonene and lemon essential oil for toxicity, mutagenicity, and
326 antimutagenicity evaluation on extracts from roasted fish skins.

327

328 3.4 Antimutagenicity of limonene and citrus essential oils on extracts from roasted fish
329 skins

330 Antimutagenicity tests using the three citrus essential oils were conducted using a
331 high mutagenicity concentration. In this experiment, limonene, cold pressed lemon oil,
332 and steam distilled extracted lemon essential oil were used as experimental samples. Table
333 3 shows the results. Under the maximum concentrations of these three samples, the
334 antimutagenicity rate and ability of these samples towards extracts from roasted fish skin
335 (200 μg/plate) were limonene: 18–23%; cold pressed lemon oil: 18–22%; and steam
336 distilled extracted lemon essential oil: 8–16%. The antimutagenicity rate exhibited
337 significant increases only under high doses of samples, which is likely attributed to the
338 overly high concentration of the fish extracts. Therefore, a lower concentration of

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339 mutagenic fish skin extract (50 μg/plate) was used for subsequent identical experiments.
340 The antimutagenicity rate and ability of these samples towards extracts from roasted fish
341 skins (50 μg/plate) were limonene: 24–35%; cold pressed lemon oil: 14–24%; and steam
342 distilled extracted lemon essential oil: 15–20%. When compared with high concentrations
343 of fish skin extracts (200 μg/plate), the antimutagenicity rates of the three samples
344 increased by 12%, 2%, and 4% for limonene, cold pressed lemon oil, and steam distilled
345 extracted lemon essential oil, respectively (data not shown).
346
347 3.5 Toxicity and mutagenicity tests before roasting
348 Table 2 shows the toxicity of extracts from fish skins after pre-treatment with citrus
349 essential oils and roasting. As the concentration the extract increased, the colony counts
350 showed a decreasing trend. No toxicity occurred when a dose of 150 μg/plate was used
351 regardless of whether liver enzymes were added.
352 The most suitable concentration for toxicity testing, 150 μg/plate, was used for
353 antimutagenic ability comparison for various groups. The results are shown in Table 4.
354 Limonene pre-treatment before roasting showed only a slight reduction in mutagenicity
355 when liver enzymes were added. However, similar results were not obtained after liver
356 enzymes were added. We speculate that limonene pre-treatment followed by roasting may

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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.

3.6 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 antimutagenic 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 antimutagenic 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 antimutagenic effects of limonene. We hypothesize that the effective

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inhibition of mutagenicity by roasting before limonene treatment, with or without addition of liver enzymes, may be attributed 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 oils groups (with or without S9 mixture) versus untreated had significantly difference, maybe caused by the evaporation of effective substances during the roasted.

4. Conclusions

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 antimutagenicity experiments, the antimutagenic 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 antimutagenicity 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 antimutagenicity 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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Table 1. Volatile compound compositions of citrus essential oil extracted by different methods

Retation 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

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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 (N=3/Group)

Amount (μg/plate)	Colony number				Number of His^+ revertant 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

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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)

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				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

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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)

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$^{\#\dagger}$
Lemon essential oils	1083 ± 121$^{\#\dagger}$	1157 ± 48$^{\#\dagger}$

***NT: Not tested**

$^{\#}$ $p < 0.05$ versus control

† $p < 0.05$ versus untreated