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RESEARCH PAPER

Antifungal Activity of Shirazi Thyme (*Zataria multiflora* Boiss.) Essential Oil against *Hypomyces perniciosus*, a causal agent of wet bubble disease of *Agaricus bisporus*

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مضادات نشاط فطر *Hypomyces perniciosus* المسبب لمرض الفقاعة الرطبة على فطر الأبيض (المشروم) *Agaricus bisporus* بإستخدام الزيت العطري من الزعتر الشيرازي (*Zataria multiflora* Boiss.)

يمنى بنت جمعة بن راشد البلوشي[1] و عبدالله بن محمد السعدي[1] و عيسى بن هاشل المهمولي[1] و ماجدة بنت محمد بن علي الحراصي[1]
و جمال بن ناصر الصباحي[2] والآء بنت خميس بن سليمان العلوي[1] وخالد الفارسي[3] و ريثيناسامي فيلازهاهن[1]

Abstract. Wet bubble disease (WBD) caused by *Hypomyces perniciosus* is a major constraint of button mushroom (*Agaricus bisporus*) cultivated worldwide. A few synthetic chemical fungicides are used to control WBD. In our study, the potential of essential oil (EO) from *Zataria multiflora* in inhibition of *H. perniciosus* was evaluated as an alternative to chemical fungicides. An isolate of *H. perniciosus* was isolated from wet bubble diseased *A. bisporus* and pathogenicity of the mycoparasite was determined under artificially inoculated conditions. The mycoparasitic fungus was identified using sequences of the internal transcribed spacer (ITS) region of ribosomal DNA. The EO was extracted from the aerial parts of *Z. multiflora* by microwave extraction method and evaluated in vitro for its antifungal activity against *H. perniciosus*. The EO of *Z. multiflora* (ZEO) at the tested concentrations (50% and 100%) inhibited the growth of *H. perniciosus* in the agar diffusion test. The minimum inhibitory concentration (MIC) of ZEO was 0.04% as assessed by the poisoned food technique. The chemical composition of ZEO was determined by gas chromatography-mass spectrometry analysis. A total of 23 compounds were identified. Among them, the most abundant compounds were Linalool (20.3%) and Bornyl acetate (15.5%). Linalool at the tested concentrations of 0.25% and 0.125% completely inhibited the mycelial growth of *H. perniciosus* in an *in vitro* assay. These results suggest that ZEO can be exploited for control of WBD.

Keywords: : *Agaricus bisporus; antifungal*; essential oil; *Mycogone perniciosa*; wet bubble disease; *Zataria multiflora.*

الملخص: مرض الفقاعة الرطبة (WBD) الناجم عن *Hypomyces perniciosus* هو أحد المعوقات الرئيسية لفطر الأبيض (*Agaricus bisporus*) المزروع في جميع أنحاء العالم. يستخدم عدد قليل من مبيدات الفطريات الكيميائية الأصطناعية للتحكم في WBD. في دراستنا، تم تقييم إمكانية الزيت العطري من *Zataria multiflora* في تثبيط *H. perniciosus* كبديل لمبيدات الفطريات الكيميائية. تم عزل *H. perniciosus* من الفطر الابيض *A. bisporus* مصاب بـ الفقاعة الرطبة وبعد ذلك تم إختبار هذه العزلة و تحديد الإمراضية للطفيل الفطري لتسبب هذا المرض على فطر أبيض سليم تحت ظروف مناخية معلومة. تم تعريف الطفيل الفطري بإستخدام التصنيف الجيني في تسلسل وحدة الريبوسومات الموجودة على فواصل النسخ الداخلي (ITS) من حمض النووي الريبوسومي .تم إستخراج الزيت العطري EO من الأجزاء العلوية لـ *Z. multiflora* بطريقة الإستخلاص بالميكروويف وتم تقييمه في المختبر لنشاطه المضاد للفطريات ضد *H. perniciosus*. أعاق EO لـ (ZEO) *Z. multiflora* بتركيزات مختبرة (٥٠٪ و ١٠٠٪) نمو *H. perniciosus* في إختبار خلط الزيت بالأجار. كان الحد الأدنى للتركيز المثبط (MIC) لـ ZEOهو ٠,٠٤٪ الذى تم تقييمه بواسطة تقنية الغذاء المسموم. تم تحديد التركيب الكيميائي لـ ZEO عن طريق تحليل كروماتوجرافيا الغاز لقياس الطيف الكتلي. كذلك ، تم تحديد إجمالي ٢٣ مركبًا. من بينها ، كانت المركبات الأكثر وفرة Linalool (٢٠,٣٪) و Bornyl acetate (١٥,٥٪). قام Linalool بتركيزات مختبرة بنسبة ٠,٢٥٪ و ٠,١٢٥٪ بتثبيط نمو الغزل الفطري (الميسيليوم) *H. perniciosus* تمامًا في إختبار في المختبر. تشير هذه النتائج إلى أنه يمكن إستغلال ZEO للتحكم في WBD.

الكلمات المفتاحية: *Agaricus bisporus*، مضاد للفطريات، زيت العطري، *Mycogone perniciosa*، مرض الفقاعة الرطبة، *Zataria multiflora*

Introduction

Mushroom farming is becoming a popular agro-based business worldwide. Over 8.99 million tons of mushrooms are produced annually worldwide (Kumla et al., 2020). White button mushroom [*Agaricus bisporus* (Lange) Imbach] is the most popular and widely cultivated edible mushroom globally (Sanchez, 2004). Diseases are serious constraints in the commercial production of button mushrooms. Several fungal diseases including wet bubble (*Hypomyces perniciosus*), dry bubble (*Lecanicillium fungicola*), cobweb (*Cladobotryum mycophilum*) and green mold (*Trichoderma aggressivum*) are reported to significantly reduce the yield and quality of mushroom crops (Gea et al., 2021). Wet bubble disease (WBD) caused by the ascomycetes fungus *Hypomyces perniciosus* Magnus [*Mycogone perniciosa* (Magnus) Delacroix] was reported to cause yield reductions ranging from 15 to 30% in

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button mushroom (Wang et al., 2016; Zhou et al., 2016; Li et al., 2019; Shi et al., 2020). Deformation of basidiome, appearance of white cottony growth of the mycelium, exudation of brown coloured liquid and appearance of flocculent mycelia on the substrate are the common symptoms of wet bubble disease (Fletcher et al., 1995; Fu et al., 2016). Since the mycoparasite directly affects the formation of caps the yield losses to the mushroom industry due to this disease is very high. In general vegetative mycelium of *A. bisporus* was not affected by *H. perniciosus*, whereas the morphogenesis of its fruiting bodies was severely affected (Zhang et al., 2017). This fungus is also known to infect *Pleurotus citrinopileatus* (Zhang et al., 2017).

The contaminated casing soil has been reported as the main source of inoculum of the fungus (Fletcher and Gaze, 2008). Pathogen also spreads through air, contaminated tools and operators (Gea et al., 2021). WBD can be prevented by good hygiene, sanitation and application of fungicides without affecting the growth of mushrooms (Gea et al., 2021). Fungicides such as Benomyl (Bollen and Fuchs, 1970), iprodione and prochloraz-Mn (Gea et al., 2010; Potocnik et al., 2010), thiabendazole, fludioxonil, diniconazole, fenbuconazole and imazalil (Shi et al., 2020) were found to be effective in controlling *H. perniciosus*. The use of natural products for the control of foodborne pathogens has been considered a safe and environmentally friendly approach. The inhibitory effect of essential oils (EOs) of a few plant species including *Lippia citriodora* and *Thymus vulgaris* (Regnier and Combrinck, 2010), *Crithmum maritimum* (Glamoclija et al., 2009), *Origanum majorana* (Tanovic et al., 2009) against *H. perniciosus* was reported. These essential oils are aromatic and volatile liquids extracted from plants through steam distillation process. In the course of screening of Omani traditional medicinal plants for in vitro antifungal activity, we observed that the essential oil of *Zataria multiflora* Boiss. (Lamiaceae) completely inhibited the growth of *Aspergillus flavus*, a common contaminant and major aflatoxin producer in a wide range of agricultural commodities (unpublished data). Considering the best knowledge of the authors, the antifungal activity of *Z. multiflora* essential oil (ZEO) against *H. perniciosus* has not been studied. In this study, the inhibitory effect of ZEO and its major constituent linalool on *H. perniciosus* was determined.

Materials and Methods

Plant Material

Zataria multiflora Boiss. (Lamiaceae) (Accession number 201100114) plants were obtained from Oman Botanic Garden, Muscat, Sultanate of Oman.

Mycoparasite isolation

Agaricus bisporus fruiting bodies showing symptoms of wet bubble disease were collected from the mushroom cultivation demonstration trials at the Department of Plant Sciences, Sultan Qaboos University.

A small piece of tissue was cut with a sterile surgical scalpel from the infected mushroom and surface sterilized with 1% sodium hypochlorite for 1 min. The tissue was then rinsed in sterile distilled water (SDW) and placed on potato dextrose agar (PDA) (Oxoid Ltd., UK) medium in a petri dish. The plate was kept at 27°C for 3-5 days. A pure culture of the fungus was obtained by hyphal tip culture method.

Molecular Identification

Mycelia were collected from 7-day-old PDA culture plate. DNA was extracted from approximately 80 mg of mycelium according to Lee and Taylor (1990). The DNA quantity and quality was checked using a NanoDrop 2000 spectrophotometers (Thermo Scientific, USA). Polymerase chain reaction (PCR) was performed in 25 µl reaction volume using ITS4 and ITS5 primers (White et al., 1990) with PuReTaq Ready-to-Go PCR bead (GE Healthcare, UK) according to Al-Rashdi et al. (2020). An aliquot (5 µl) of the PCR product was analyzed by 1.2% (w/v) agarose gel electrophoresis and amplified product (~600 bp) was sequenced at Macrogen Inc. (Seoul, Korea). The DNA sequence from this study was compared with reference sequences of fungal species in the NCBI database (www.ncbi.nlm.nih.gov) using BLAST search.

Testing Pathogenicity

Polyethylene bags (25×30 cm) were filled with approximately 1 kg of Phase III compost spawned with *A. bisporus* (obtained from Gulf Mushroom Products Company, Barka, Oman). Casing soil (obtained from Gulf Mushroom Products Company) was applied as a layer (30-40 mm) on the surface. Ten ml of spore suspension (1×10^4 spores/ml) of *H. perniciosus* was mixed with the casing soil. The bags were kept at 25°C for 2-3 weeks and checked for the disease development.

Extraction of Essential Oil

One kg of *Z. multiflora* leaves and stem was transferred to a glass reactor followed by the addition of 1.5 L of distilled water. The essential oil (EO) was extracted using ETHOS X microwave extraction system (Milestone Inc., Shelton, CT, USA). The essential oil was stored in small amber glass vials in a freezer at -20°C.

Testing Antifungal Activity

The antifungal activity of the EO of *Z. multiflora* against *H. perniciosus* was tested using agar diffusion assay (Al-Maawali et al., 2021). Briefly, sterile filter paper discs (6-mm diameter) were placed on the surface of PDA medium in Petri dishes and 10 µl of EO (50% and 100%) was

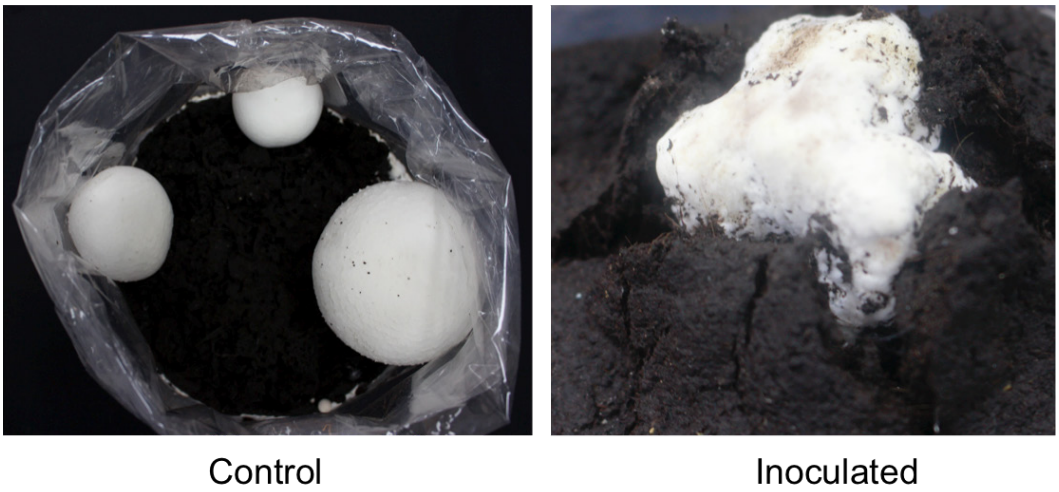

Figure 1. *Agaricus bisporus* showing symptoms of wet bubble disease, 14 days after artificial inoculation with *Hypomyces perniciosus*.

applied on the discs. Then a 7-mm mycelial disc obtained from a 7-day-old *H. perniciosus* culture was placed in the center of the Petri dish and incubated at 27°C for 5-7 days. The formation of inhibition zone around the filter paper discs was observed. The assay was conducted in triplicate.

Minimal Inhibitory Concentration (MIC)

The MIC of ZEO was determined according to Kiran et al. (2016). Briefly, calculated quantity of the ZEO was diluted in 0.5 ml of 5% Tween-20 and mixed with 19.5 ml of molten PDA and poured into sterile Petri dishes to obtain the final concentrations of 0.1-1.0 µl/ml. In the center of the Petri dish, a 7-mm mycelial disc obtained from a 7-day-old *H. perniciosus* culture was placed. The Petri plates were incubated at 27°C for 5-7 days and observed for the mycelial growth inhibition and MIC (the lowest concentration that causes no visible growth of the fungus). Petri plate containing PDA amended with 0.5 ml of 5% Tween-20 alone was used as negative control. Four replications were maintained per treatment. The data were analyzed using SAS v8, (SAS Institute, NC, USA) and the values were compared by "Duncan's multiple range test, DMRT" at $P \leq 0.05$. Purified linalool (≥ 95.0%) (Fluka, Sigma-Aldrich Chemie GmbH, Switzerland) was diluted in ethanol and mixed with PDA medium to obtain final concentrations of 0.25% and 0.125% and evaluated for its antifungal activity against *H. perniciosus* as described above.

Analysis of Essential Oil

Shimadzu GC-2010 Plus gas chromatography machine fitted with Rtx-5MS capillary column (30 m × 0.25 mm; 0.25 µm), coupled to a GCMS-QP2010 ULTRA MS was used for analysis of *Z. multiflora* essential oil (Hanif et al., 2011). The data were obtained by collecting the full-scan mass spectra with the scan range of 40-550 amu.

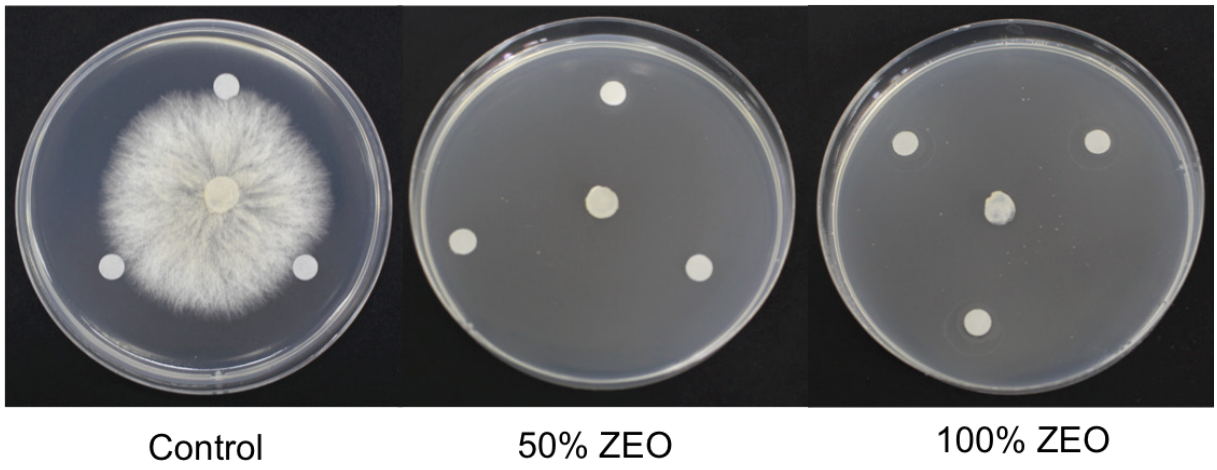

Figure 2. Inhibition of mycelial growth of *Hypomyces perniciosus* by essential oil of *Zataria multiflora*.

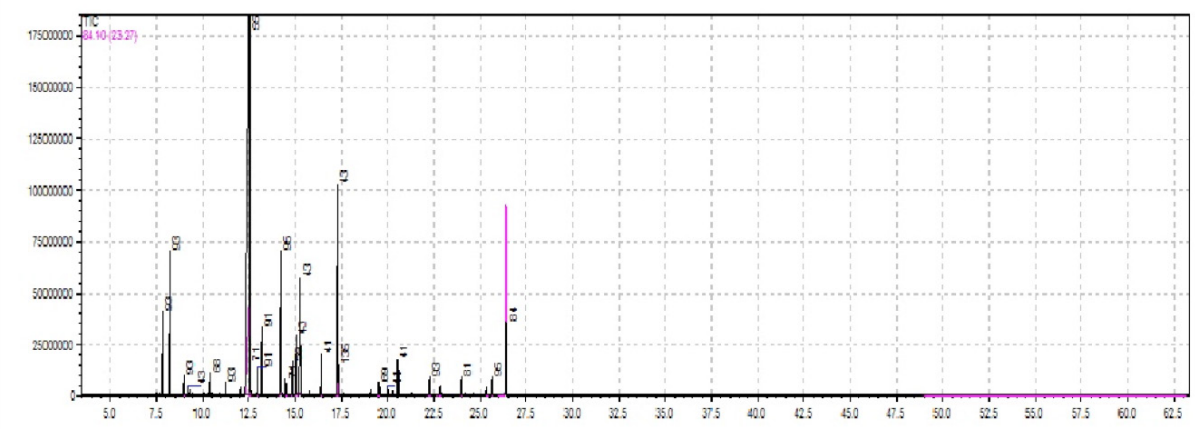

Figure 3. Gas chromatogram of *Zataria multiflora* essential oil.

The total run time was 63.5 min. The National Institute of Standards and Technology (NIST) v.2.3 and Wiley 9th edition mass spectrum libraries were used for identification of the compounds.

Results and Discussion

Hypomyces perniciosus was isolated from *Agaricus bisporus* showing the symptoms of WBD and pure culture was obtained. The fungus was identified based on the analysis of PCR-amplified ITS regions. The fungal isolate showed 100% identity to sequences of over 30 strains of *Hypomyces perniciosus* stored in the GenBank database. The sequence of the fungus was deposited in the GenBank under the accession number MZ149255. The pathogenicity of the fungus was confirmed by artificial inoculation of casing soil with the spore suspension of *H. perniciosus*. The mycoparasite induced typical symptoms of WBD on the emerging mushrooms approximately 14 days after inoculation (Figure 1).

The EO extracted from *Z. multiflora* (ZEO) aerial parts had a very strong antifungal activity and completely stopped the growth of *H. perniciosus* at the tested concentrations (50% and 100%) in agar diffusion test, whereas the control recorded 4.8 cm diameter growth after 7 days of incubation (Figure 2). The MIC value of ZEO was 0.4 µl/ml as assessed by poisoned food technique (Table 1). Antifungal activities of EOs of a few plants against *M. perniciosa* have been reported in previous studies (Regnier and Combrinck, 2010; Glamoclija et al., 2009). Glamoclija et al. (2009) demonstrated that the EO of *Critmum maritimum* and its major components viz., limonene and α-pinene effectively inhibited the growth of *M. perniciosa*. Preventive application of EOs of *Lippia citriodora* and *Thymus vulgaris* at a concentration of 40 µl/L was demonstrated to control the development of WBD in a simulated commercial trial (Regnier and Combrinck, 2010).

Different modes of action of EOs on fungi have been reported. Cox et al. (2000) while studying the mode of action of essential oil on yeast (*Candida albicans*) and bacteria (*Staphylococcus aureus* and *Escherichia coli*) reported that the EO of *Melaleuca alternifolia* suppressed the respiration and augmented the permeability of plasma membranes of yeast and cytoplasmic membranes of bacteria. Tian et al. (2012) demonstrated that EO of *Anethum graveolens* induced morphological alterations in *Aspergillus flavus* cells and reduced the ergosterol content and activities of ATPase and dehydrogenase and increased the mitochondrial membrane potential and production of reactive oxygen species. Chaudhari et al. (2020) reported that the EO of *Pimenta dioica* completely inhibited the growth of *A. flavus* and the production of aflatoxin B1. The oil caused reduction of methylglyoxal, a signaling molecule that can trigger aflatoxin biosynthesis gene *aflR*, increased ions leakage from the cells and ergosterol content of fungal plasma membrane, suggesting plasma membrane of fungi as the action site. The inhibition of mycelial growth of *M. perniciosa* by ZEO as observed in this study might be due to the presence of antifungal compounds in ZEO (Nazzaro et al., 2017).

Phytochemical profile of ZEO by GC-MS analysis in

Table 1. Minimum inhibitory concentration of *Zataria multiflora* essential oil against *Hypomyces perniciosus.*

Concentration of ZEO (µl/ml)	Diameter of mycelial growth (cm)
0	5.3 a
0.1	2.5 b
0.2	1.8 c
0.3	1.2 d
0.4	0 e
0.5	0 e
0.6	0 e
0.7	0 e
0.8	0 e
0.9	0 e
1	0 e

Values followed by the same alphabetical letter, do not differ significantly (P = 0.05; Duncan's multiple range test).

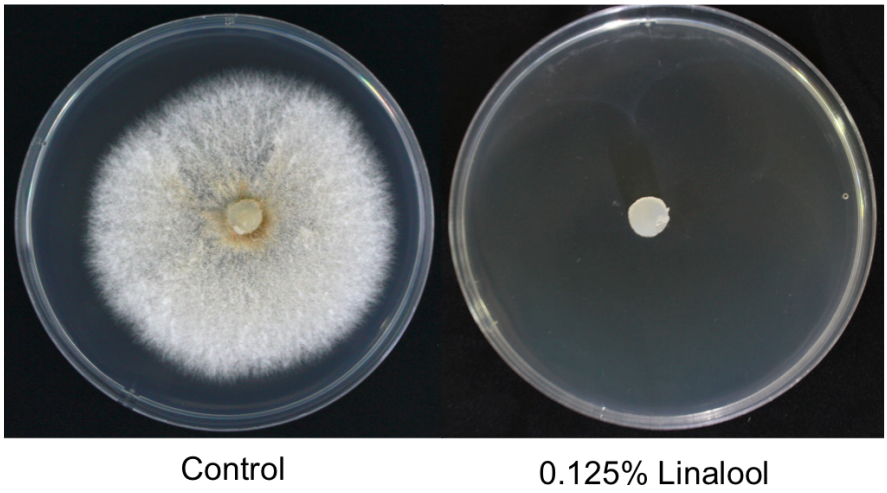

Figure 4. Inhibition of mycelial growth of *Hypomyces perniciosus* by linalool.

this study identified 23 compounds (Figure 3). Among these, linalool (20.3%) and bornyl acetate (15.5%) were the major components (Table 2). Antimicrobial effect of linalool, an acyclic monoterpene (Park et al., 2012) has been reported earlier (Peana et al., 2002). Bornyl acetate has been identified as the major component of EO of *Tetraclinis articulata* that showed antibacterial activities (Rabib et al., 2020). Several reports indicated the chemical composition of EO of *Z. multiflora* (Shafiee and Javidnia, 1997; Moosavy et al. 2008; Mahboubi and Bidgoli, 2010; Raeisi et al., 2016). Carvacrol (71.20%), γ-terpinene (7.34%) and α-pinene (4.26%) were reported as the major components of ZEO (Moosavy et al. 2008). Mahboubi and Bidgoli (2010) reported thymol (38.7%), carvacrol (15.3%) and p-cymene (10.2%) as the major components in ZEO. In another study, Carvacrol (63.2%) and thymol (15.1%) were reported as the main constituents of ZEO (Raeisi et al., 2016). Saleem et al. (2004) reported higher thymol concentration in fresh plant (73.21%) and carvacrol in dry plant (62.87%) tissues. The difference in the composition of ZEO might be due to plant samples collected at varying growth stages, geographical locations, prevailing climatic conditions and habitat (Ruiz-Navajas et al., 2012; Abd-ElGawad et al., 2019). The results of this study also revealed that linalool, one of the major components of ZEO also inhibited the growth of *H. perniciosus* even at a concentration of 0.125% under laboratory conditions (Figure 4). The inhibitory effect of ZEO on *H. perniciosus* in this study could be attributed to the presence of linalool.

Table 2. Chemical composition of essential oil of *Zataria multiflora*.

S.No.	Name of the compound	Rt (min)	Area %	Calculated KI	NIST KI
1	Camphene	7.83	6.195	941	935
2	trans-.beta.-Ocimene	8.24	10.703	956	958
3	.beta.-Pinene	8.99	1.454	984	970
4	3-Octanone	9.23	0.727	993	962
5	.(-)-Limonene	10.41	1.593	1036	1020
6	.gamma.-Terpinene	11.24	0.954	1066	1047
7	Linalool	12.53	20.332	1113	1081
8	1,5,7-Octatrien-3-ol, 3,7-dimethyl-	12.57	2.567	1114	1115
9	2,6-Dimethyl-1,3,5,7-octatetraene, E,E-	12.96	2.169	1129	1134
10	Isoborneol	14.26	10.696	1177	1146
11	Terpinen-4-ol	14.49	1.209	1185	1161
12	.alpha.-Terpineol	14.84	2.518	1198	1172
13	2,6-Dimethyl-3,5,7-octatriene-2-ol, ,E,E-	15.07	4.368	1207	1187
14	Linalyl acetate	16.43	3.043	1260	1236
15	Bornyl acetate	17.31	15.564	1294	1269
16	Thymol	17.37	2.269	1296	1262
17	(-)-.beta.-Elemene	19.53	0.858	1385	1377
18	Caryophyllene	20.00	0.720	1404	1424
19	.gamma.-Elemene	20.54	2.611	1428	1425
20	Humulene	22.28	1.393	1504	1456
21	Germacren D-4-ol	23.98	1.289	1581	1570
22	.alpha.-Cadinol	25.62	1.323	1659	1641
23	Shyobunol	26.43	5.434	1793	1709

Conclusion

Z. multiflora is commonly used as a flavor ingredient in foods and has a wide range of biological and medicinal properties including antibacterial, antiseptic, anesthetic, antioxidant and immunomodulatory activities. This study demonstrated the antifungal activities of ZEO and its major constituent linalool against *H. perniciosus*. The results of this study suggest that ZEO is a safe, environmentally friendly natural product for control of WBD. However, further research is required to investigate the inhibitory effect of ZEO on *A. bisporus*.

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