Inhibitory Effects of Citrus Essential Oils and Their Components on the Formation of *N*-Nitrosodimethylamine

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Twenty-eight kinds of citrus essential oils and their components were studied for inhibitory effects on the formation of *N*-nitrosodimethylamine (NDMA). The reaction mixture consisted of dimethylamine and sodium nitrite adjusted at pH 3.6, in addition to essential oils and an emulsifying agent. The quantification was determined by high-performance liquid choromatography monitored at 220 nm. All of the essential oils inhibited the formation of NDMA in the range of 20−85%. The oils of ujukitsu (*Citrus ujukitsu* Hort. ex Shirai), yuzu (*C. junos* Tanaka), mochiyu (*C. inflata* Hort. ex Tanaka), and ponkan (*C. reticulata* Blanco cv. F-2426) inhibited the formation of NDMA much more effectively than other citrus oils. The inhibitory proportions of components of citrus essential oils such as myrcene, α-terpinene, and terpinolene were as high as 80%.

Keywords: *Nitrosodimethylamine; citrus essential oil; nitrite; carcinogen; terpenoids*

INTRODUCTION

Citrus flavor is a favorite in most parts in the world, and the aroma essence is important in the food flavor, cosmetics, and perfume industries.

Considering long-standing customs, we generally consume meat, fish, and/or their processed forms together with vegetables at the same time. This pattern of dietary combination has long been considered to be good for health. However, these foods contain substantial amounts of several kinds of amines and nitrates or nitrites. It is well-known that saliva is always a major source of nitrite for humans because the nitrates found in vegetables and other sources are readily converted by nitrate reductase in nature or in the human body (Vogtmann and Biedermann, 1985). Therefore, it is probable that nitrosoamines, including tertiary amines and quaternary ammonium compounds as well as secondary amines (Fiddler et al., 1972), would be formed in our organs during the process of digesting the diet or in blood after absorption. However, the food we consume in a usual meal also contains a number of foodstuffs that inhibit the formation of carcinogens, promote immunological functions, and supply nutrients. Besides, we possess natural defense mechanisms such as detoxification of potentially harmful compounds and immunological and biological responses. It is known that ascorbic acid and tocopherols, for instance, inhibit the formation of *N*-nitrosodimethylamine (NDMA) in vivo (Walters, 1981; Ohshima and Bartsch, 1981). In some cases NDMA can be metabolized by peroxidase from horseradish (Stiborova et al., 1997). Citrus fruits are rich in essential oils including terpene hydrocarbons, alcohols, aldehydes, and esters. It is not uncommon to consume the essential oils coincidentally when we eat fresh fruits or dishes seasoned with citrus fruits.

The authors have studied citrus essential oils from the aspect of the functional property of flavor or fragrance. The present study aims to investigate the occurrence of another function of citrus essential oils. We studied the effects of citrus essential oils and their components on the inhibition of formation of NDMA in vitro.

MATERIALS AND METHODS

Citrus Essential Oils and Authentic Compounds. The citrus samples used in the present study are shown in Table 1. The 26 kinds of citrus fruits and 1 kind of kumquat were obtained from the Kochi Prefectural Fruit Tree Experimental Station and the Ministry of Agriculture, Forestry and Fisheries, Okitsu Branch Experimental Station in Shizuoka. Bergamot (Fantastico) was collected in Reggio Calabria in Italy. Kumquat is different from but close to the *Citrus* genus in the family Rutaceae. The classification of citrus fruits in Table 1 is based on the taxonomy of Swingle (1943). The essential oil samples were prepared by cold-pressing (Njoroge et al., 1994) and kept at −25 °C until analyzed. The authentic reagents were available as follows: α-terpinene from Sigma Chemical Co. (St. Louis, MO); myrcene and terpinen-4-ol from Aldrich (Milwaukee, WI); γ-terpinene from Fluka Chemie AG (Buchs, Switzerland); β-pinene, *p*-cymene, geraniol, citronellol, linalol, 1-octanol, 1-octanal, 1-decanal, and geranyl acetate from Wako Pure Chemical Industries (Osaka, Japan); α-pinene, terpinolene, *d*-limonene, α-terpineol, citronellal, 1-decanol, and citral (a mixture of neral and geranial) from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Nootkatone was provided by Ogawa & Co., Ltd., Tokyo. An emulsifier, Tween 20, was purchased from Wako Pure Chemical Industries.

Assay for NDMA. The assay for NDMA was performed by modifying the method used by Achiwa et al. (1997). Each 0.5 mL of 50 mM dimethylamine and sodium nitrite, the pH values of which were adjusted at 3.6 with 5% acetic acid in advance, and 10 μL of Tween 20 were mixed with 10 μL of essential oil or authentic compound in a brown vessel (3 mL) with a tightly screwed cap with an inner seal made of Teflon. Water was used for the control instead of either essential oil or authentic compound. It was reported that Tween 20 was a good emulsifier in an O/W type emulsion (Sirendi et al., 1998). Incubation was carried out for 24 h at 37 °C with shaking at 50 strokes/min. After a 24-h period, the sample solutions were

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Table 1. Samples of *Citrus* Genus

no.	botanical name	common name	species[a]
1	*C. junos* Sieb. ex Tanaka	yuzu	*C. ichangensis*
2	*C. junos* Sieb. ex Tanaka	mukakuyuzu or seedless yuzu	*C. ichangensis*
3	*C. sudachi* Hort. ex Shirai	sudachi	*C. ichangensis*
4	*C. inflata* Hort. ex Tanaka	mochiyu	*C. ichangensis*
5	*C. yuko* Hort. ex Tanaka	yuko	*C. ichangensis*
6	*C. ozu* Hort. ex Y. Tanaka	oyu	*C. ichangensis*
7	*C. wilsonii* Tanaka	ichanlemon	*C. ichangensis*
8	*C. aurantifolia* Swingle	lime	*C. aurantifolia*
9	*C. latifolia* Tanaka	Tahiti lime	*C. aurantifolia*
10	*C. bergamia* Risso var. Fantastico	bergamot	*C. aurantifolia*
11	*C. bergamia* Risso var. Balotin	bergamot	*C. aurantifolia*
12	*C. limon* Burm. f. cv. Eureka	Eureka lemon	*C. limon*
13	*C. limon* Burm. f. cv. Lisbon	Lisbon lemon	*C. limon*
14	*C. grandis* Osbeck forma Tosa-buntan Tanaka	Tosa-buntan	*C. grandis*
15	*C. paradisi* Macfadyen	grapefruit	*C. paradisi*
16	*C. hassaku* Hort. ex Y. Tanaka	hassaku	*C. paradisi*
17	*C. natsudaidai* Hayata	natsudaidai	*C. paradisi*
18	*C. aurantium* Linn. var. Cyathifera Y. Tanaka	daidai	*C. aurantium*
19	*C. aurantium* Linn. forma Kabusu	kabusu	*C. aurantium*
20	*C. sinensis* Osbeck forma Valencia	Valencia orange	*C. sinensis*
21	*C. sinensis* Osbeck var. Sanguinea Tanaka forma Tarocco	Tarocco orange	*C. sinensis*
22	*C. iyo* Hort. ex Tanaka	Iyokan	*C. sinensis*
23	*C. tamurana* Hort. ex Tanaka	Hyuganatsu	*C. sinensis*
24	*C. ujukitsu* Hort. ex Shirai	ujukitsu	*C. sinensis*
25	*C. unshiu* Marcov. forma Miyagawa-wase	unshumikan or Satsuma mandarin	*C. reticulata*
26	*C. unshiu* Marcov. forma Imamura	unshumikan or Satsuma mandarin	*C. reticulata*
27	*C. reticulata* Blanco cv. F-2426	ponkan	*C. reticulata*
28	*Fortunella japonica* Swingle[b]	kinkan or kumquat	*Fortunella japonica*

[a] Classified by Swingle. [b] Another genus in the Rutaceae family comprising the *Citrus* genus.

filtered through a 0.22 μm nitrate cellulose membrane (Fuji Photo and Film Co. Ltd., Tokyo) prior to high-performance liquid chromatography (HPLC) injection. The measurement was triplicated. The wastes were treated with 2 N NaOH to break down NDMA.

HPLC Operating Conditions. The measurement of NDMA was carried out by HPLC. The content of NDMA was determined by means of a Jasco HPLC composed of an 880-PU pump, an 875-UV detector, and a Rheodyne Model 7125 injector with a 10 μL loop. Absorbance was monitored at 220 nm. A reverse-phase column, Wakosil 5C18 (4.6 mm i.d. × 250 mm, from Wako Pure Chemical Industries), was used. The column temperature was kept at 40 °C, and 5% methanol was used isocratically as the mobile phase at 1.0 mL/min. All of the chemical reagents used for HPLC were of the highest purity of Wako Pure Chemical Industries. Water used in the experiment was Milli-Q.

Gas Chromatography (GC) and GC/Mass Spectrometry (MS) Conditions. The major volatile components of cold-pressed oils were determined by GC and GC/MS. Data calculation was based on the relative peak area percent method. A Shimadzu GC-14A equipped with a flame ionization detector and a Shimadzu GC/MS QP-5000 were used for quantitative determination and identification of the peaks, respectively. These analytical conditions were the same as those in the previous paper (Njoroge et al., 1996).

RESULTS AND DISCUSSION

Inhibition of the Formation of NDMA by Citrus Essential Oils. The formation of NDMA in the presence of citrus essential oils in model solutions was examined.

The optimum pH of this reaction is ~3.5 to yield NDMA (Walters, 1981). The reactivity is extremely low at pH 1 and 2. The foodstuffs we eat pass successively the stomach, duodenum, small intestine, and large intestine after mastication in the mouth. Some foods are even absorbed into the blood after digestion in each organ. Thus, the foodstuffs taken into the body are assumed to be in the pH range of 1−7. Considering the occurrence of carcinogens in the conditions in vivo, we preferred employing the optimum pH of 3.6 in this reaction.

The incubation temperature for the NDMA assay was 37 °C, whereas the HPLC was carried out at 40 °C, because we intended to reduce the analysis time to as short as possible. We checked primarily that there was no different peak on each chromatogram regulated at 37 and 40 °C.

The results are shown in Figure 1. It was demonstrated that all kinds of citrus essential oils used in the present experiment inhibited the formation of NDMA to some extent. The essential oils presenting an inhibition ratio between 20 and 50% were as follows: oyu; Tahiti lime; Eureka lemon; Tosa-buntan; grapefruit and hassaku; daidai; Tarocco and Hyuganatsu. The second category, with between 50 and 70% inhibition, included the following: seedless yuzu, sudachi, yuko, and ichanlemon; lime, bergamot (Balotin), and Lisbon lemon; natsudaidai; kabusu; Valencia orange; Satsuma mandarin (Imamura); kumquat. The third category, with an inhibitory ratio of >70%, includes the following essential oils: yuzu and mochiyu; bergamot (Fantastico); Iyokan and ujukitsu; Satsuma mandarin (Miyagawa-wase) and ponkan. The essential oil of ujukitsu showed an inhibition ratio as high as 85%. Although it seems to be difficult to find a relationship between species and inhibitory effect, it is clear that citrus essential oils affect the formation of NDMA.

Inhibition of the Formation of NDMA by Authentic Compounds. The results are shown in Figure 2. Terpene hydrocarbons such as myrcene, α-terpinene, terpinolene, γ-terpinene, α-pinene, and β-pinene showed a ratio of inhibition of formation of NDMA >50%. However, the inhibitory ratio of limonene was 34% and that of *p*-cymene was only a few percent. The chemical structures of those compounds are given in Figure 3. Myrcene, an open-chain monoterpene with three double bonds, presented the highest inhibition among all of the compounds examined, 87%. Most oxygenated compounds showed a slight inhibition, and the ratios of terpinen-4-ol, 1-decanol, and nootkatone were almost 0%.

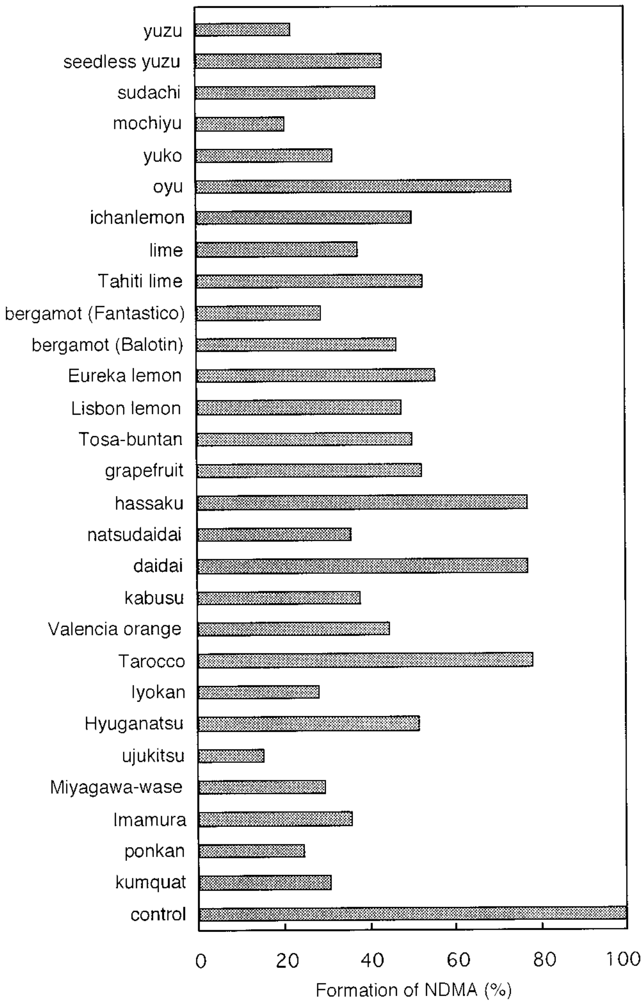

Figure 1. Effects of citrus essential oils on the formation of NDMA from dimethylamine and nitrite.

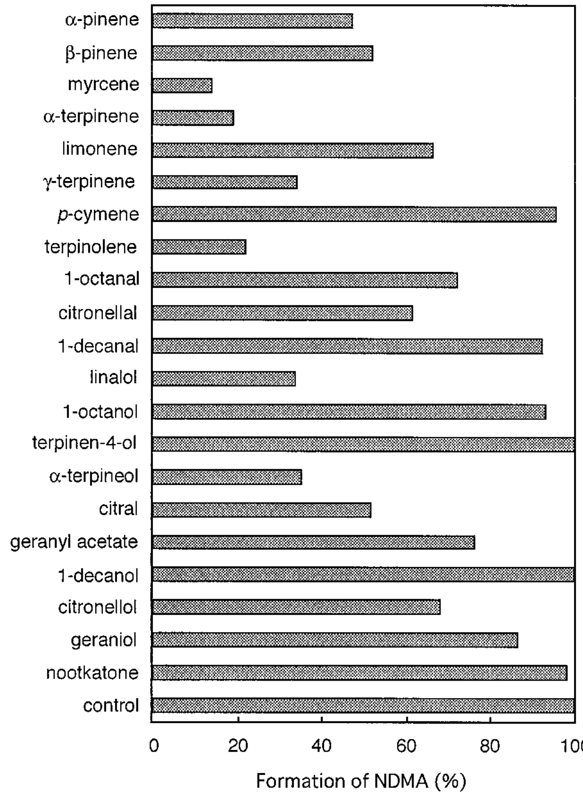

Figure 2. Effects of the authentic compounds relating to citrus essential oils on the formation of NDMA from dimethylamine and nitrite.

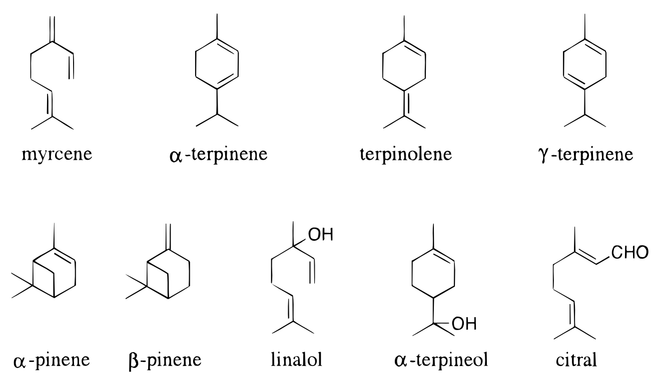

Figure 3. Citrus essential oil components with remarkable inhibitory effect of NDMA formation.

The inhibitory ratios were ∼65% in linalol and α-terpineol and ∼50% in citral.

The reaction of dimethylamine with nitrite is conducted by electrophilic substitution to form NDMA. The N atom on HNO_2 is so electrophilic that it attacks the N atom on dimethylamine, the electron density of which is apt to increase with two alkyl groups. It is considered that hydrocarbons consisting of two or three double bonds such as α-terpinene, γ-terpinene, terpinolene, and myrcene would compete with dimethylamine to react with nitrite.

Inhibitory Effects of Essential Oils and Authentic Compounds. The major volatile compositions of cold-pressed oils are shown in Table 2. Ujukitsu (sample 24) and mochiyu (sample 4) were exceedingly abundant in myrcene compared with others, 28.3 and 20.6%, respectively. The inhibitory effect of myrcene on the formation of NDMA was the highest, as shown in Figure 2. It is suggested that the inhibitory efficiency of most essential oils depends on composition. In the family of *C. ichangensis* (samples 1−7) except for oyu (sample 6), the higher efficiency may be caused by the composition of essential oils having a total of >10% myrcene, α-terpinene, and γ-terpinene. The higher efficiency of yuko (sample 5) may be caused by the composition of 21.3% γ-terpinene and 0.5% α-terpineol. In *C. aurantifolia* and *C. limon* (samples 8−13), the combined percent of β-pinene and γ-terpinene ranged from 12.4 to 31.1%. It is also considered that the characteristic composition of neral and geranial in limes and that of linalol and linalyl acetate in bergamots contributed to their inhibitory efficiency. As the essential oils of samples 16−28, except sample 24, were mainly composed of limonene in a proportion of ≥90%, limonene would play the principal role of inhibitory efficiency for these species. In addition to the limonene component, the inhibitory efficiency of the essential oils from samples 22−27 is likely to be further enhanced by a considerable amount of γ-terpinene and linalol. The essential oils of oyu (sample 6), hassaku (sample 16), daidai (sample 18), and Tarocco (sample 21), which had poor inhibitory efficiencies, contained little of the more effective compounds such as α-terpinene, terpinolene, linalol, and α-terpienol. Kumquat (sample 28) showed a strong inhibition (Figure 2), although the component content was low except for limonene. Factors other than essential oil components that contribute to the inhibition

Table 2. Volatile Components[a] of *Citrus* Essential Oils

compound	sample[b] 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
α-pinene	1.8	1.8	1.6	0.3	3.3	0.7	3.3	3.6	3.2	1.6	1.3	2.3	2.6	1.1	0.5	1.2	1.2	0.5	0.6	0.5	0.5	1.2	1.4	1.4	1.0	1.2	1.2	0.4
β-pinene	0.7	0.7	0.5	0.1	1.8	*	1.8	13.4	13.0	8.9	6.8	10.5	14.0	0.6	*[c]	0.4	0.3	0.7	0.9	*	*	0.3	0.8	0.4	0.2	0.3	0.3	*
myrcene	2.2	2.1	1.3	20.6	1.3	0.3	0.7	1.4	1.3	0.9	0.7	1.6	1.4	1.2	1.3	1.8	1.7	1.6	1.7	1.8	1.8	1.8	1.8	28.3	1.8	1.8	1.8	1.8
α-terpinene	0.2	0.3	1.4	*	0.3	0	2.4	0.3	0.3	0.2	0.1	*	*	0.1	0	0	0	0	0	0	0	0	*	0.1	0	0	0	0
limonene	78.1	78.1	69.1	77.2	66.6	93.9	68.8	50.5	52.2	38.8	24.3	69.7	64.6	75.3	83.1	89.8	90.2	94.7	92.0	95.8	96.6	88.2	84.5	59.2	90.6	89.7	89.9	96.7
γ-terpinene	9.3	9.1	7.5	0.8	21.3	*	16.0	17.7	17.0	8.3	5.6	8.2	10.3	4.9	0.1	5.6	4.9	0.1	0	*	*	5.4	7.5	7.6	3.5	4.6	4.6	0.1
p-cymene	0.4	0.4	0.4	*	0.2	*	*	0.1	0.1	0.3	*	*	*	0.1	0	*	0.1	0	0	0	0	0.1	*	0.1	0.2	0.2	0.1	*
terpinolene	0.4	0.5	0.3	*	0.9	*	0.7	0.7	0.7	0.3	0.2	0.3	0.4	0.4	*	0.2	0.2	*	*	*	*	0.2	0.3	0.3	0.2	0.2	0.2	*
1-octanal	*	*	*	0	0.1	0	0	*	*	0	*	*	0.1	0.2	0.5	0.1	0.3	0.1	0.2	0.3	0.1	0.1	*	*	0.1	*	0.2	*
citronellal	*	*	*	*	*	*	0	0.1	0.1	0	0	0.1	*	0.2	0.1	*	0	0	0	*	*	*	0.2	*	*	*	0.1	0
1-decanal	*	*	0.1	*	0.1	0	0	*	0.1	0.1	*	*	*	0.4	0.5	0.1	0.2	0.1	0.1	0.1	0.1	0.1	*	0	0.1	0.1	0.1	*
linalol	1.8	1.8	0.3	*	0.2	0.1	0.2	*	0.2	4.2	18.2	0.2	0.2	0.2	0.2	0.1	0.1	0.2	1.1	0.4	0.3	1.3	1.3	0.7	0.4	0.3	0.6	0.1
1-octanol	0	0	*	0	0.1	0	0	0	*	0	0	0	0	0	0	0	*	0	0	*	*	*	*	0	0	0	*	0
linalyl acetate	0	0	0	0	0	0	0	0	0	32.1	39.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
terpinen-4-ol	0	0	0	0	0.1	*	*	*	0	0.1	0.1	*	0	0.1	0.2	*	*	0	0	*	0	*	*	*	*	*	0	0
neral	*	0	0	*	0.1	*	*	1.0	1.2	0.2	0.2	1.1	0.6	0.1	0	*	*	*	0.1	0.1	*	*	*	0.1	*	0	*	0
α-terpineol	0.1	0.1	0.2	*	0.5	0.1	*	0.3	0.4	0.2	0.1	0.2	0.3	0.1	0.1	0.1	0.1	*	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	*
geranial	*	*	*	*	*	0	0	2.1	2.3	0.3	0	1.0	1.13	0.2	0.1	0.1	0	*	0.1	0.1	0.1	0	0	0	0	0	*	0
geranyl acetate	0	0	0	0	0	0	*	0.7	1.0	0.3	0.2	0.16	0.23	0.1	0.1	*	0.1	0.1	0.1	0	*	0.1	*	*	*	0.1	*	0.1
1-decanol	*	*	0	0	0	0	0	0	0	*	0	0	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
citronellol	0	0	0	0	0	*	0	*	0	0	*	*	*	*	*	*	0	*	0	*	*	*	*	0	*	*	*	0
nerol	0	0	0	0	0	*	*	0.1	0	*	0.1	*	*	*	0	0	0	*	0	*	*	0	0	0.1	0	0	*	0
geraniol	0	0	*	*	*	0	*	0.1	*	*	*	*	0.1	*	*	0	0	*	0	*	0	0	0	*	0	0	0	0
nootkatone	0	0	0	0	0	0	0	*	0	0.1	*	0	0	0.4	0.1	*	*	0	0	0	0	0	0	0	*	0	0	0

[a] Given in relative peak area percent. [b] The sample number is coincident with that in Table 1. [c] *, <0.1%.

of the formation of NDMA may exist. Achiwa et al. (1997) reported that the juices of lemon, Satsuma mandarin, and grapefruit demonstrated an NDMA inhibitory capacity. This suggested that ascorbic acid in those juices did not always play a major role in inhibition and that there existed other compounds which could cause inhibition. One of the factors may be the presence of some essential oil in those juices. Recently, terpenoids such as *d*-limonene, farnesol, geraniol, and perillyl alcohol have been reported to be effective in the inhibition of pancreatic or mammary cancer growth (Burke et al., 1997; Crowell, 1997; Gould, 1997; Nakaizumi et al., 1997). It was also reported that the administration of terpene hydrocarbons such as limonene and carvone to mice reduced the occurrence of cancer because of increased activity of glutathione *S*-transferase (Zheng et al., 1992a,b). Terpenes and citrus essential oils containing high levels of them seem to have the potential to act through multiple mechanisms in the chemoprevention of cancers, although the mechanisms of the inhibition have not been clarified. Even if the inhibition of NDMA formation might not be cancer chemopreventative, it is substantially preferable in our diet to minimize the factors that act in the formation ofcarcinogens, Although the present study was carried out in a model solution, this is the first proof of inhibition of NDMA formation by citrus essential oils and their components. The present work also encompassed another functional property, that is, the inhibitory effects of citrus essential oils and terpenes on the formation of NDAM. This knowledge will contribute to the improvement of our daily life and applications for food processing.

In conclusion, it was demonstrated that citrus essential oils inhibited the formation of NDMA in a given reaction medium and that the compositions of citrus essential oils were also related to the inhibitory efficiency.

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