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Chemical Constituents, Antifungal and Antioxidative Effects of Ajwain Essential Oil and Its Acetone Extract

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GC and GC-MS analysis of ajwain essential oil showed the presence of 26 identified components which account for 96.3% of the total amount. Thymol (39.1%) was found as a major component along with *p*-cymene (30.8%), γ-terpinene (23.2%), β-pinene (1.7%), terpinene-4-ol (0.8%) whereas acetone extract of ajwain showed the presence of 18 identified components which account for 68.8% of the total amount. The major component was thymol (39.1%) followed by oleic acid (10.4%), linoleic acid (9.6%), γ-terpinene (2.6%), *p*-cymene (1.6%), palmitic acid (1.6%), and xylene (0.1%). Moreover, the oil exhibited a broad spectrum of fungitoxic behavior against all tested fungi such as *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus oryzae*, *Aspergillus ochraceus*, *Fusarium monoliforme*, *Fusarium graminearum*, *Pencillium citrium*, *Penicillium viridicatum*, *Pencillium madriti*, and *Curvularia lunata* as absolute mycelial zone inhibition was obtained at a 6-μL dose of the oil. However, the acetone extract showed better antioxidative activity for linseed oil as compared with synthetic antioxidants such as butylated hydroxyl toluene and butylated hydroxyl anisole.

KEYWORDS: ***Trachyspermum ammi* (L.); antioxidant; antifungal; thymol; acetone extract; *Aspergillus*; *Penicillium*; *Fusarium***

INTRODUCTION

Ajwain, *Trachyspermum ammi* (L.) Sprague, is an erect annual herb with striate stem, originated in eastern regions of Persia and India. The most utilized part of ajwain is the small carawaylike fruit, which is particularly popular in Indian savory recipies, savory pastries, and snacks. In Ayurvedic medicines, it is used as a medicinal plant for its antispasmodic, stimulant, tonic, and carminative properties (*1*). Aroma chemicals present in spices have wide application in aromatherapy since ancient times suggesting that they have some beneficial health effects in addition to pleasant flavor (*2*). Furthermore, they inhibit (*3*−*5*) other undesirable changes in food affecting its nutritional quality, flavor, and texture. However, due to possible toxicological (*6*, *7*) side effects of synthetic food additives on human health, the general trend toward reducing the use of synthetic food additives resulted in an expansion of the search for natural substances (*8*−*11*) possessing antimicrobial and antioxidant properties and hence they are valuable in preventing cellular damage, the cause of aging, and human diseases. For this purpose, essential oils and extracts are widely used globally because it has a complex composition, containing constituents of mostly hydrocarbons and oxygenated compounds. On the basis of this background, many studies are in progress (*12*−*15*). In literature, there are many reports available on chemical composition and antimicrobial studies (*16*−*22*) of volatile oil of ajwain. However, the anti- oxidative properties of its volatile oil and acetone extract seem not to have been reported before. As a part of our ongoing research program (*20*−*24*), chemical constituents and antifungal and antioxidative studies of volatile oil and acetone extract used as a natural food additive have been undertaken. The objective of present investigation is to compare the chemical composition of volatile oil and its extract as well as to determine the antifungal and antioxdative behavior on linseed oil using as additive by various methods.

MATERIALS AND METHODS

Plant Material. Fruits of ajwain were purchased from the local market of Gorakhpur during April, and voucher specimens were deposited at the Herbarium of the Science Faculty of DDU Gorakhpur University, Gorakhpur.

Extraction of the Essential Oil. The fruits of ajwain were powdered (800 mesh size) using domestic model mixi and were subjected to hydrodistillation in a Clevenger type apparatus for 6 h in accordance with European pharmacopeia procedure (*25*). Yellow-colored oil (yield 2.2%) with characterstic odor and sharp taste was obtained. It was dried over anhydrous sodium sulfate to remove traces of moisture and stored in refrigerator in dark at 4 °C until use.

Isolation of the Acetone Extract. After the isolation of the essential oil, the powdered fruits were dried at 25 °C. Extract was obtained by extracting 20 g of dried fruits with 900 mL of acetone for 6 h in a Soxhlet apparatus. The extract was concentrated up to 20 mL. The

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remaining acetone was evaporated by placing the samples in a vacuum drier under reduced pressure. The viscous extract, known as oleoresin (yield 3.6%), were stored in a freezer at 4 °C until use.

Chemical Investigation. Qualitative and quantitative analysis of volatile oil and acetone extract were undertaken by gas chromatography (GC) and gas chromatography-mass spectroscopy (GC-MS) techniques.

GC. Using a Hewlett-Packard 5890 series II gas chromatograph equipped with flame ionization detector (FID) and silica column, the gas chromatograms of the oil and acetone extract were obtained. The column was an HP-5 (5% phenyl methyl silicone, 30-m × 0.32-mm × 0.25-μm) whose injector and detector temperatures were maintained at 250 and 270 °C, respectively. The amount of the samples injected was 0.1 μL (in split mode 80:1 for oil and 85:1 for acetone extract). Carrier gas used for oil and acetone extract was N_2 with a flow rate 1.1 mL min^{-1}. The oven temperature was programmed as follows: 60 °C (1 min), 60−85 °C (0.5 °C min^{-1}), 85 °C (1 min), 85−105 °C (1 °C min^{-1}), 105 °C (2 min), 105−275 °C (10 °C min^{-1}), and 275 °C (2 min). That for acetone extract was as follows: 100 °C (1 min), 100−280 °C (5 °C min^{-1}), and 280 °C(20 min).

GC-MS. The volatile oil and acetone extract were subjected to GC-MS analysis using a Hewlett-Packard mass detector (model 5973) and an HP-5 MS column (length, 30 m; inner diameter, 0.25 mm; film thickness, 0.25 μm). The injector, GC-MS interphase, ion source and selective mass detector temperatures were maintained at 270, 280, 230, and 150 °C, respectively. Carrier gas used for oil and acetone extract was He with a flow rate of 1.1 and 1.5 mL min^{-1}, respectively. The oven temperature programmed for the volatile oil was as follows: 60° C (1 min), 60−85 °C (0.5 °C min^{-1}), 85 °C (1 min), 85−105 °C (1 °C min^{-1}), 105 °C (2 min), 105−275 °C (10 °C min^{-1}), and 275 °C (2 min). That for acetone extract was as follows: 100 °C (1 min), 100−280 °C (5 °C min^{-1}), and 280 °C (20 min).

Identification of Components. The percentages of components were obtained from electronic integration measurements using flame ionization detection (FID). The retention indices were calculated for all volatile constituents using a homologous series of *n*-alkanes C8−C16. Chemical constituents were identified by comparing their mass spectra with the library (*26*, *27*) NBS 75K and/or by co-injection with authentic samples, and the results of volatile oil and its acetone extract were reported in **Table 1**.

Antifungal Investigations. To determine the antifungal efficacy of the volatile oil and its extract, the pathogenic fungus *Aspergillus niger* (AN), *Aspergillus flavus* (AF), *Aspergillus oryzae* (AO), *Aspergillus ochraceus* (AO′), *Fusarium monoliforme* (FM), *Fusarium graminearum* (FG), *Pencillium citrium* (PC), *Penicillium viridicatum* (PV), *Pencillium madriti* (PM), and *Curvularia lunata* (CL) were undertaken. These fungi were isolated from food materials such as onion, vegetable waste, curd, wheat straw, fruits of *Musa* species, sweet potato, decaying vegetation, vegetable, and cheese, respectively, and purchased from Microbial Type Culture Collection (MTCC), Chandigarh, India. The MTCC code numbers of these strains are 2479, 1884, 1846, 1810, 1893, 2088, 2553, 2007, 3003, and 2073, respectively. Cultures of each of the fungi were maintained on Czapek (DOX) agar media with adjusting pH 6.0−6.5 and slants were stored at 5 °C. The antifungal activity of the volatile oil and acetone extract against fungi were undertaken using inverted Petriplate and food poison techniques (*28*). In inverted Petriplate method, the required dose (2, 4, and 6 μL) of undiluted sample were soaked on a small piece (diameter 12 mm) of Whatman No. 1 filter paper, and it was kept on the lid of Petriplate, which is in inverted position, whereas in food poison technique, the required doses (2, 4, and 6 μL) of the undiluted sample were mixed with the 20 mL of culture medium. Each test was performed at three concentrations (2, 4, and 6 μL) and replicated three times. The results taken by inverted Petriplate and food poison technique are given in **Tables 2** and **3**, respectively.

Antioxidant Activity. To assess the antioxidant activity (*29*−*31*) of ajwain volatile oil and acetone extract, crude linseed oil, having initial peroxide value 4.2 meq kg^{-1} was taken for present investigation. The oil was selected due to its high degree of unsaturation and generally used as edible oil in Central Europe and Asia.

Peroxide Value Method. For measuring the peroxide value (*32*, *33*) a modified oven test (*34*) was used. The antioxidant activity of volatile oil and acetone extract were compared with synthetic antioxidants such as BHA and BHT. The calculated quantities of each (200 ppm) were added to 30 g of linseed oil in an open mouthed beaker. The mixtures were thoroughly homogenized and placed into an 80 °C thermostat. The peroxide values were measured every 7 days, and the test was replicated three times. A control sample was prepared under

Table 1. Chemical Composition of Ajwain Essential Oil and Acetone Extract[a]

compound	essential oil %FID	essential oil KI[b]	% FID of acetone extract
2-methyl but-3-ene-2-ol	tr	0807	
2-methyl(methyl butanoate)	tr	0872	
alpha-thujene	0.2	0931	
alpha-pinene	0.2	0941	tr
camphene	tr	0953	
sabinene	tr	0975	
beta-pinene	1.7	0980	0.2
myrcene	0.4	0993	
alpha-phellandrene	tr	1007	
delta-3-carene	tr	1013	
alpha-terpinene	0.2	1020	
p-cymene	30.8	1026	1.6
beta-phellandrene	0.6	1030	1.3
1,8-cineole	tr	1035	
gamma-terpinene	23.2	1064	2.6
terpinolene	0.2	1088	
trans-sabinenhydrate	0.1	1098	
linalool	0.1	1099	
terpinen-4-ol	0.8	1177	tr
alpha-terpineol	0.1	1189	tr
thymol	39.1	1291	39.1
carvacrol	0.3	1298	0.3
beta-selinene	0.1	1492	
alpha-selinene	tr	1499	
caryophyllene oxide	tr	1583	
delta-dodecalactone	tr		
4-methylpent-3-ene-2-one			0.5
4-hydroxy-4-methylpent-2-one			1.1
xylene (isomer not identified)			tr
undecane			0.1
thymoquinone			0.2
2,3,4,6-tetramethyl phenol			0.1
palmitic acid			1.6
linoleic acid			9.6
oleic acid			10.4
total	**96.3%**		**68.8%**

[a] Percentages are the mean of three runs and were obtained from electronic integrations measurements using flame ionization detection (FID). tr = trace, < 0.01%. [b] The retention indices were calculated for all volatile constituents using a homologous series of *n*-alkanes C8–C16.

Table 2. Antifungal Investigations of Ajwain Essential Oil and Acetone Extract by Using Inverted Petriplate Technique

fungus[b]	%mycelial zone inhibition at different dose of sample[a]: ajwain essential oil 2 μL	4 μL	6 μL	ajwain acetone extract 2 μL	4 μL	6 μL
Aspergillus niger (AN)	68.7	87.5	100	12.5	25.0	37.5
Aspergillus flavus (AF)	37.5	56.3	100	12.5	50.0	52.5
Aspergillus oryzae (AO)	75.0	87.5	100	25.0	13.7	21.3
Aspergillus ochraceus (AO′)	87.5	100	100	11.3	62.5	87.5
Fusarium graminearum (FG)	50.0	75.0	100	0.0	50.0	75.0
Fusarium monoliforme (FM)	75.0	87.5	100	12.5	37.5	50.0
Penicillium citrium (PC)	87.5	100	100	12.5	18.7	43.8
Penicillium viridicatum (PV)	100	100	100	6.25	12.5	25.0
Penicillium madriti (PM)	100	100	100	18.7	26.3	38.7
Curvularia lunata (CL)	87.5	93.7	100	43.7	50.0	75.0

[a] Average of three replicates. [b] For all tested fungi, the data was found to be highly significant ($p < 0.05\%$).

Table 3. Antifungal Investigations of Ajwain Essential Oil and Acetone Extract Using Food Poison Technique

	%mycelial zone inhibition at different dose of sample[a]					
	ajwain essential oil			ajwain acetone extract		
fungus[b]	2 μL	4 μL	6 μL	2 μL	4 μL	6 μL
Aspergillus niger (AN)	100.0	100.0	100.0	18.7	26.3	38.7
Aspergillus flavus (AF)	87.5	100.0	100.0	27.5	41.3	46.3
Aspergillus oryzae (AO)	87.5	100.0	100.0	25.0	43.8	62.5
Aspergillus ochraceus (AO′)	25.0	37.5	100.0	25.0	31.3	50.0
Fusarium graminearum (FG)	18.7	50.0	56.3	6.3	50.0	75.0
Fusarium monoliforme (FM)	100.0	100.0	100.0	62.5	77.5	87.5
Penicillium citrium (PC)	6.2	13.8	40.0	25.0	62.5	87.5
Penicillium viridicatum (PV)	100.0	100.0	100.0	50.0	52.5	60.0
Penicillium madriti (PM)	50.0	75.0	87.5	37.5	50.0	75.0
Curvularia lunata (CL)	18.7	26.3	46.3	18.8	26.3	46.3

[a] Average of three replicates. [b] For all tested fungi, the data was found to be highly significant ($p < 0.05\%$).

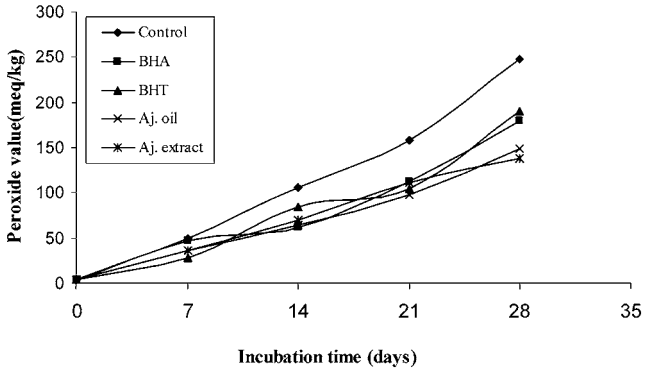

Figure 1. Inhibitory effect on peroxide accumulation of ajwain Oil and its acetone extract for linseed oil at 80 °C.

similar conditions without any additive. The effect of oil and acetone extract in terms of linseed oil peroxidation at 80 °C are shown in **Figure 1**.

Thiobarbituric Acid Value (TBA). The test was performed according to the methods previously stated by some authors (*35*,*36*) with small changes. The same sample as prepared for the peroxide value method was used. To 10 g of sample, 0.67% aq thiobarbituric acid (20 mL) and benzene (25 mL) solution were added. This mixture was shaken continuously for 2 h, using a mechanical shaker. After 2 h, the supernatant was taken and placed in a boiling water bath for 1 h. After cooling, absorbance of supernatant was measured at 500 nm with Hitachi-U-2000 spectrophotometer. The TBA value (meq/g) was calculated using the following formula:

$$\text{TBA value} = \frac{3.5 \times \text{OD}}{0.15 \times W}$$

where OD = absorbance of supernatant solution and W = amount of sample (in grams)

The results in the form of plot of incubation time verses TBA value are shown in **Figure 2**.

The effect of oil and acetone extract on linseed oil in terms of incubation time versus TBA value at 80 °C is shown in **Figure 2**.

Determination of Antioxidant Activity in Linoleic Acid System. Antioxidant activity was carried out using the method (*37*) proposed by Osawa and Namiki (*38*) with small changes. Samples (4 mg) in 99.5% ethanol were mixed with 2.5% linoleic acid in 99.5% ethanol (4.1 mL), 0.05M phosphate buffer (pH = 7, 8 mL) and distilled water (3.9 mL) and kept in screw cap containers under dark conditions at 40 °C. To 0.1 mL of this solution was added to 9.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate. After 3 min, 0.1 mL of 0.02 M ferrous chloride in 3.5% hydrochloric acid was added to the reaction mixture, the absorbance of red color was measured at 500 nm

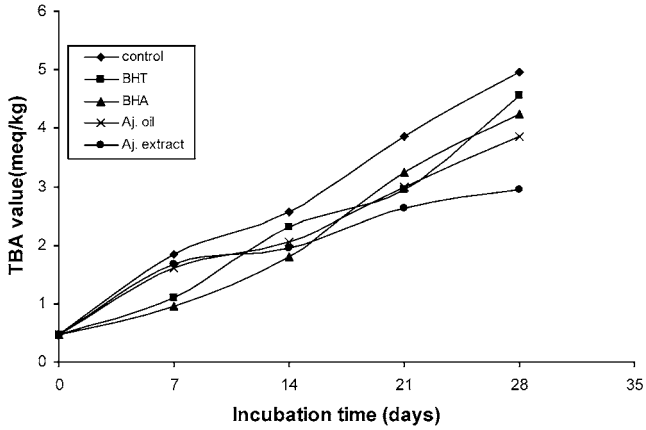

Figure 2. Inhibitory effect of ajwain oil and its acetone extract for linseed oil measured using thiobarbituric acid value method at 80 °C.

in the spectrophotometer every 2 days. The control and standard were subjected to the same procedure except for the control, where there was no addition of sample, and for the standard, 4 mg of sample was replaced with 4 mg of BHA and BHT. The incubation time versus absorbance is plotted in **Figure 3**.

Statistical Analysis. The quantitative data of major components of oil and acetone extract were statistically examined by analysis of variance (ANOVA), and significant differences among several groups of data were examined by Ducan's multiple range test.

RESULTS AND DISCUSSION

The investigation of aroma compounds of the essential oils and acetone extract of dried fruits of ajwain was carried out by means of GC and GC-MS to identify the odorous target component responsible for characteristic odor of valuable spice and food flavoring products. The analysis of oil and acetone extract of ajwain was undertaken by using previously stated HP-5 series. The volatile oil on analysis showed the presence of 26 identified components (**Table 1**), which account for 96.3% of the total amount. Thymol (39.1%) was found as a major component along with *p*-cymene (30.8%), γ-terpinene (23.3%), β-pinene (1.7%), terpinene-4-ol (0.8%), and several other components in minor percentages. Moreover, acetone extract showed the presence of 18 identified components (**Table 1**), which account for 68.8% of the total amount. The major component was thymol (39.1%), followed by oleic acid (10.4%), linoleic acid (9.6%), γ-terpinene (2.6%), *p*-cymene (1.6%), palmitic acid (1.6%), and 4-hydroxy-4-methylpenta-2-one (1.1%). It is interesting to note that thymol content in both the oil and acetone extract was almost the same. Chialva et al. (*16*) have already reported that thymol is a major component of this oil and there is a compositional variation between a steam distilled oil and oleoresin.

Using inverted Petriplate technique, the oil was found to be 100% antifungal against all the tested fungi at a 6-μL dose (**Table 2**). It was highly effective in controlling mycelial growth of tested *Penicillium* species even at a 2-μL dose of the oil. Moreover, the oil was also found to be active against AO′, PC, and CL at a 2-μL dose as more than 80% mycelial zone inhibition was obtained, whereas using the same method, acetone extract was found less effective than the volatile oil, as only more than 50% mycelial zone inhibition of AF, AO′, FG, FM, and CL was obtained even at a 6-μL dose. Moreover, using food poison technique (**Table 3**), the oil was found highly effective against AN, AF, AO, AO′, FM, and PV as absolute mycelial zone inhibition was obtained at 6 μL. It is very interesting to note that 100% activity of the fungi AN, FM,

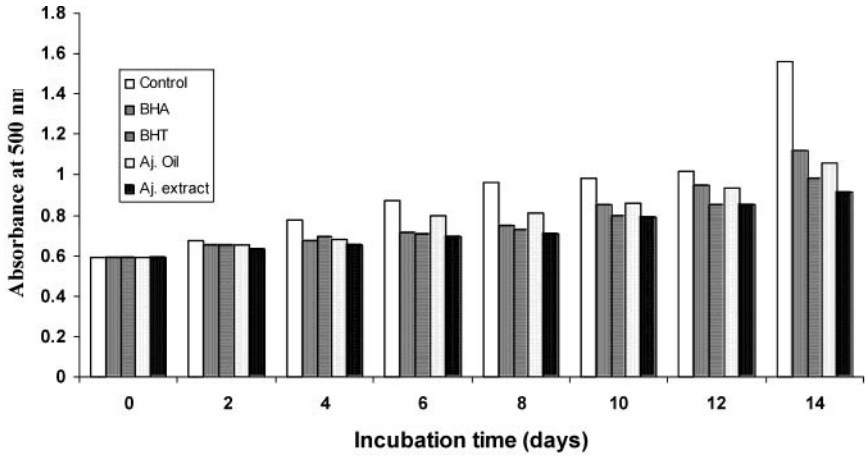

Figure 3. Determination of antioxidant activity by linoleic acid method at 40 °C.

and PV was obtained even at a 2-μL dose of the oil, whereas using the same method acetone extract was found to be comparatively less effective than the volatile oil, as only more than 70% mycelial zone inhibition was obtained for FG, FM, PC, and PM. Various researchers (*17*, *19*) have already reported that this oil exhibited a broad fungitoxic spectrum, inhibiting the mycelial growth of a number of fungi at 100, 200, and 300 ppm. The oil was thermostable and more efficacious than various synthetic fungicides. Moreover, the data was found to be highly significant ($p < 0.05$).

The oxidation of lipids has been classified as a major deterioration process affecting both the sensory and nutritional quality of food. Hydroperoxides are primary oxidation products. It should be mentioned that peroxide value method is a widely used measure of primary lipid oxidation, indicating the amount of peroxides formed in fats and oils during oxidation. The curves in **Figure 1** demonstrate peroxide value (meq/kg) changes in linseed oil treated with volatile oil, acetone extract, BHA, and BHT. Peroxide value was measured at time periods of 28 days of storage. During this time, peroxide value of control sample (without additive) increased to 239 meq/kg. The results in **Figure 1** show that all samples reduce the oxidation rate of linseed oil at 80 °C in terms of formation of peroxide. The samples with volatile oil and acetone extract were found to be significantly ($p < 0.05$) more effective than the control.

During the oxidation process, peroxides are generally decomposed to lower molecular weight compounds. One such compound is malonaldehyde, which is measured by the TBA method. Malonaldehyde, the compound used as an index of lipid peroxidation, was determined by selective third order derivative spectrophotometric method previously developed by some authors (*39*). **Figure 2** shows that volatile oil and acetone extract had significantly ($P < 0.05$) lower TBA values than the control up to 28 days of incubation at 80 °C. Their activity is quite comparable to that of BHA and BHT. It is interesting to note that, after a certain duration (21 days), BHA and BHT become less effective than acetone extract in stabilizing linseed oil.

The initial stage of peroxide level of lipid oxidation was measured using linoleic acid system. Low absorbance values indicate high levels antioxidative activity. The inhibitor activities against lipid peroxidation in linoleic acid caused by additives were evaluated by measuring concentration of ferric thiocynate. The values obtained without additives were taken for 100% lipid peroxidation. **Figure 3** shows absorbance values measured for oil, acetone extract, BHA, and BHT, along with the control, for 12 days. These results are well correlated with those obtained previously by peroxide value and TBA methods.

It is known that the effectiveness of added antioxidants varies depending on the food and on the processing and storage conditions (*30*). BHT is very effective in animal fat but less effective in vegetable oils. Volatile oil and BHT may also be lost during heating because of their volatility. BHA is known to be very effective antioxidant for vegetable oils, and it is more stable at high temperatures than BHT (*40*). Most likely, acetone extracts are more effective than BHA and BHT at high temperature. In terms of retarding the formation of primary and secondary oxidation products, the effectiveness of samples added to the concentration of 200 ppm can be put into the following order: acetone extract > oil > BHA > BHT > control.

Several factors can induce changes in the antioxidant activity during the distillation of volatile constituents from spices. The volatile oil and acetone extract both contain thymol (39.1%) as a major component, which refers, in general, antioxidative properties of these extracts. In acetone extract, oleic acid (10.4%) and linoleic acid (9.6%) do not possess antioxidant activity. Probably other substances, which could be present in acetone extract and not identified by GC, can contribute to improve antioxidant activity of acetone extract, which needs further investigations. Furthermore, hydrolysis and other cleavage processes can release such compounds. Moreover, heat- and water-induced chemical reactions can also change the activity of a complex extract system consisting of numerous compounds with different chemical and physical properties.

Concluding these results, we can say that the ajwain volatile oil, which is rich in thymol, exhibited a broad fungitoxic spectrum by inhibiting the mycelial growth of all tested fungi. Besides this, the volatile oil and acetone extract have been proven to be alternative sources of natural antioxidants and more efficacious than various synthetic antioxidants, BHA and BHT.

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