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Research Article

Potential of new fungicide production from phthalides of *Kelussia odoratissima*

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Abstract: A bioassay-guided fractionation on repeated silica gel column chromatography was used to identify natural antifungal agents in the ethanol extract of *Kelussia odoratissima* leaves. The ethyl acetate fraction of the extract has significant inhibitory activity against *Macrophomina phaseolina* (Tassi) Goid, whereas the water fraction showed no effect. The main compound responsible for antifungal activity was determined by NMR and Mass Spectrometry techniques and identified as (Z)-3-butylidenephthalide. It exhibited the highest mycelial growth inhibition with IC_{50} = 270 mg/L. Additionally, it inhibited the microsclerotia production and dark pigments formation. (Z)-ligustilide was identified as a moderately active compound with 62% inhibition. These findings revealed the crucial role of natural phthalides as sources of bioactive compounds that might be used in natural pesticide discovery.

Keywords: *Macrophomina phaseolina*, (Z)-3-butylidenephthalide, (Z)-ligustilide, natural products, Lead compound

Introduction

Aiming to discover novel agrochemicals without side effects on non-target organisms is an enormous challenge in pesticide chemistry (Dutta, 2015). Recently, efforts have been made to find new and alternative strategies in crop protection, such as using natural products as potential sources of bioactive compounds (Dutta, 2015; Kagale *et al*., 2004; Amadioha, 2000). Increasingly, aromatic plants are becoming more critical in the medical, agricultural, and industrial fields (De Silva, 1997; Al-Samarrai *et al.*, 2012). The chemical compounds with natural sources have almost less adverse effects and lead to protecting crops and the environment from synthetic pesticide hazards (Al-Samarrai *et al.*, 2012). Numerous literatures showed that natural products might have either direct antimicrobial activity (Ansari, 1995; Jakubczyk and Dussart, 2020; Karas *et al.*, 2020: Feknous and Boumendjel, 2022) or induce host plant defense leading to the reduction of disease development (Schneider and Ullrich, 1994; Jamiołkowska, 2020; Rani *et al.*, 2023).

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There is a large number of wildly used aromatic plants belonging to the Apiaceae family, for example, *Kelussia odoratissima* Mozaff, which is an Iranian endemic plant, and it is the only species in *Kelussia* genus (Sajjadi et al., 2009; Mozaffarian, 2003). This aromatic plant, with the local names of "keloss" or "Karafs-e-koohi" (Mozaffarian, 2003) grows in the central Zagros Mountain chain with a size between 20 to 60 cm in normal and can reach to two-meter height (Asgary et al., 2004; Ahmadi *et al.*, 2007; Rabbani *et al.*, 2011). It has medicinal properties against hypertension, inflammation, and cardiovascular diseases and is consumed as a sauce or food additive in some regions of the country (Ahmadi *et al.*, 2007). The plant's essential oil is rich in phthalide structures (Shojaei *et al.*, 2011), and the plant leaf extract was used to synthesize antibacterial silver nanoparticles (Azizi *et al.*, 2017).

Macrophomina phaseolina, a highly damaging plant pathogen causing charcoal rot disease, poses a significant economic threat to numerous plant species due to its broad host range (Lodha and Mawar, 2020). Its capacity to persist in the soil for extended periods presents a continuous risk to agricultural productivity (Vibha, 2016; Lodha and Mawar, 2020).

In this study, we investigated the antifungal effect of *K. odoratissima* plant extract on the plant pathogenic fungus *Macrophomina phaseolina*. Bioassay fractionation and structure elucidation revealed two active metabolites in the Kelussia ethanol extract. These molecules could serve as natural lead compounds for developing new antifungal agents.

Materials and Methods

Plant material

Leaves of *K. odoratissima* were collected from the Koohrang (32°30'N, 50°02'E, 2700m alt.) in Chaharmahal-Bakhtiari province of Iran in June 2018. The voucher specimen was deposited in the herbarium of Medicinal Plants and Drugs Research Institute of Shahid Beheshti University with the number of MPH-1835. The plant materials dried in shade (25 ± 3 °C) and ground into a powder. The dried and ground (50 mesh) plant material was subsequently utilized for extraction.

Fungal isolate

We used *Macrophomina phaseolina* Mph44, which was isolated and identified previously by Mahdizadeh et al. (2011). This strain was among the most virulent and was initially isolated from melons with charcoal rot disease in Khorasan province (Mahdizadeh *et al.*, 2011). The pure cultures were obtained by hyphal tip technique, maintained on sterile toothpicks at room temperature (Edmunds, 1964), and cultured on PDA at 4 °C for further use.

Preparation of the plant extracts

300 g of dried aerial parts of *K. adoratissima* were macerated with 3 liters of ethanol 96% for 24 hours; then, the ethanol extract (EtE) was concentrated with a vacuum using a rotary evaporator (Heidolph laborota-4001 efficient, Germany). The dried extract was suspended in water (450 ml) and successively partitioned with ethyl acetate (EtOAc) (3 × 150 ml) using a separating funnel. The ethyl acetate (EaF) and water fractions (WtF) and fractions were also dried with a rotary evaporator. The dried material was kept in the dark at 4 °C before use.

Preparation of stock solutions and poisoned food technique

A $5 \times 10^{+4}$ mg/L stock solution of the plant extract was prepared by dissolving 500 mg of the dried plant within 1.5 ml of Dimethyl sulfoxide (DMSO, Merck, Germany) and making up the volume 10 ml with sterile distilled water. The final concentration was prepared based on pre-test results of the extract by dissolving a specific volume of the stock solution with 20–25 ml of cooled, molten (45 to 50 °C) and autoclaved potato dextrose agar (Merck, Germany), and allowed to solidify at room temperature in Petri plates. In the poisoned food technique (Schmitz, 1930), a mycelial disc 6mm in diameter, cut out from the periphery of 3 to 5-day old cultures of fungus, was aseptically inoculated onto the

center of the Petri plate containing the plant extract. The inoculated plates were incubated at 25 °C and colony diameter was measured and recorded after 3 to 4 days. The percentage of mycelial growth inhibition is given by the equation (Eq.1) (Pandey *et al.*, 1982).

Mycelial growth inhibitGI) % = [C-T/C]×100 (Eq.1)

C is the diameter of the fungal colony (mean) in negative control (solvent without extract), and T is the diameter of the fungal colony (mean) in the presence of plant extract. Thiophanate-methyl 70% WP ($C_{12}H_{14}N_4O_4S_2$) was used as a positive control based on its good effect against a wide range of fungal pathogens (Siddiqui *et al.,* 1999). Microsclerotia inhibition was observed by microscopic examination using the previously reported method (Xiong *et al.,* 2016) with modifications. After measuring the mycelial growth inhibition, an agar plug (1 cm2) was taken from each plate. Then, it was examined using an Olympus DP72 digital camera attached to an Olympus BX51 microscope for microsclerotia counting.

Bioassay-guided fractionation of plant extracts

To purify the active compounds, the extract was separated using bioassay-guided fractionation (Wedge et al., 2009), so EaF was separated by silica gel column chromatography 60 (0.063–0.200 mm, Merck, Germany), eluted with *n*-hexane/EtOAc (9:1, 8:2, 7:3, 6:4, 5:5, 3:7, 0:10 v/v). Fifteen fractions (MB1 to MB15) were collected and monitored by TLC (silica gel 60 F254; Merck; *n*-hexane/EtOAc 7:3 and 6:4; detection with methanolic sulphuric acid 3% reagent). Fractions were dried with a rotary evaporator, then stock solution having 15000 mg/L was prepared by dissolving specific amounts of the dried fractions with sterile distilled water having no more than 15% of DMSO, then added to PDA medium to get a final concentration of 750 mg/L. The MB4 fraction was separated by column chromatography in a small glass column with silica gel 60 (0.063–0.200 mm, Merck), eluted with *n*-hexane/EtOAc (15:1, 12:1, 8:1). Four fractions were collected, monitored by TLC (*n*-hexane/ EtOAc 12:1 and 15:1; detection with methanolic sulphuric acid 3% reagent). The percentage of mycelial growth and microsclerotia inhibition for all fractions were evaluated using the poisoned food technique.

Structural elucidation of bioactive compounds

The structures of the isolated, active compounds were elucidated by ^{1}HNMR and electron ionization mass spectrometry in GCMS. The mass data were confirmed with the library and also compared with the retention index. The condition for the GCMS and NMR were presented in supporting information.

Statistical analysis

All statistical analyses were performed using SPSS 16.0 software (SPSS Inc., 2007). Means and standard errors were computed for variables. Mycelial growth and microsclerotia inhibition mean comparison has been done by LSD test using completely random design with three replicates.

Results

Fungicidal activity of the ethanol extract (EtE)

The fungicidal activity of the ethanol extract (EtE) from *K. odoratissima* against *M. phaseolina* was evaluated using a poisoned food technique. Results indicated a 77 ± 2.3% inhibition of mycelial growth at a concentration of 2.5 g/L, along with suppression of microsclerotia production. A bioassay-guided fractionation technique was used to purify the main antifungal compounds in the *K. odoratissima* extract. EaF and WtF fractions of EtE were tested against fungus. WtF displayed no antifungal activity, while EaF had a significant activity against the mycelia growth in a concentration-dependent manner (Table 1). EaF exhibited distinct activity against the microsclerotia production and dark pigments at any of the concentrations tested. It showed 91 ± 2.64% of mycelial growth inhibition at 2.5 g/L (Fig. 1, Table 1).

Table 1 Mycelial growth inhibition of ethanol extract (EtE) ethyl acetate (EaF) and water fractions (WtF) against *Macrophomina phaseolina*.

Sample name	Concentration (g/L)	MGI (%)
EtE	2.5	77 ± 2.30
EaF	2.5	91 ± 2.64
EaF	2.0	72 ± 1.52
EaF	1.0	45 ± 0.57
EaF	0.5	34 ± 2.08
WaF	2.5	0
NC	-	0
PC	0.15	100

NC is the negative control (DMSO with water), and PC is the positive control (Thiophanate-methyl).
Inhibition was measured experimentally (mean ± SE), replicate number $n = 3$.

Bioassay-guided fractionation of EaF and structure structural elucidation of the isolated compounds

Column chromatography was used to further isolate the individual antifungal compounds from EaF. Fifteen fractions (MB1 to MB15) were collected and tested against the fungus. They exhibited varying degrees of mycelial growth inhibition (0% to 87%) at 0.75 g/L (Fig. 2). MB4 and MB5 fractions displayed full inhibition of microsclerotia production. MB4 (most active fraction) was subjected to another column chromatography, and four main fractions (F1–F4) were collected. The first fraction (F1) had the highest mycelial growth inhibition by 85% ± 2.9 (IC_{50} = 270 mg/L) with full microsclerotia and dark pigmentation inhibition. The second fraction exhibited moderate activity with 62% ± 3 at 0.75 g/L. F1 had a constant antifungal activity against the microsclerotia formation even after seven days, while in the case of F2, the microsclerotia appeared after three days of treatment (Figs. 3, 4).

Both F1 and F2 fractions showed one pure spot in TLC, and they were introduced for structure elucidation in ^{1}HNMR and Mass spectrometry. Results showed that F1 was (Z)-3-butylidenephthalide, while F2 was (Z)-ligustilide (Figs. 5, S1–S4).

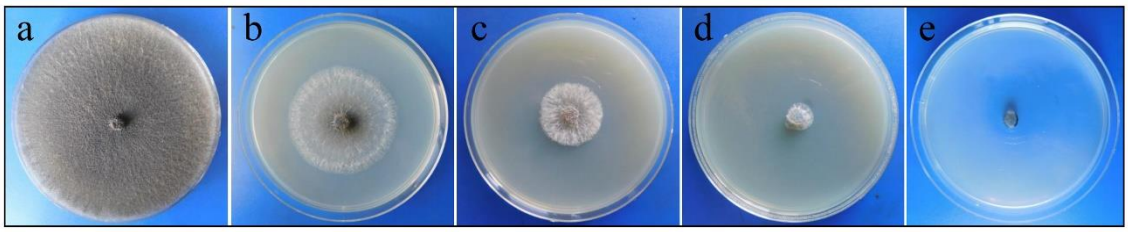

Figure 1 The inhibition of mycelial growth and microsclerotia using ethyl acetate (EaF) with three concentrations. (a) Negative control treatment (DMSO with water). (b) 1 g/L of EaF. (c) The concentration of 2 g/L of EaF. (d) The concentration of 2.5 g/L of EaF. (e) Positive control thiophanate-methyl 70 % WP (0.15 g/L).

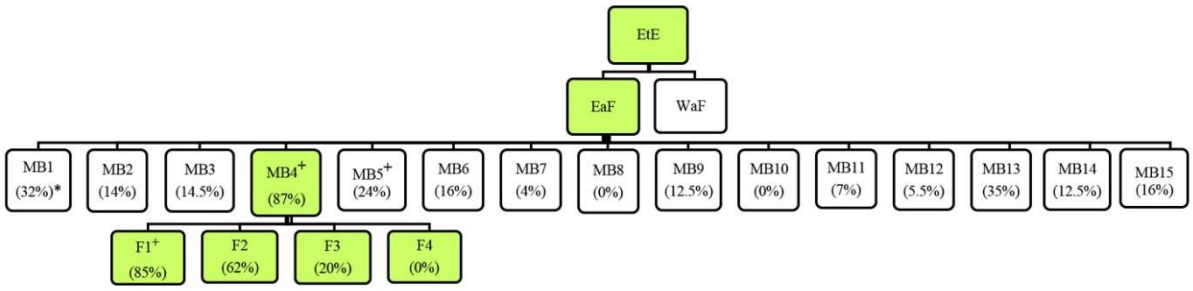

Figure 2 Schematic for bioassay-guided fractionation to purify the antifungal compounds sequentially against *Macrophomina phaseolina*. (*) percentage of mycelial growth inhibition (+) represents full inhibition of microsclerotia production.

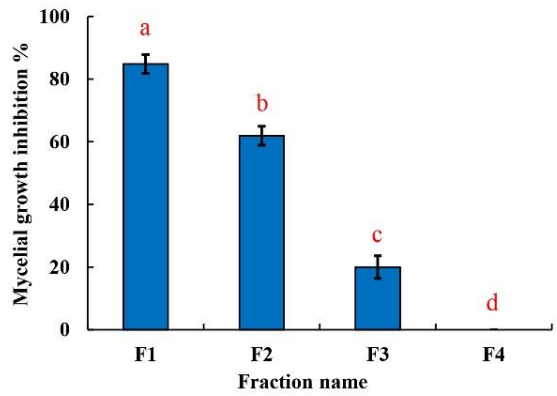

Figure 3 Means comparison of *Macrophomina phaseolina* mycelial growth inhibition by last four fractions (F1, F2, F3, F4) of *Kelussia odoratissima* using completely random design with three replicates (at 0.75 g/L). Different letters indicate a significant difference (LSD test, $p < 0.05$).

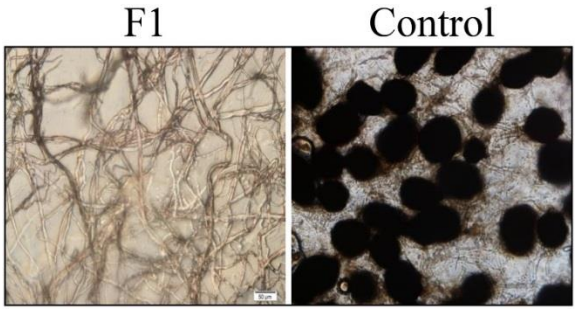

Figure 4 Microsclerotia formation after 7 days in F1 (0.75 g/L), and control (DMSO with water) plates. F1 displayed constant inhibition of microsclerotia and dark pigments.

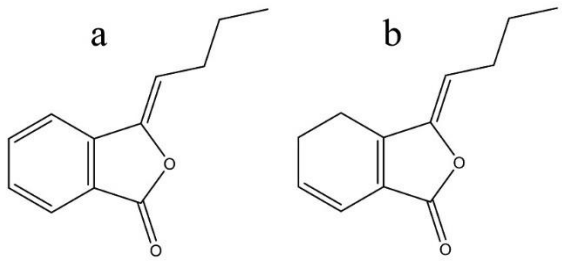

Figure 5 Structure of antifungal compounds isolated from *Kelussia odoratissima* ethanol extract. (a) (Z)-3-butylidenephthalide, (b) (Z)-ligustilide.

Discussion

Several studies reported the bioactivity of phthalide derivatives (Tsukamoto et al., 2005; Meepagala et al., 2005); for example, the ethanol extract of *K. odoratissima* leaves had a significant inhibitory effect against *Enterobacter aerogenes* strains (Sureshjani *et al.*, 2014). Z-ligustilide showed moderate activity against *Botrytis cinerea* and had an anti-mosquito activity. Butylidene phthalide isolated from *Ligusticum chuanxiong* Hort. showed potent vasorelaxant effects (Chan et al., 2006); also, it exhibited acaricidal activity against *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* (Kwon and Ahn, 2002). 3-butylidenephthalide exhibited *in silico* activity toward trihydroxynaphthalene reductase and *Drosophila melanogaster* acetylcholinesterase (Abbod *et al.*, 2020).

(Z)-3-butylidenephthalide and (Z)-ligustilide belong to the 3-substituted not alkaloid phthalides group (Lin *et al.*, 2005). The commercial fungicide 4,5,6,7-tetrachlorophthalide inhibits the fungal melanin biosynthesis pathway in rice blast disease (Chida *et al.*, 1982; Yamaguchi, 1982; Liao *et al.*, 2001). It was observed that the microsclerotia and dark pigments were strongly inhibited by the (Z)-3-butylidenephthalide, which implies that this compound may inhibit the melanin biosynthesis pathway within the fungus (Liao *et al.*, 2001; Andersson *et al.*, 1996).

Conclusions

In our study, we report the antifungal activity of natural (Z)-3-butylidenephthalide isolated from *Kelussia odoratissima* against *M. phaseolina*. (Z)-3-butylidenephthalide was more effective than (Z)-ligustilide; also, it significantly inhibited the microsclerotia and dark pigments within the fungus. These results showed the crucial role of the *K. odoratissima* as a botanical source of bioactive compounds that may be used in the pesticide industry and drug design. The authors suggest (Z)-3-butylidenephthalide as a natural lead compound for fungicide development.

Further assays, such as bioinformatics and *in vitro* tests, are necessary to identify the potential biological targets of these molecules in the pathogen. Developing appropriate formulations of these molecules may be necessary to boost their fungicidal effectiveness.

Supporting material

^{1}HNMR and EI-MS data and instuments for the isolated compounds, Supporting figures (S1,S2,S3,S4) are available as supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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پتانسیل تولید قارچکش جدید از فتالیدهای کرفس کوهی

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چکیده: یک جداسازی مبتنی زیست‌سنجی بر روی کروماتوگرافی ستونی ژل سیلیکا برای شناسایی عوامل ضدقارچی طبیعی در عصاره اتانولی برگ‌های کرفس کوهی *Kelussia odoratissima* استفاده شد. بخش استات‌اتیل عصاره فعالیت مهاری قابل‌توجهی علیه قارچ بیمارگر *Macrophomina phaseolina* نشان داد، درحالی‌که بخش آبی عصاره هیچ اثری نداشت. ترکیب اصلی مسئول فعالیت ضدقارچی با استفاده از تکنیک‌های NMR و طیف‌سنجی جرمی تعیین شد و به‌عنوان (Z)-۳-بوتیلیدن‌فتالید شناسایی شد. این ترکیب بالاترین مهار رشد میسلیوم را با IC50=270 mg/L نشان داد؛ علاوه‌براین، تولید میکروسکلروتیا و تشکیل رنگدانه‌های تیره را مهار کرد. (Z)-لیگوستیلید به‌عنوان یک ترکیب با فعالیت متوسط با ۶۲% مهار شناسایی شد. این یافته‌ها نقش حیاتی فتالیدهای طبیعی به‌عنوان منابع ترکیبات زیست‌فعال که ممکن است در کشف آفتکش‌های طبیعی استفاده شوند را نشان دادند.

واژگان کلیدی: *Macrophomina phaseolina*، (Z)-۳-بوتیلیدن‌فتالید، (Z)-لیگوستیلید، محصولات طبیعی، ترکیب رهبر