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 mercapta, 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 mercapta. Metabolite analysis suggested that the deodorizing activity may be generated by the addition of a reactive quinone molecule to methyl mercapta. 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 mercapta, 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 and rosemary have similar biological activity. Therefore, many reviews have already been published and several biological active compounds, mainly the menthone-type monoterpenes thymol, carvacrol, thymoquinone, and dihydromethoxyquinone, have been identified.

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, >
2.3 Evaluation of deodorizing activity

The deodorizing activities of the test compounds against methyl mercaptan were examined using the head space method. 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 an Inert Cap Pure Wax capillary column (3 mm i.d., 20 μm film thickness) and a Flame Photometric Detector.

2.4 Constituents and identification of active compounds

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/minute to 240°C for 50 min; injector and detector temperature, 250°C; carrier gas, helium 1.2 mL/minute; 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 MHz) 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 MHz) 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 Rf 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 MHz) 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](image-url)
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 (EC_{50}: 7.3 µg/mL) was ca. 200-fold that of sodium copper chlorophyllin (EC_{50}: 1.4 mg/mL), which is commonly used as a deodorant, and ca. 14-fold that of green tea extract (EC_{50}: 0.104 mg/mL) (Fig. 3). In addition, the safety of black cumin seed essential oil has already been demonstrated by animal experiments^{9}. No significant difference was noted in the organs, enzymes, and protein values of the rats used in the test, compared with those in the control group. Additionally, the LD_{50}s 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 rats^{10}.

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, \( \delta \)-cymene, thymol, and \( \alpha \)-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 \( \delta \)-cymene and thymol. These oxidized monoterpenes are further oxidized to generate thymoquinone. The monoterpenic compositions of the black cumin extract and essential oil collected in Morocco and Algeria showed slightly different profiles from those collected in India. A comparison of Morocco/Algeria and Indian black cumin components demonstrated twice the amount of \( \delta \)-cymene and half the amount of thymoquinone^{11, 12}. This may be explained by the fact that \( \delta \)-cymene-containing hydrocarbons, 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 habitat^{13}. The dimeric compound, dithymoquinone was detected by a HPLC analysis in the essential oil of black cumin^{14}.

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
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 \( EC_{50} = 17.6 \mu M(2.9 \mu g/mL) \), and was ca. 110-fold that of sodium copper chlorophyllin, \( EC_{50} = 1932 \mu M \) (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-tumor\(^{15}\) and hepatoprotective activities\(^{16}\) 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 \( \mu M \). The results of 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 \( \alpha \)-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 shikoknin in the roots of Lithospermum erythrorhizon, which...
are phytochemicals, also exhibited deodorizing activities against methyl mercaptan\(^1\). 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 mushrooms\(^3\). In addition, \(o\)-quinones were found to be intermediates that produced methylthio adducts in the case of catechins, such as epigallocatechin gallate (EGCG)\(^6\). 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 products\(^20\). In addition, the safety of black cumin seed essential oil has already been demonstrated by animal experiments\(^5\). Indeed, commercially available oral washes and toothpastes are hardly absorbed into the body.

References

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