Deodorizing Substance in Black Cumin (*Nigella sativa* L.) Seed Oil

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Abstract: A deodorizing substance in black cumin (*Nigella sativa* L.), a spice for curry and vegetable foods in Southwest Asia, was examined. The essential oil prepared from the seeds of this plant exhibited strong deodorizing activity against methyl mercaptan, which is a main factor in oral malodor. After purification with silica gel column chromatography, the active substance in black cumin seed oil was identified as thymoquinone. This monoterpenic quinone functions as the main deodorizing substance in this oil against methyl mercaptan. Metabolite analysis suggested that the deodorizing activity may be generated by the addition of a reactive quinone molecule to methyl mercaptan. In the present study, the menthane-type quinone and phenol derivatives exhibited deodorizing activities via this mechanism.

Key words: black cumin, thymoquinone, oral deodorant

1 INTRODUCTION

Oral malodor is caused by food debris with strong odor or dry mouth due to smoking. In addition, its fundamental cause is food residues decomposed by bacteria in the oral cavity. Oral malodor affects communication in everyday life. The causative substances of oral malodor include methyl mercaptan, hydrogen sulfide, and dimethyl sulfide generated through food decomposition by bacteria in the oral cavity. Therefore, oral malodor may be ameliorated by reducing the amount of sulfur compounds. Oral malodor has recently been treated using mouthwash and brushing with toothpaste. A green tea extract and sodium cupper chlorophyllin have been used as commercial deodorants in mouthwash and toothpaste. Screening tests using plant extracts have been conducted in order to identify more effective deodorants from plant sources than sodium cupper chlorophyllin. Some herb extracts including thyme (*Thymus vulgaris* L.) and rosemary (*Rosmarinus officinalis* L.) have similar biological activity¹⁾. In the present study, we focused on the essential oil of black cumin seeds for the discovery of new botanical deodorizing agents against the sulfur compounds that cause oral malodor.

Black cumin (*Nigella sativa* L., Ranunculaceae) is an annual plant that is distributed from Mediterranean regions to Western Asia. The seeds of this plant species were traditionally used as a spice as well as a folk medicine as a

natural remedy for various diseases from ancient times. These seeds have been reported to exhibit anti-tumor²⁾, anti-inflammatory³⁾, antioxidant⁴⁾, and antibacterial activities⁵⁾. Therefore, many reviews have already been published and several biological active compounds, mainly the menthane-type monoterpenes thymol, carvacrol, thymoquinone, and dihydrothymoquinone, have been identified^{6, 7)}.

2 EXPERIMENTAL

2.1 Plant materials and extraction

Black cumin (*Nigella sativa* L.) seeds were purchased from a spice market in India. The seeds (500 g) were ground into a powder using a mill (SK-M10, Kyoritsu-Riko Co., Japan). The black cumin seed powder prepared was subjected to steam distillation. The obtained distillate (750 mL) was extracted twice using ethyl acetate (150 mL). The ethyl acetate phase was concentrated with a Vigreux column at around 87°C and subsequently a rotary evaporator under reduced pressure at <45°C. A reddish-brown oil (3.6 g, yield 0.72%) was obtained.

2.2 Chemicals

The authentic reference compounds thymoquinone (>98%), thymohydroquinone (2,5-dihydroxy-*p*-cymene, >

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95%), thymol (>98%), and carvacrol (>95%) were purchased from Sigma-Aldrich Co., CHEMOS GmbH & Co. KG, Kanto Chemical Co., and Tokyo Chemical Industry Co., respectively. Methyl mercaptan standard solution (1 µg/µL in benzene) was purchased from Wako Pure Chemical Industries, Ltd.. Sodium methyl mercaptan (ca. 15% in water) was purchased from Tokyo Chemical Industry Co.. Hexane and ethyl acetate were purchased from Kanto Chemical Co. and used without purification. Silica gel for column chromatography and TLC plates were purchased from MERCK Co..

2.3 Evaluation of deodorizing activity

The deodorizing activities of the test compounds against methyl mercaptan were examined using the head space method⁸⁾. The procedure involved 1.0 mL of ethanol or an aqueous solution of the test compound and 1.5 mL of 0.1 M phosphate buffer (pH 7) being mixed for 5 sec in a 23-mL test tube, which was then sealed with a silicon cap. After the addition of 0.5 mL of ethanolic methyl mercaptan solution (1 µg/mL) prepared from the reagent purchased from Wako Chemical Industries, Ltd., the test tube was mixed for 5 sec using a vortex mixer. The test tube was incubated at 37°C for 6 min and then mixed for 5 sec using the vortex mixer. Methyl mercaptan released into the head space of the test tube was measured by GC equipped with a Flame Photometric Detector (FPD). GC analyses were performed using a Shimadzu GC-17A instrument equipped with a packed column (6 m × 3 mm i.d., 20% dinonyl phthalates, Uniport HP, 60-80 mesh) at 70°C; injector temperature at 120°C; detector temperature at 230°C; carrier gas, nitrogen 40 mL/min; splitless injection; sample injection volume; 5 mL of the head space. Deodorizing activity was calculated from the following equation⁸⁾. Data shown in figures are the mean results obtained with means of three replicates.

$$\text{Deodorizing activity (\%)} = (C - S)/C (\%)$$

C: Peak area of methyl mercaptan for the control

S: Peak area of methyl mercaptan for the test sample

Black cumin seed essential oil was compared with green tea extract and sodium cupper chlorophyllin, which are used as commercial oral deodorants. Thymoquinone was compared with other menthanes: thymohydroquinone, thymol, carvacrol, *p*-cymene (>98%, chemical for flavor), terpinen-4-ol (>90%, chemical for flavor), *l*-carvone (>99%, chemical for flavor), limonene (>90%, chemical for flavor), and piperitone (>98%, chemical for flavor), in order to assess the structure-activity relationship of deodorizing activity against methyl mercaptan.

2.4 Constituents and identification of active compounds in the essential oil of black cumin seeds

The essential oil of black cumin seeds was subjected to gas chromatography (GC) with FID. The GC analysis was performed using an Agilent Technology 6890N instrument equipped with an Inert Cap Pure Wax capillary column (60 m × 0.25 mm i.d., 0.25 µm film thickness) under the following temperature program: 50°C for 2 min, ramp of 2.5/min to 240°C for 50 min; injector and detector temperature, 250°C; carrier gas, helium 1.2 mL/min; split ratio 1/100. Components were identified by matching the retention time and the GC-MS data in the library search of the NIST file.

The black cumin seed essential oil (28 g) was separated using silica gel (Silica gel 60, MERCK) column chromatography (600 mm × 45 mm i.d.) with hexane and ethanol as eluents. The hexane eluent fraction (4.6 g) and ethanol eluent fraction (20.4 g) were obtained. As a deodorizing activity guide, the ethanol fraction (4.5 g) was separated using a silica gel flash column chromatography system (Isorela, Biotage) with a linear gradient from 10% to 90% ethyl acetate in hexane in order to obtain thymoquinone (**1**) (155 mg).

Thymoquinone (**1**) ¹H NMR (CDCl₃ 400 Mz) δ (ppm): 6.55 (1H q, *J* = 1.6 Hz, 6-H), 6.48 (1H, d, *J* = 1.2 Hz, 3-H), 2.98 (1H, dq, *J* = 6.9, 1.2 Hz, 8-H), 1.99 (3H, d, *J* = 1.6 Hz, 7-CH₃), 1.08 (6H, d, *J* = 6.9 Hz, 9-CH₃, 10-CH₃); ¹³C NMR (CDCl₃ 100 Mz) δ (ppm): 188.5 (C4), 187.3 (C1), 154.8 (C2), 145.0 (C5), 133.7 (C6), 130.2 (C3), 26.4 (C8), 21.3 (C9, C10), 15.2 (C7) (Fig. 1).

2.5 Structural elucidation of metabolites formed through the deodorizing activity of thymoquinone and methyl mercaptan

Several metabolites were obtained by mixing 600 mL of the ethanol solution of compound **1**, 0.1 M phosphate buffer (pH 7), and 12 mL of sodium methyl mercaptan (ca. 15% in water) at 37°C for 15 minutes. A reddish-brown oil (816 mg) was obtained. The crude metabolite was separated by silica gel column chromatography and PTLC to obtain two metabolites **2**, **3**. The R_f values of **1**, **2**, and **3** were 0.32, 0.67, and 0.55, respectively, developed with a hexane:ethyl acetate = 20:1 solvent system, respectively (Fig. 2).

Metabolite **2** ¹H NMR (CDCl₃ 400 Mz) δ (ppm): 3.43 (1H, q, *J* = 7.0 Hz, 8-H), 2.44 (3H, s, -SCH₃), 2.41 (3H, s, -SCH₃),

Fig. 1 Thymoquinone from black cumin seed oil.

Fig. 2 Thymoquinone and metabolites generated from methyl mercaptan on deodorization.

2.06 (3H, s, 7-CH₃), 1.20 (6H, d, J = 7.0 Hz, 9-CH₃, 10-CH₃); ¹³C NMR (CDCl₃ 100 Mz) δ (ppm): 180.5 (C4), 180.4 (C1), 153.2 (C2), 146.2 (C6), 144.2 (C5), 142.6 (C3), 31.6 (C8), 20.5 (C9, C10), 17.9 (-SCH₃), 17.2 (-SCH₃), 14.5 (C7)

Metabolite 3 ¹H NMR (CDCl₃ 400 Mz) δ (ppm): 6.42 (1H, d, J = 1.2 Hz, 3-H), 3.00 (1H, dq, J = 6.8, 1.2 Hz, 8-H), 2.48 (3H, s, -SCH₃), 2.09 (3H, s, 7-CH₃), 1.05 (6H, d, J = 6.8 Hz, 9-CH₃, 10-CH₃); ¹³C NMR (CDCl₃ 100 Mz) δ (ppm): 185.0 (C4), 183.2 (C1), 155.8 (C2), 145.5 (C6), 142.7 (C5), 130.2 (C3), 27.2 (C8), 21.4 (C9, C10), 17.5 (-SCH₃), 14.1 (C7)

3 RESULTS AND DISCUSSION

3.1 Evaluation of deodorizing activity

The most effective sample in the deodorizing activity test using two plant extracts, the essential oil of black cumin seeds and green tea extract, and sodium copper chlorophyllin against methyl mercaptan was the essential oil of black cumin seeds. The deodorizing activity of black cumin essential oil (EC₅₀: 7.3 μ g/mL) was *ca.* 200-fold that of sodium copper chlorophyllin (EC₅₀: 1.4 mg/mL, 1.9 mM), which is commonly used as a deodorant, and *ca.* 14-fold that of green tea extract (EC₅₀: 0.104 mg/mL) (Fig. 3). In addition, the safety of black cumin seed essential oil has already been demonstrated by animal experiments⁹. No significant difference was noted in the organs, enzymes, and protein values of the rats used in the test, compared

Fig. 3 Comparison of deodorizing activity of plant extracts and Cu-chlorophyllin-Na.

with those in the control group. Additionally, the LD₅₀s of 1 were 104.7 mg/kg (*i.p.*) and 870.9 mg/kg (*p.o.*) for mice and 57.5 mg/kg (*i.p.*) and 794.3 mg/kg (*p.o.*) for rats¹⁰.

3.2 Constituents and identification of active compounds in the essential oil of black cumin seeds

There were many peaks on the FID-GC chart, and the major constituents were thymoquinone, *p*-cymene, thymol, and *l*-carvone (Fig. 4). These monoterpenes were considered to be precursors in the metabolism of thymoquinone in this plant, namely hydrocarbons, and limonene was oxidized to generate *p*-cymene and thymol. These oxidized monoterpenes are further oxidized to generate thymoquinone. The monoterpene compositions of the black cumin extract and essential oil collected in Morocco and Algeria showed slightly difference profiles from those collected in India. A comparison of Morocco/Algeria and Indian black cumin components demonstrated twice the amount of *p*-cymene and half the amount of thymoquinone^{11, 12}. This may be explained by the fact that *p*-cymene-containing hydrocarbon fractions, which eluted at the initial stage of distillation, were excluded from the Indian products. The amount of thymoquinone was dependent on the withholding of irrigation in the plant habitat¹³. The dimeric compound, dithymoquinone was detected by a HPLC analysis in the essential oil of black cumin¹⁴.

The most effective sample was the essential oil of black cumin seeds, and the active compound was disclosed by bioassay-guided fractionation. Thymoquinone (1) was identified as a causative agent for the deodorizing activity by

Fig. 4 GC-FID profile of black cumin essential oil.

Fig. 5 The deodorizing activity of thymoquinone.

fractionation purification in the biological activity test using the methyl mercaptan reduction rate as an indicator. The deodorizing activity of **1** showed the most active $EC_{50} = 17.6 \mu\text{M}$ ($2.9 \mu\text{g/mL}$), and was *ca.* 110-fold that of sodium copper chlorophyllin, $EC_{50} = 1932 \mu\text{M}$ (Fig. 5). However, this value for **1** was similar to that of the essential oil of black cumin seeds (Fig. 3). This demonstrated that compound **1** was the active compound in black cumin seed essential oil, involved in the deodorizing activity on methyl mercaptan.

Major compound **1** in black cumin seeds has already been shown to exhibit various pharmaceutical activities including anti-tumor¹⁵⁾ and hepatoprotective activities¹⁶⁾ based on the findings of animal studies and/or *in vivo* tests using cells.

3.3 Structure-activity relationship of **1** to deodorizing activity against methyl mercaptan

The deodorizing activity of various menthanes against methyl mercaptan were evaluated at $50 \mu\text{M}$. The results of

Fig. 7 Comparison of deodorizing activity with thymoquinone and related compounds at $50 \mu\text{M}$.

the present study showed that compound **1** and its reductant, thymohydroquinone exhibited significant biological activities, with reducing rates of 80% and 61%, respectively. The monophenols, thymol and carvacrol also exhibited weak methyl mercaptan reducing activities. The other menthanes tested did not exhibit this biological activity in this test (Figs. 6 and 7). These results suggest that certain quinone and phenolic structures, particularly quinone structures and quinone precursors that are converted to quinone, are essential for the biological (deodorizing) activity. Carvacrol and thymol produce their corresponding *o*-quinone methides when oxidized. These molecular species behave in the same manner as *p*-quinone *in vitro* and are finally added to methyl mercaptan. Consequently, the amount of methyl mercaptan decreased in this test. Rosmanol in rosemary (*Rosmarinus officinalis* L.) and shikoinin in the roots of *Lithospermum erythrorhizon*, which

Fig. 6 Menthanes tested for the structure-activity relationship of deodorizing activity.

are phytochemicals, also exhibited deodorizing activities against methyl mercaptan¹⁷⁾. These natural products with *o*-phenol and *p*-phenol moieties, respectively, were similar to active monoterpenes in this test.

3.4 Structure elucidation of metabolites formed during deodorization with compound 1 and methyl mercaptan

The deodorizing activity of compound 1 may be explained by quinone binding to methyl mercaptan, because two metabolites 2, 3 could be isolated from the ethanol solution of sodium methyl mercaptan and thymoquinone. Both metabolites were the methylthio adducts of 1, mono- and di- methylthio substituents, respectively based on 2D-NMR data (Fig. 2). Similarly, *o*-quinone generated by polyphenol oxidase have been shown to play a critical role in the methyl mercaptan capturing properties of mushroom¹⁸⁾. In addition, *o*-quinones were found to be intermediates that produced methylthio adducts in the case of catechins, such as epigallocatechin gallate (EGCG)¹⁹⁾. These reactions were considered to be the main factors responsible for the deodorizing activities observed.

4 CONCLUSION

The results obtained for the essential oil of black cumin seeds suggested that this botanical resource is applicable to the control of oral malodor. Since the biological activities of the essential oil of black cumin seeds and compound 1 comprise antibacterial and deodorizing activities, these seeds represent a good botanical resource for the development of new oral health care products²⁰⁾. In addition, the safety of black cumin seed essential oil has already been demonstrated by animal experiments⁹⁾. Indeed, commercially available oral washes and toothpastes are hardly absorbed into the body.

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