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Full Length Research Paper

Antifungal and biochemical effects of pseudoguaianolide sesquiterpenes isolated from *Ambrosia maritima* L.

S. A. M. Abdelgaleil[1]*, M. E. I. Badawy[1], T. Suganuma[2] and K. Kitahara[2]

[1]Department of Pesticide Chemistry and Technology, Faculty of Agriculture, 21545-El-Shatby, Alexandria University, Alexandria, Egypt.
[2]Department of Biochemistry and Applied Biosciences, Faculty of Agriculture, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan.

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Five pseudoguaianolide sesquiterpenes, namely neoambrosin, damsinic acid, damsin, ambrosin and hymenin isolated from the aerial parts of *Ambrosia maritima*, were tested for their antifungal activity against the most economic plant pathogenic fungi *Botrytis cinerea* and *Fusarium oxysporum* which cause grey mold and root rot diseases, respectively. In mycelial radial growth inhibition assay, neoambrosin and damsin were the most potent, while hymenin and damsinic acid were the lowest effective among the tested compounds. *F. oxysporum* was more sensitive for inhibition than *B. cinerea*. On the other hand, the sesquiterpenes caused significant reduction in spore germination of both fungi at 50, 100 and 200 mg/L compared with the control. Complete inhibition was observed when the spores treated with damsinic acid and ambrosin at 200 mg/L. *In vivo* activities of polyphenol oxidase, polygalacturonase and pectin-lyase in both fungi that treated with 0.5 and 1 fold of the EC_{50} value were also carried out in order to investigate the biochemical influences of the tested compounds. The compounds showed significant inhibitory effects on the enzyme activities compared with the control however; the activity was not consistently related to the *in vitro* inhibitory effects.

Key words: Sesquiterpenes, *Ambrosia maritime,* antifungal activity, *Botrytis cinerea, Fusarium oxysporum,* biochemical effects.

INTRODUCTION

Nowadays there is a clear tendency towards the utilization of natural products as safe alternatives to hazardous pesticides for pest and plant disease control with negligible risk to human health and the environment. Because of continuing development of microbial resistance in medicine and agriculture, introducing new antimicrobial substances is an urgent research objective. Therefore, the search and use of phytochemicals as natural antimicrobial agents commonly called ‘‘biocides’’ is gaining popularity worldwide. The phytochemicals provide unlimited opportunities for novel antimicrobial agents with different modes of action than existing antimicrobial agents and consequently, lack cross-resistance to chemicals currently used (Kirst, 1992; McChesney, 1993).

Sesquiterpenes are plant origin C-15 terpenoids which occur as hydrocarbons or in oxygenated forms such as alcohols, ketones, aldehydes, acids or lactones in nature. They are mainly found in several genera of Asteraceae, but also in Umbelliferae, Acanthaceae, Amaranthaceae, Apiaceae and Magnoliaceae. Over 5000 sesquiterpene have been described so far (Fraga, 1999, 2000).

These compounds are known for their different biological activities, such as anti-inflammatory, phytotoxic, antimicrobial, antiprotozoal, and antitumoral properties (Galindo et al., 1999; Neerman, 2003; Picman, 1986).

Ambrosia maritima L. (Asteraceae) is a widely distributed weed in southern parts of Egypt, Sudan, Senegal, and neighboring countries. In Egypt, it is a popular medicinal plant for the treatment of renal colic and calculi. It acts also as antispasmodic, diuretic and

*Corresponding author. E-mail: samir1969us@yahoo.com. Fax: +20 3 592 0067.

useful in bronchial asthma, spasms and frequent urination (Ghazanfar, 1994). This plant showed promising potential as a source of molluscicidal agent (El-Sawy et al., 1984).

The antifungal properties of some sesquiterpenes isolated from different plants have been previously reported. For example, sesquiterpenes, sesquiterpene lactones and eudesmanolide sesquiterpenes isolated from *Centaurea thessala*, *Centaurea attica*, *Centaurea deusta* and *Bazzania trilobata* were described to possess antifungal activities against phytopathogenic fungi (Skaltsa et al., 2000; Karioti et al., 2002; Scher et al., 2004). Likewise, Wedge et al. (2000) evaluated the antifungal activity of 36 natural and synthetic sesquiterpene lactones with guaianolide, *trans, trans*-germacranolide, *cis, cis*-germacranolide, melampolide, and eudesmanolide carbon skeletons against the phytopathogenic fungi *Colletotrichum acutatum*, *C. fragariae*, *C. gloeosporioides*, *F. oxysporum*, *B. cinerea*, and *Phomopsis* sp. The highly oxygenated guaianolide sesquiterpenes isolated from *Ajania fruticulosa* revealed inhibitory effect against human pathogenic fungus *Candida albicans* (Meng et al., 2001). In our previous study, the sesquiterpene lactones isolated from the stem bark of *Magnolia grandiflora* showed potent antifungal activity against phytopathogenic fungi of *Alternaria alternata*, *Helminthosporium* spp, *Nigrospora* spp, *F. oxysporum*, *F. culmorium* and *Rhizocotonia solani* (Ahmed and Abdelgaleil, 2005).

However, few studies have been reported on the bioactivities of sesquiterpenes isolated from *A. maritima*. For example, the sesquiterpenes damsin, ambrosin and tribromoambrosin were found to have toxic effect against the fresh water snails *Biomphalaria alexandrina* and *Bulinus truncates* (Shoeb and El-Emam, 1976). Our recent data indicated the insecticidal, herbicidal and molluscicidal activities of some sesquiterpenes isolated from *A. maritima* (Abdelgaleil, 2010). In addition, the sesquiterpenes isolated from this plant possessed cytotoxic effect on Chinese hamster lung cells (V-79) (Nagaya et al., 1994).

Up to date, no studies are available on the antifungal properties of sesquiterpenes isolated from *A. maritima*. Therefore, this study was aimed to evaluate the antifungal activity of sesquiterpenes (1) neoambrosin, (2) damsinic acid, (3) damsin (4) ambrosin and (5) hymenin against phytopathogenic fungi *B. cinerea* and *F. oxysporum*. We also studied the *in vivo* effects of these sesquiterpenes on polyphenol oxidase, polygalacturonase and pectin-lyase enzymes to investigate their biochemical influences.

MATERIALS AND METHODS

Instruments

Nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$ on a JEOL FX-600 spectrometer operating at 600 MHz for ^1H and 150 MHz for ^{13}C. Mass spectra (EIS-MS) were measured on QSTAR-XL LC/MS.

Test microorganisms

Fungi of *B. cinerea* (Family: Moniliaceae; Class: Deuteromycetes) and *F. oxysporum* (Family: Tuberculariaceae; Class: Deuteromycetes) those cause the grey mold root rot diseases, respectively were provided by Microbiology Laboratory, Department of Plant Pathology, Faculty of Agriculture, Alexandria University, Egypt. The fungi were maintained during the course of the experiments on potato dextrose agar (PDA) medium at 25°C.

Extraction and isolation of sesquiterpenes from *A. maritima*

The pseudoguaianolide sesquiterpenes of (1) neoambrosin, (2) damsinic acid, (3) damsin (4) ambrosin and (5) hymenin were isolated from chloroform extract of *A. maritima* as previously described by Abdelgaleil (2010). The chemical structure (Figure 1) of these sesquiterpenes (1–5) was confirmed on the basis of their spectroscopic data of ultraviolet (UV), infrared (IR), nuclear magnetic resonance (NMR), and mass spectrometry (MS) (Iskander et al., 1988; Nagaya et al., 1994).

Neoambrosin

Colourless needels; ^1H NMR ($CDCl_3$): δ 5.97 (1H, t, *J* = 2.3 Hz, H-2), 3.16 (1H, dd, *J* = 2.3 and 22.9 Hz, H-3a), 2.80 (1H, dd, *J* = 2.3 and 22.5 Hz, H-3b), 4.43 (1H, d, *J* = 8.7 Hz, H-6), 3.40 (1H, m, H-7), 2.00~2.12 (2H, m, H-8 and H-9), 1.71~1.81 (2H, m, H-8 and H-9), 2.89~2.93 (1H, m, H-10), 6.25 (1H, d, *J* = 3.7 H-13a), 5.54 (1H, d, *J* = 3.2 Hz, H-13b), 1.17 (3H, d, *J* = 7.0 Hz, H-14), 1.18 (3H, s, H-15); ^{13}C NMR ($CDCl_3$): δ 148.9 (s, C-1), 124.1 (d, C-2), 39.7 (t, C-3), 213.8 (s, C-4), 58.5 (s, C-5), 79.6 (d, C-6), 43.2 (d, C-7), 23.6 (t, C-8), 29.9 (t, C-9), 38.5 (d, C-10), 138.5 (s, C-11), 169.7 (s, C-12), 119.7 (t, C-13), 20.9 (q, C-14), 14.6 (q, C-15); $C_{15}H_{18}O_3$; ESIMS (positive ion mode) *m/z* (rel. int.): 247.1720 (22) $[M+H]^+$ (calculated for $C_{15}H_{19}O_3$ 247.1329), 229.1585 (29), 211.1473 (24), 201.1584 (100), 183.1481 (33), 173.1148 (61), 159.1098 (69).

Damsinic acid

Colourless needels; ^1H NMR ($CDCl_3$): δ 2.19 (1H, m, H-1), 1.57~1.63 (1H, m, H-2a), 1.65 (1H, m, H-2b), 2.26 (1H, m, H-3a), 2.07 (1H, m, H-3b), 2.16 (1H, dd, *J* = 7.7 and 17.4 Hz, H-6a), 2.46 (1H, dd, *J* = 8.3 and 19.7 Hz, H-6b), 2.73 (1H, m, H-7), 1.78 (1H, m, H-8a), 1.94 (1H, m, H-8b), 1.61~1.67 (2H, m, H-9a and H-9b), 1.87 (1H, m, H-10), 6.24 (1H, s, H-13a), 5.63 (1H, s, H-13b), 1.05 (3H, d, *J* = 7.3 Hz, H-14), 1.05 (3H, s, H-15); ^{13}C NMR ($CDCl_3$): δ 45.8 (d, C-1), 22.5 (t, C-2), 35.8 (t, C-3), 221.9 (s, C-4), 51.0 (s, C-5), 37.6 (t, C-6), 35.9 (d, C-7), 30.9 (t, C-8), 33.7 (t, C-9), 34.7 (d, C-10), 146.3 (s, C-11), 172.1 (s, C-12), 124.5 (t, C-13), 17.3 (q, C-14), 21.2 (q, C-15); $C_{15}H_{22}O_3$; ESIMS (positive ion mode) *m/z* (rel. int.): 251.1997 (7) $[M+H]^+$ (calculated for $C_{15}H_{23}O_3$ 251.1854), 233.1877 (90), 215.1761 (44), 187.1769 (100), 161.1578 (43), 145.1251 (49), 105.0873 (55).

Damsin

Colourless needels; ^1H NMR ($CDCl_3$): δ 2.28 (1H, dd, *J* = 9.6 and 11.0, H-1), 1.60~2.13 (7H, m, 2H-2, 2H-8, 2H-9 and 1H-10), 2.45 (1H, ddd, *J* = 1.9, 8.7 and 17.8, H-3a), 2.23 (1H, m, H-3b), 4.55 (1H, d, *J* = 8.7 Hz, H-7), 3.35 (1H, m, H-7), 6.25 (1H, d, *J* = 3.2 Hz, H-13a), 5.57 (1H, d, *J* = 2.7 Hz, H-13b), 1.08 (3H, d, *J* = 7.3 Hz, H-

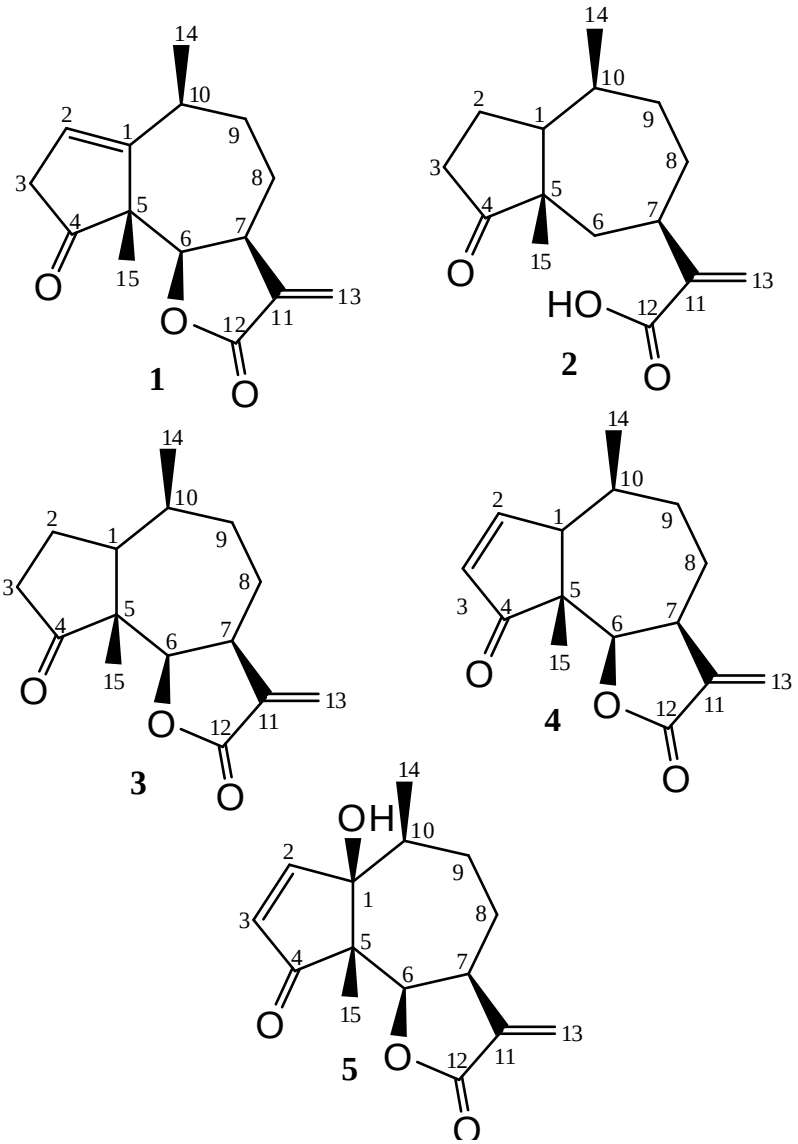

Figure 1. Chemical structure of esquiterpenes: (1) neoambrosin, (2) damsinic acid (3) damsin, (4)ambrosin and (5) hymenin isolated from *A. maritima*.

14), 1.06 (3H, s, H-15); ^{13}C NMR ($CDCl_3$): δ 45.7 (d, C-1), 23.6 (t, C-2), 35.9 (t, C-3), 218.9 (s, C-4), 54.6 (s, C-5), 81.5 (d, C-6), 44.0 (d, C-7), 25.3 (t, C-8), 33.0 (t, C-9), 33.9 (d, C-10), 139.3 (s, C-11), 170.0 (s, C-12), 120.6 (t, C-13), 15.6 (q, C-14), 13.5 (q, C-15); $C_{15}H_{20}O_3$; ESIMS (positive ion mode) *m/z* (rel. int.): 249.1947 (12) $[M+H]^+$ (calculated for $C_{15}H_{21}O_3$ 249.1591), 231.1794 (100), 213.1687 (23), 203.1810 (40), 185.1679 (75).

Ambrosin

Colourless needels; ^{1}H NMR ($CDCl_3$): δ 3.06 (1H, m, H-1), 7.58 (1H, dd, *J* = 1.8 and 5.9 Hz, H-2), 6.13 (1H, dd, *J* = 2.8 and 6.0 Hz, H-3), 4.68 (1H, d, *J* = 8.2 Hz, H-6), 3.50 (1H, m, H-7), 1.90 (1H, m, H-8a), 2.25 (1H, m, H-8b), 1.71 (1H, m, H-9a), 1.99 (1H, m, H-9b), 2.47 (1H, m, H-10), 6.27 (1H, d, *J* = 3.6 H-13a), 5.55 (1H, d, *J* = 3.2 Hz, H-13b), 1.05 (3H, d, *J* = 7.3 Hz, H-14), 1.18 (3H, s, H-15); ^{13}C NMR ($CDCl_3$): δ 47.2 (d, C-1), 163.7 (d, C-2), 130.4 (d, C-3), 210.6 (s, C-4), 55.6 (s, C-5), 79.8 (d, C-6), 44.0 (d, C-7), 24.1 (t, C-8), 29.0 (t, C-9), 33.1 (d, C-10), 137.6 (s, C-11), 170.2 (s, C-12), 119.5 (t, C-13), 17.0 (q, C-14), 16.7 (q, C-15); $C_{15}H_{18}O_3$; ESIMS (positive ion mode) *m/z* (rel. int.): 247.1503 (26) $[M+H]^+$ (calculated for $C_{15}H_{19}O_3$ 247.1334), 229.1576 (100), 211.1464 (35), 201.1576 (96), 183.1468 (42), 173.0913 (43), 159.1062 (32).

Hymenin

Colourless needels; ^{1}H NMR ($CDCl_3$): 7.65 (1H, d, *J* = 6.0 Hz, H-2), 6.21 (1H, d, *J* = 5.5 Hz, H-3), 4.91 (1H, d, *J* = 9.6 Hz, H-6), 3.31 (1H, m, H-7), 1.72~1.84 (2H, m, H-8 and H-9), 2.02~2.18 (2H, m, H-8 and H-9), 2.40 (1H, m, H-10), 6.28 (1H, d, *J* = 3.7 H-13a), 5.60 (1H, d, *J* = 3.2 Hz, H-13b), 1.18 (3H, d, *J* = 5.5 Hz, H-14), 1.10 (3H, s, H-15); ^{13}C NMR ($CDCl_3$): δ 82.9 (s, C-1), 164.9 (d, C-2), 129.9 (d, C-3), 209.0 (s, C-4), 57.4 (s, C-5), 79.9 (d, C-6), 41.5 (d, C-7), 31.4 (t, C-8), 25.4 (t, C-9), 37.6 (d, C-10), 138.8 (s, C-11), 170.6 (s, C-

12), 120.7 (t, C-13), 17.4 (q, C-14), 14.6 (q, C-15); $C_{15}H_{18}O_4$; ESIMS (positive ion mode) *m/z* (rel. int.): 263.1678 (5) $[M+H]^+$ (calculated for $C_{15}H_{19}O_4$ 263.1263), 245.1544 (60), 227.1412 (38), 217.1559 (100), 199.1425 (86), 189.0864 (88), 171.1424 (42), 121.0870 (34).

Antifungal assay on mycelial growth of *B. cinerea* and *F. oxysporum*

The inhibitory effect of the isolated sesquiterpenes (1-5) on the mycelial growth of *B. cinerea* and *F. oxysporum* was tested using a radial growth technique (Zambonelli et al., 1996). The isolated sesquiterpenes were dissolved in dimethyl sulfoxide (DMSO) and added to PDA medium immediately before it was poured into the Petri dishes at a temperature 40-45°C. The compounds were tested at concentrations ranged from 100 to 800 mg/L. Each concentration was tested in triplicate. Parallel controls were maintained with water and DMSO mixed with PDA medium. The discs of mycelial culture (0.5 cm diameter) of fungi, taken from 8-day-old cultures on PDA plates, were transferred aseptically to the centre of the Petri dishes. The plates were incubated in the dark at 25°C. The mycelial growth diameter was measured when the fungal growth in the control had completely covered the Petri dishes. Inhibition percentage of mycelial growth was calculated as follows:

$$\text{Mycelial growth inhibition } (\%) = \left[\frac{DC - DT}{DC} \right] \times 100$$

Where; DC and DT are average diameters of fungal mycelia of control and treatment, respectively. Effective concentration that caused 50% inhibition of a mycelial growth (EC_{50}) and its corresponding 95% confidence limits was estimated by a probit analysis (Finney, 1971).

Spore germination assay of *B. cinerea* and *F. oxysporum* in liquid medium

Spores of *B. cinerea* and *F. oxysporum* were harvested from a 2-week-old PDA cultures grown under fluorescent lights in 9 cm diameter Petri dishes at 25°C. An amount of 5 ml of sterile water was added to a Petri plate culture. The spores were gently dislodged from the surface with a sterile glass rod and the suspension was filtered through three layers of cheesecloth to remove mycelia fragments. The suspension was diluted with sterile water to an absorbance of 0.25 at 425 nm. This suspension contained about 1.0×10^6 conidia/ml. Aliquots of 50 µl of a spore suspension were placed in eppendorf tubes containing 500 µl of Potato Dextrose Broth (PDB) medium treated with sesquiterpenes at concentrations of 50, 100 and 200 mg/L. The tubes were incubated at 25°C for 16 h. The samples were placed on both chambers of a hemocytometer by carefully touching the edges of cover slip with the pipette tip and allowed capillary action to fill the counting chambers and observed under the microscope for spore germination. Spore counting was done using a Neubauer haemocytometer and light microscopy at 40x. All experiments were conducted in four replicates. A spore was considered germinated when the length of the germ tube equaled or exceeded the length of the spore (Griffin, 1994).

***In vivo* biochemical studies**

Biochemical interactions were conducted *in vivo* on fungi of *B. cinerea* and *F. oxysporum* using liquid medium experiments. The PDB medium was prepared in conical flasks containing the tested concentrations to be 0, 0.5 EC_{50} and EC_{50} values of the tested compounds on each fungus. An inoculums disc of the tested fungus was located on the surface of the medium in each flask. Parallel controls were maintained with water and DMSO mixed with PDB medium. The flasks were incubated for five days at 26°C. The contents of each flask were homogenized and then centrifuged at 5000 rpm for 15 min at 4°C. The supernatant was used in determination of protein and enzymes.

Total protein assay

The Lowry et al. (1951) method was used to determine the protein content of fungi. 100 µl of protein extract was added to 2 ml of alkaline copper reagent (48 ml of 2% (w/v) sodium carbonate in 0.1 N sodium hydroxide + 1 ml of 1% (w/v) sodium-potassium tartarate + 1 ml of 0.5% (w/v) copper sulphate) and immediately mix well. After 10 min, 0.2 ml of Folin-Ciocalteu phenol reagent was added and the samples were thoroughly mixed then the absorbance of the developed blue color was measured at 600 nm using Unico 1200-Spectrophotometer. The sample protein content was determined by comparing to the standard curve of bovine serum albumin (BSA).

Polyphenol oxidase (PPO) activity assay

The activity of PPO (EC 1.10.3.2) was determined according to Zhi-qing et al. (2008) by mixing of 1.5 ml of 0.2 mol/L pyrocatechol, 1.4 mL of 0.05 mol/L phosphate buffer (pH 6.8) and 0.1 ml enzyme extract, respectively. The mixture was incubated at 25°C for 25 min and the absorbance was measured at 420 nm by spectrophotometer (Unico 1200-Spectrophotometer, USA). The specific activity of PPO was calculated and expressed as OD_{420}. 30 min^{-1} protein.

Polygalacturonase and pectin-lyase activities assay

Polygalacturonase (PGase) and pectin-lyase (PLase) activities were determined colorimetrically by thiobarbituric acid (TBA) reagent according to the method of Nelson (1944) and that modified by Nedjma et al. (2001) and Aboaba (2009). The reaction mixture containing 0.3 ml enzyme extract and 0.8 ml (1.0%) of citrus pectin in 0.2M acetate buffer (pH 4.5) was incubated for 1 h at 30°C. Following incubation, 2.5 ml of a mixture comprising 2.5 ml of 1 N HCl and 5 ml of 0.04 M thiobarbituric acid was added and the reaction mixture was mixed thoroughly. The tested tube was then placed into a water bath for 30 min, cooled and the absorbance was read at 515 nm for PG activity and at 550 nm for PL activity by a Unico 1200-Spectrophotometer. An untreated control sample was used for blank reading. One unit per ml (U/ml)/mg protein of PGase and PLase defined as the amount of enzyme which produced an increase of 0.01 in absorbance at 515 and 505 nm, respectively per 1 min, under optimal conditions.

Statistical analysis

Statistical analysis was performed using the SPSS 12.0 software program (Statistical Package for Social Sciences, USA). The log dose-response curves allowed determination of the EC_{50} values for the fungal bioassay according to the probit analysis. The 95% confidence limits for the range of EC_{50} values were determined by the least-square regression analysis of the relative growth rate (% control) against the logarithm of the compound concentration. The data of spore germination and enzyme activities were analyzed by one-way analysis of variance (ANOVA). Mean separations were

Table 1. *In vitro* antifungal activity of sesquiterpenes isolated from *A. maritima* against *B. cinerea* and *F. oxysporum* using mycelial growth inhibition method.

Compound	EC_{50}[a] (mg/L)	95% confidence limits (mg/L)		Slope[b] ± SE	Intercept[c] ± SE	(χ^2)[d]
		Lower	Upper			
B. cinerea						
1	316.6	273.6	369.1	2.13±0.22	-5.32±0.54	1.75
2	488.0	427.0	570.4	2.57±0.25	-6.92±0.65	0.12
3	332.5	287.3	388.2	2.13±0.22	-5.37±0.55	0.70
4	413.9	363.6	478.3	2.54±0.24	-6.64±0.61	0.31
5	495.3	444.3	559.3	3.37±0.31	-9.08±0.83	2.33
F. oxysporum						
1	204.3	165.1	254.5	1.67±0.31	-3.86±0.71	1.52
2	464.8	399.7	556.3	2.18±0.23	-5.82±0.58	1.18
3	230.2	183.6	303.2	1.50±0.31	-3.55±0.71	0.87
4	246.6	194.3	340.7	1.41±0.31	-3.37±0.71	0.09
5	444.1	381.7	530.7	2.13±0.23	-5.65±0.57	0.02

[a]The concentration causing 50% mycelial growth inhibition, [b]Slope of the concentration-inhibition regression line ± standard error, [c]Intercept of the regression line ± standard error, [d]Chi square value.

performed by Student-Newman-Keuls (SNK) and differences at $P \leq 0.05$ were considered as significant.

RESULTS

Effect of sesquiterpenes on fungal mycelial growth

The *in vitro* antifungal activity of sesquiterpene compounds (1-5) against the plant pathogenic fungi *B. cinerea* and *F. oxysporum* is presented in Table 1 and the results of this assay are expressed as effective concentration that inhibited 50% of mycelial growth (EC_{50}). As can be seen that all of the tested compounds showed inhibitory effect against both fungi. Neoambrosin (compound 1) was the most potent among the tested compounds with EC_{50} of 316.6 and 204.3 mg/L against *B. cinerea* and *F. oxysporum*, respectively. Compounds of damsin and ambrosin (3 and 4) also showed relatively high inhibitory effect. In contrast, damsinic acid (2) and hymenin (5) were the lowest active compounds against both fungi. In addition when we consider the susceptibility of the microorganisms, another point deserves attention; the compounds had more effective inhibition on *F. oxysporum* than *B. cinerea*.

Effect of sesquiterpenes on spore germination of fungi

The effect of ssquiterpenes (1-5) on spore germination of *B. cinerea* and *F. oxysporum* was examined at 50, 100 and 200 mg/L and the data are presented in Table 2. As can be seen that the spore germination was affected significantly at the tested concentrations and all of the compounds had a better inhibition compared to the control. Generally, the reduction in spore germination of *B. cinerea* was higher than of *F. oxysporum*. At 200 mg/L, the tested compounds strongly inhibited spore germination with inhibition greater than 90% except with damsin (3) against *F. oxysporum*. At this concentration, compounds 2 and 4 achieved complete inhibition of spore germination in both fungi.

Inhibitory effect of sesquiterpenes on PPO enzyme

The *in vivo* inhibitory effect of sesquiterpenes (1-5) on PPO activity in fungi treated with concentrations of 0.5 EC_{50} and EC_{50} is shown in Table 3. The results showed that the highest value of the specific activity was found in the untreated fungus of *F. oxysporum* (10.08) compared to 8.38 in untreated *B. cinerea*. The tested sesquiterpenes significantly inhibited the enzyme activity in *B. cinerea* and *F. oxysporum* at both concentrations. The enzyme activity in all treatments was concentration dependant and it was significantly declined to the lowest value with the highest concentration (EC_{50} value). It can be noted that the ambrosin (4) proved significantly higher inhibition in PPO activity than the other treatments and the untreated controls at treatment of EC_{50} where that 1.89 and 2.29 specific activities were found in *B. cinerea* and *F. oxysporum*, respectively. At concentration of 0.5 EC_{50}, compound 2 exhibited the highest inhibitory effect on enzyme activity of *B. cinerea* (specific activity = 4.17),

Table 2. Effect of sesquiterpenes isolated from *A. maritima* on spore germination of *B. cinerea* and *F. oxysporum.*

Treatments	Concentration (mg/L)	Inhibition of spore germination (%) ± SE	
		B. cinerea	***F. oxysporum***
Control	0	18.5^{d}±1.44	13.7^{e}±4.62
1	50	48.7^{c}±6.63	34.0^{d}±3.29
	100	66.0^{b}±6.18	50.0^{bcd}±4.08
	200	95.1^{a}±1.66	93.8^{a}±6.25
2	50	66.0^{b}±5.35	37.4^{cd}±2.41
	100	69.8^{b}±2.86	53.5^{bc}±5.64
	200	100.0^{a}±0.00	100.0^{a}±0.00
3	50	63.8^{b}±3.96	31.8^{d}±4.76
	100	66.4^{b}±3.06	44.1^{cd}±3.95
	200	96.5^{a}±1.22	67.1^{b}±3.07
4	50	69.6^{b}±2.85	43.7^{cd}±4.56
	100	71.6^{b}±2.53	64.2^{b}±6.29
	200	100.0^{a}±0.00	100.0^{a}±0.00
5	50	66.3^{b}±5.73	40.0^{cd}±7.07
	100	69.1^{b}±4.61	54.2^{bc}±4.17
	200	97.6^{a}±2.38	95.8^{a}±4.17

Data are average of four replicates ± SE. Values within a column bearing the same letter are not significantly different (P ≤ 0.05) according to Student-Newman-Keuls (SNK) test.

Table 3. *In vivo* effect of sesquiterpenes isolated from A. maritima on polyphenol oxidase activity of *B. cinerea* and *F. oxysporum* grown in liquid medium treated with 0.5 and EC50 values

Treatments	Concentration	Specific activity ± SE ($OD_{\lambda 420}$.mg protein^{-1}. 30 min^{-1})	
		B. cinerea	***F. oxysporum***
Control	0	8.38^{a}±0.13	10.08^{a}±0.07
1	0.5 EC_{50}	6.28^{c}±0.08	7.48^{b}±0.03
	EC_{50}	5.84^{d}±0.22	6.89^{c}±0.03
2	0.5 EC_{50}	4.17^{f}±0.17	5.23^{d}±0.06
	EC_{50}	3.71^{g}±0.05	4.94^{e}±0.09
3	0.5 EC_{50}	5.39^{e}±0.07	7.02^{c}±0.07
	EC_{50}	4.50^{f}±0.11	6.79^{c}±0.07
4	0.5 EC_{50}	5.32^{e}±0.09	4.56^{f}±0.06
	EC_{50}	1.89^{h}±0.08	2.29^{h}±0.14
5	0.5 EC_{50}	7.05^{b}±0.07	4.21^{g}±0.10
	EC_{50}	5.54^{de}±0.08	2.44^{h}±0.01

Data are average of three replicates ± SE. Values within a column bearing the same letter are not significantly different (P ≤ 0.05) according to Student-Newman-Keuls (SNK) test.

Table 4. *In vivo* effect of sesquiterpenes isolated from *A. maritima* on the activity of PGase and PLase of *B. cinerea* and *F. oxysporum* grown in liquid medium treated with 0.5 and EC_{50} values.

Treatments	Concentration	(U/mL)/mg protein of PGase ± SE		(U/mL)/mg protein of PLase ± SE	
		B. cinerea	***F. oxysporum***	***B. cinerea***	***F. oxysporum***
Control	0	60.5^{a}±1.24	54.7^{a}±0.77	49.7^{b}±1.62	44.9^{a}±0.95
1	0.5 EC_{50}	62.6^{a}±1.11	44.1^{b}±1.57	58.7^{a}±0.21	35.7^{b}±0.51
	EC_{50}	55.6^{b}±0.19	30.2^{e}±0.24	44.8^{bc}±0.88	22.9^{d}±0.41
2	0.5 EC_{50}	53.8^{bc}±1.92	52.8^{a}±1.27	47.7^{b}±1.45	42.5^{a}±1.58
	EC_{50}	46.9^{d}±2.21	49.9^{a}±1.76	39.3^{d}±1.87	40.8^{a}±1.03
3	0.5 EC_{50}	53.9^{bc}±0.76	40.7^{bc}±1.97	44.3^{bc}±0.44	34.1^{bc}±1.64
	EC_{50}	49.7^{cd}±0.49	36.0^{cd}±0.51	41.9^{cd}±1.69	30.7^{bc}±0.71
4	0.5 EC_{50}	53.7^{bc}±1.04	36.9^{cd}±1.82	48.9^{b}±1.29	29.8^{c}±1.55
	EC_{50}	35.7^{f}±2.09	29.5^{e}±0.80	33.8^{e}±2.12	21.0^{d}±1.43
5	0.5 EC_{50}	40.8^{e}±1.28	45.2^{b}±1.73	38.0^{de}±1.61	35.5^{b}±1.56
	EC_{50}	38.4^{ef}±0.73	32.1^{de}±1.14	34.4^{e}±0.50	21.7^{d}±1.93

Data are average of three replicates ± SE. Values within a column bearing the same letter are not significantly different ($P \leq 0.05$) according to Student-Newman-Keuls (SNK) test. One unit per ml (U/ml)/mg protein of PGase and PLase defined as the amount of enzyme which produced an increase of 0.01 in absorbance at 515 and 505nm, respectively per 1 min, under optimal conditions).

while compound 5 was significantly the most potent against *F. oxysporum* (specific activity = 4.21).

Inhibitory effect of sesquiterpenes on PGase and PLase enzymes

The *in vivo* inhibitory effects of sesquiterpenes on PGase and PLase enzymes that isolated from fungi treated with concentrations of 0.5 EC_{50} and EC_{50} is shown in Table 4. Generally, the result revealed that the enzymes activity was higher in *B. cinerea* than *F. oxysporum*. It can be seen that compounds 5 and 4 were the most potent inhibitors for enzyme activity of both fungi at 0.5 EC_{50} and EC_{50}. Ambrosin (4) proved significantly higher inhibition in PGase and PLase than the other compounds and the untreated controls at treatment of EC_{50}, where that 35.7 and 29.5 (U/ml)/mg protein of PGase were found in *B. cinerea* and *F. oxysporum*, respectively compared to 60.5 and 54.7 in the untreated control, respectively. It was also the most significant inhibitor against PLase at treatment of EC_{50} with 33.8 and 21.0 (U/ml)/mg protein in *B. cinerea* and *F. oxysporum*, respectively compared to 49.7 and 44.9 in the untreated control, respectively. In contrast, neoambrosin (1) was the lowest active compound in inhibition of PGase and PLase of *B. cinerea* however, compound 2 was the lowest active in inhibition of these enzymes in *F. oxysporum*.

DISCUSSION

The present study shows that the sesquiterpenes (1-5) isolated from *A. maritima* possess antifungal potential against plant pathogenic fungi *B. cinerea* and *F. oxysporum*. The sesquiterpenes significantly inhibited mycelial growth and spore germination of the tested fungi. It was observed that the compounds had inhibition effect on spore germination greater than mycelial growth. The isolated sesquiterpenes were more effective against *F. oxysporum*, a fungus known to be highly resistant to antifungal agents, than sesquiterpenes vernolide and vernodalol isolated from *Vernonia amygdalina* (Erasto et al., 2006) and costunolide and parthenolide isolated from *Magnolia grandiflora* (Ahmed and Abdelgaleil, 2005). It also reported that sesquiterpenes isolated from some plants had antifungal activity against other phytopathogenic fungi (Skaltsa et al., 2000; Karioti et al.,

2000; Wedge et al., 2000; Scher et al., 2004).

The data of enzyme activities of PPO, PGase and PLase revealed that the sesquiterpenes had inhibitory effects of the tested enzymes. However the inhibitory effects of compounds were not correlated to their anti-fungal activity. This finding indicated that these enzymes are not the main targets of the tested sesquiterpenes and the modes of action of these compounds are different. It is known that bioactivities of sesquiterpenes are mainly due to their reactions with -SH group of amino acids, proteins and enzymes (Picman, 1986). It is also important to note that the antifungal activity of the tested sesquiterpenes is based on their permeability of the mycelial and spore walls of the tested fungi.

With regard to the structure-antifungal relationship, sesquiterpenes: (1) neoambrosin, (3) damsin and (4) ambrosin with α-methylene-γ-lactone are more active than (2) damsinic acid in which the lactone ring is opened and formed carboxyl group. Similarly, Rodriguez et al. (1976) stated that the presence of α-methylene-γ-lactone is essential for potent bioactivity of sesquitrpenes. The introduction of hydroxyl group at C1 in (5) hymenin decreased the antifungal activity of this compound comparing with (4) ambrosin. This finding is in a good agreement with those reported on the antifungal activity of sesquiterpene melampolides and sesquiterpene lactones in which the least polar compounds showed the greatest activity (Inoue et al., 1995; Barrero et al., 2000). It could be concluded that, besides the, α-methylene-γ-lactone, a relatively low polarity of (1) neoambrosin, (3) damsin and (4) ambrosin comparing with (2) damsinic acid and (5) hymenin is actually responsible for their strong antifungal activity. The low polarity of these compounds is matched with optimum lipophility degree required for passing through the fungal cell wall.

In summary, the results of the present study indicated that the sesquiterpenes (1-5) isolated from *A. maritima* possessed pronounced antifungal activity against plant pathogenic fungi *B. cinerea* and *F. oxysporum*. In addition, the sesquiterpenes significantly inhibited the activities of polyphenol oxidase, polygalacturonase and pectin-lyase. Moreover, these results could be useful in the research for newer more selective, biodegradable and natural antifungal compounds or can be used as lead compounds for the development new antimicrobial agents.

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