JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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pubs.acs.org/JAFC

Pinus pinaster Knot: A Source of Polyphenols against *Plasmopara viticola*

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ABSTRACT: Pine knot extract from *Pinus pinaster* byproducts was characterized by UHPLC-DAD-MS and NMR. Fourteen polyphenols divided into four classes were identified as follows: lignans (nortrachelogenin, pinoresinol, matairesinol, isolariciresinol, secoisolariciresinol), flavonoids (pinocembrin, pinobanksin, dihydrokaempferol, taxifolin), stilbenes (pinosylvin, pinosylvin monomethyl ether, pterostilbene), and phenolic acids (caffeic acid, ferulic acid). The antifungal potential of pine knot extract, as well as the main compounds, was tested *in vitro* against *Plasmopara viticola*. The ethanolic extract showed a strong antimildew activity. In addition, pinosylvins and pinocembrin demonstrated significant inhibition of zoospore mobility and mildew development. These findings strongly suggest that pine knot is a potential biomass that could be used as a natural antifungal product.

KEYWORDS: *pine knot, polyphenols, UHPLC-MS, NMR, downy mildew*

INTRODUCTION

Pinus pinaster, also known as maritime pine, is a conifer widespread in Europe, especially in the Atlantic and Mediterranean regions.[1] Its transformation into pulp and paper generates several byproducts such as pine knots, which are undesirable owing to their content of lipophilic constituents.[2] For example, a French paper factory uses between 1000 and 2000 tons of wood pine per day generating 1−2% of pine knots that represents a biomass of 10−40 tons of knots per day.[3] Pine knots are generally used as a combustible fuel in factories, even though they are known to be rich in polyphenols such as lignans, flavonoids, and stilbenes and have potential as a biomass.

Lignans are phenolic compounds arising from a secondary plant metabolism with a $(C6-C3)_2$ structure that results from the oxidative dimerization of two phenylpropanoid units.[4] Lignans are particularly known for their bioactivity in human health with anticancer properties, anti-inflammatory activity, and an antioxidant effect.[5−7] In addition, some lignans exhibit antibacterial activities against Gram-positive bacteria and antifungal activities against fungi of white rotting and wood staining.[8] Flavonoids from the phenylpropanoid pathway are secondary metabolites with a C6−C3−C6 structure. Present in the *Pinus* genus and especially in *Pinus pinaster*, flavonoids such as pinocembrin and pinobanksin have demonstrated antioxidant, anti-inflammatory, anticancer, and neuroprotective activities.[9−11] Furthermore, flavonoids have been found to have antimicrobial properties against bacteria, yeasts, and filamentous fungi.[12] Stilbenes, which are also known as pinosylvins in *Pinus genus*, are formed by a C6−C2−C6 skeleton and are known for their antifungal properties. A previous study demonstrated inhibition of *Candicans albicans* and *Saccharomyces cerevisiae* by pinosylvin and its methyl derivative while another work reported the efficacy of pinosylvins against white-rot and brown-rot fungi.[13,14] Thus, the range of polyphenols contained in pine and especially in the knots exhibits a wide spectrum of antifungal activities.

One of the most devastating diseases in cultivated grapevines is caused by *Plasmopara viticola*, an oomycete responsible for downy mildew. *Plasmopara viticola* is an obligate biotroph which develops its mycelium in host tissues and emits through stomata arbuscles called sporangiophores that have sporangia containing mobile zoospores at their extremities. At maturity, these zoospores are released, allowing rapid dissemination of *P. viticola*.[15] Particularly suited to rainy and humid conditions, the disease attacks flowers, leaves, and berries. Currently, the main antimildew agent is "Bordeaux mixture", a preventive contact product containing copper.[15,16] Despite its wide range of actions, its intensive use has several negative effects on the environment such as accumulation in the soil and metabolic disorders in berries.[17,18] Chemical products with a good bioavailability are also efficient at low doses, but they generally suffer from problems of resistance.[19] Consequently, the development of alternative or complementary strategies to pesticide treatments is a major economic challenge for agriculture and viticulture in particular. The use of natural plant extracts might be a solution owing to the huge availability of such compounds, which are less toxic than traditional phytosanitary products and are biodegradable.

The purpose of this study was to characterize *Pinus pinaster* knot extract and to evaluate *in vitro* its ability to inhibit

Received: September 4, 2017
Revised: September 18, 2017
Accepted: September 19, 2017
Published: September 19, 2017

DOI: 10.1021/acs.jafc.7b04129
J. Agric. Food Chem. 2017, 65, 8884−8891

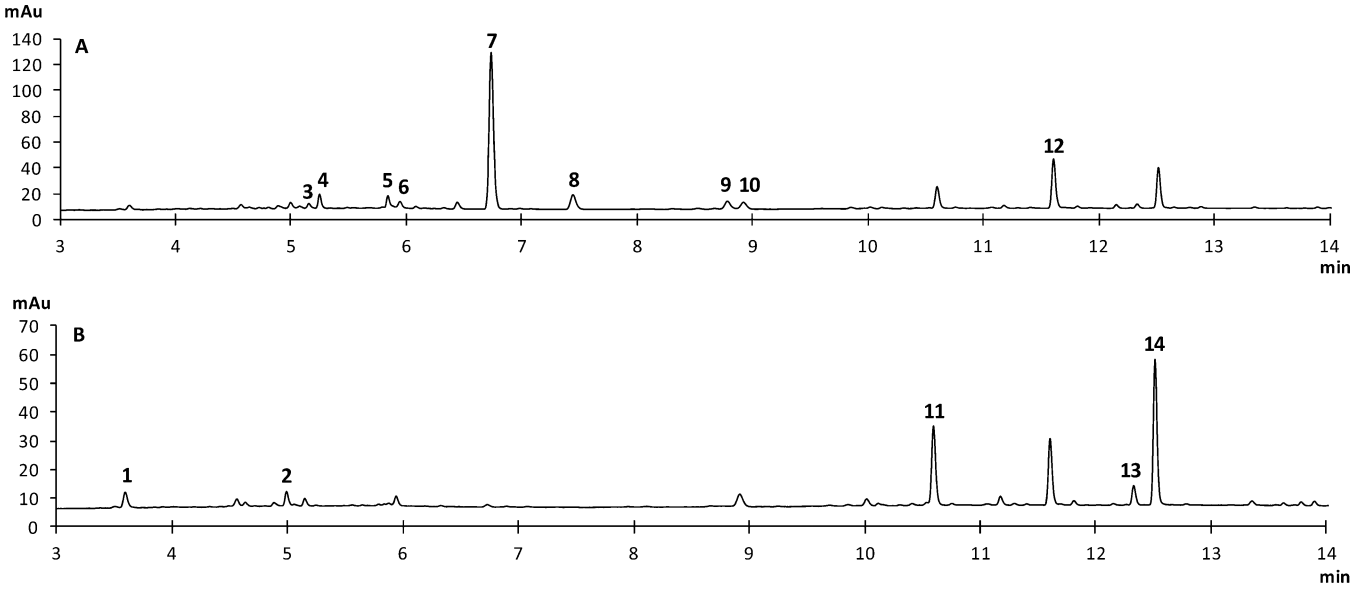


Figure 1. UHPLC-DAD chromatograms of pine knot extract at (A) 280 nm and (B) 320 nm. **1**, caffeic acid; **2**, ferulic acid; **3**, taxifolin; **4**, isolariciresinol; **5**, secoisolariciresinol; **6**, dihydrokaempferol; **7**, nortrachelogenin; **8**, pinoresinol; **9**, matairesinol; **10**, pinobanksin; **11**, pinosylvin; **12**, pinocembrin; **13**, pterostilbene; **14**, pinosylvin monomethyl ether.

Plasmopara viticola growth. The antifungal property of the main compounds of extract was also tested. The results provide new insights into the fight against downy mildew, potentially leading to the development of natural and sustainable anti-downy mildew products.

MATERIALS AND METHODS

Chemicals and Standards. For analysis, LC-MS-grade acetonitrile (VWR, Fontenay-Sous-Bois, France), formic acid (Fisher Scientific, Loughborough, U.K.), HPLC-grade acetonitrile, trifluoroacetic acid (TFA), and HPLC-grade methanol (Sigma-Aldrich, St. Louis, MO) were purchased. Water was purified by an Elga water purification system (High Wycombe, U.K.).

Ferulic acid, caffeic acid, taxifolin, dihydrokaempferol, and pterostilbene were bought (Sigma-Aldrich, St. Louis, MO). Other standards were isolated and purified in our laboratory (isolariciresinol, secoisolariciresinol, nortrachelogenin, pinoresinol, matairesinol, pinocembrin, pinobanksin, pinosylvin, pinosylvin monomethyl ether) from pine knot extract. The identity and purity of these compounds were analyzed by UV/vis diode-array detector (DAD), LC-MS, and NMR. Purity of compounds was estimated ≥90%.

Biological Material. Grapevine plants of *Vitis vinifera* cv. Cabernet-Sauvignon, supplied by UMR SAVE (INRA, Villenave d'Ornon, France), were cultivated from wood cuttings in a greenhouse. Rooted cuttings were potted after 21 d in a sandy soil and were grown under controlled conditions (25 °C day/20 °C night temperatures with relative humidity at 75% and a 15 h light/9 h dark photoperiod). Two-month-old plants with 10−12 leaves were used for bioassays by picking the fourth leaf below the apex. Downy mildew isolate (ANN-01) was harvested on *Vitis vinifera* cv. Ugni blanc in a commercial vineyard located in Charente (France) in 2015. As previously described in literature, *Plasmopara viticola* isolate was multiplied every week by inoculation of 15 μL drops of a 15000 sporangia/mL inoculum on grapevine leaves cv. Cabernet Sauvignon.[20] After 7 d of incubation in a growth chamber (22 °C, 16 h day/8 h night photoperiod), the inoculum was suitable for bioassays.

Pine Knot Extract. Pine knots (*Pinus pinaster*) were collected in the South-West region of France (Gironde), crushed to a powder, and dried at room temperature. Extraction was performed on 1 kg of pine knot with 10 L of ethanol/water (85:15; v/v) under agitation at 60 °C. The filtrate was concentrated *in vacuo* to one-tenth (ca. 1 L) followed by decantation in order to obtain two phases, one comprising a supernatant and the other comprising apolar compounds. The supernatant was recovered, concentrated, and then lyophilized to obtain 75 g of a purified extract.

Characterization by UHPLC-DAD-MS. The apparatus was a 1290 series UHPLC (Agilent Technologies, Santa Clara, CA) with a UV/vis diode-array detector (DAD). The column used was a 100 mm × 2.1 mm i.d., 1.8 μm, Zorbax SB-C18, with a 2.1 mm × 5 mm i.d. guard column of the same material (Agilent Technologies, Santa Clara, CA). The chromatographic parameters were as follows: solvent A, water acidified with 0.1% formic acid; solvent B, acetonitrile acidified with 0.1% formic acid; flow rate, 0.4 mL/min; solvent gradient, 0−1.7 min, 10% B; 1.7−3.4 min, 10−20% B; 3.4−5.1 min, 20−30% B; 5.1−6.8 min, 30% B; 6.8−8.5 min, 30−35% B; 8.5−11.9 min, 35−60% B; 11.9−15.3 min, 60−100% B; 15.3−17 min, 100% B; 17−17.3 min, 100−10% B. An Esquire 6000 ion trap (IT) mass spectrometer using an ESI source (Bruker Daltonics, Billerica, MA) was coupled to the UHPLC apparatus. Mass spectrometry parameters were programmed as previously mentioned in literature data.[21] Pine knot extract was dissolved in methanol/water (1:1, v/v) at 1 mg/mL, filtered on 0.45 μm PTFE, and injected at 1 μL. Analyses were performed in triplicate. Standards were injected at several concentrations (0, 5, 10, 50, 100 μg/mL) in independent quintuplicate to obtain calibration and equation curves. Bruker Data Analysis 3.2 software was used.

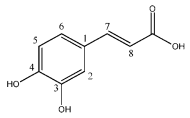
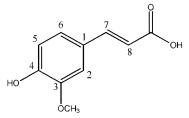
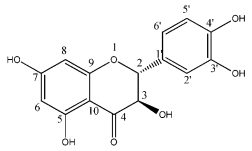
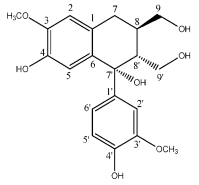
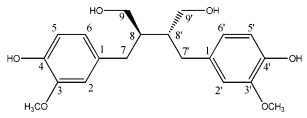
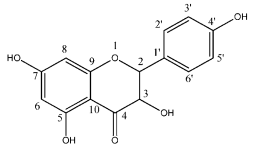
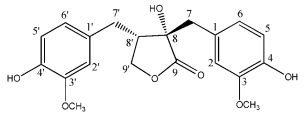
Polyphenol Purification by Preparative HPLC. The apparatus was a Varian Pro Star HPLC equipped with a UV/vis diode-array detector (DAD). The column used was a 250 mm × 21.2 mm i.d., 7 μm, Zorbax SB-C18 PrepHT (Agilent Technologies, Santa Clara, CA). Pine knot extract was dissolved at 50 mg/mL in methanol−water (50:50; v/v) and filtered on PTFE 0.45 μm. The chromatographic parameters were as follows: solvent A, water acidified with 0.025% TFA; solvent B, acetonitrile acidified with 0.025% TFA; flow rate, 19 mL/min; solvent gradient, 0−3 min, 25% B; 3−33 min, 25−60% B; 33−34 min, 60−100% B; 34−39 min, 100% B; 39−40 min, 100−25% B; 40−43 min, 25% B.

NMR Experiments. ^{1}H and 2D nuclear magnetic resonance (COSY, ROESY, HSQC, HMBC) analyses were carried out on a Bruker Ultrashield 600 MHz NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a cryogenic TXI probe head. All isolated polyphenols were dissolved in 3 mm tubes with d_4-methanol as solvent. Bruker Topspin software version 3.2 was used for analysis of NMR spectra.

Bioassays against *Plasmopara viticola*. *Pine Knot Extract.* Pine knot extract was prepared with a range of eight concentrations (0, 50,

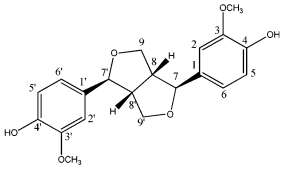
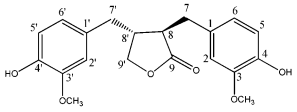
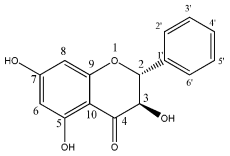
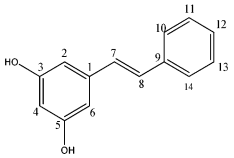
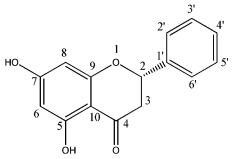
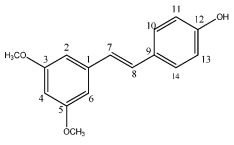
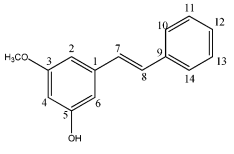
DOI: 10.1021/acs.jafc.7b04129
J. Agric. Food Chem. 2017, 65, 8884−8891

Table 1. Name, Peak Number, Structure, Retention Time, UV Data (λ_{max} in nm), MS Data (Molecular Ion, Negative Mode), and ^{1}H NMR (in d_4-Methanol) Data of Polyphenols Identified in Pine Knot Extract (*Pinus pinaster*)

Compounds	t_R (min)	λ_{max}	(m/z)	δ_H
caffeic acid **1** 	3.6	325	179	7.52 (1H, d, *J* = 15.9 Hz, H-7), 7.03 (1H, d, *J* = 2.0 Hz, H-2), 6.93 (1H, dd, *J* = 2.0, 8.0 Hz, H-6), 6.77 (1H, d, *J* = 8.0 Hz, H-5), 6.21 (1H, d, *J* = 15.9 Hz, H-8)
ferulic acid **2** 	5.0	325	193	7.59 (1H, d, *J* = 15.9 Hz, H-7), 7.19 (1H, d, *J* = 2.0 Hz, H-2), 7.06 (1H, dd, *J* = 2.0, 8.0 Hz, H-6), 6.81 (1H, d, *J* = 8.0 Hz, H-5), 6.31 (1H, d, *J* = 15.9 Hz, H-8), 3.89 (3H, s, 3-OCH_3)
taxifolin **3** 	5.2	289	303	6.96 (1H, d, *J* = 2.0 Hz, H-2'), 6.84 (1H, dd, *J* = 8.0, 2.0 Hz, H-6'), 6.80 (1H, d, *J* = 8.0 Hz, H-5'), 5.92 (1H, d, *J* = 2.0 Hz, H-8), 5.88 (1H, d, *J* = 2.0 Hz, H-6), 4.91 (1H, d, *J* = 11.5 Hz, H-2), 4.50 (1H, d, *J* = 11.5 Hz, H-3)
isolariciresinol **4** 	5.3	285	359	6.74 (1H, d, *J* = 8.0 Hz, H-5'), 6.67 (1H, d, *J* = 2.0 Hz, H-2'), 6.65 (1H, s, H-5), 6.61 (1H, dd, *J* = 8.0, 2.0 Hz, H-6'), 6.17 (1H, s, H-2), 3.80 (1H, br s, H-7'), 3.80 (3H, s, 3-OCH_3), 3.77 (3H, s, 3'-OCH_3), 3.70 (1H, dd, *J* = 11.0, 4.8 Hz, H-9'a), 3.66 (2H, m, H-9), 3.40 (1H, d, *J* = 11.0, 4.0 Hz, H-9'b), 2.78 (1H, d, *J* = 7.8 Hz, H-7), 2.00 (1H, m, H-8), 1.76 (1H, m, H-8')
secoisolariciresinol **5** 	5.9	281	361	6.65 (2H, d, *J* = 8.0 Hz, H-5, 5'), 6.58 (2H, d, *J* = 2.0 Hz, H-2, 2'), 6.54 (2H, dd, *J* = 8.0, 2.0 Hz, H-6, 6'), 3.73 (6H, s, 3, 3'-OCH_3), 3.60 (2H, dd, *J* = 11.0, 4.9 Hz, H-9b, 9b'), 3.56 (2H, dd, *J* = 11.0, 5.1 Hz, H-9a, 9a'), 2.66 (2H, dd, *J* = 14.0, 6.9 Hz, H-7b, 7'b), 2.55 (2H, dd, *J* = 14.0, 7.8 Hz, H-7a, 7'a), 1.90 (2H, m, H-8, 8')
dihydrokaempferol **6** 	6.0	290	287	7.35 (2H, d, *J* = 8.6 Hz, H-2', H-6'), 6.83 (2H, d, *J* = 8.6 Hz , H-3', H-5'), 5.92 (1H, d, *J* = 2.0 Hz, H-8), 5.88 (1H, d, *J* = 2.0 Hz, H-6), 4.98 (1H, d, *J* = 11.6 Hz, H-2), 4.54 (1H ,d, *J* = 11.6 Hz, H-3)
nortrachelogenin **7** 	6.8	281	373	6.70 (1H, d, *J* = 8.0 Hz, H-5), 6.69 (1H, d, *J* = 8.0 Hz, H-5'), 6.68 (1H, d, *J* = 2.0 Hz, H-2), 6.67 (1H, d, *J* = 2.0 Hz, H-2'), 6.59 (1H, dd, *J* = 8.0, 2.0 Hz, H-6), 6.57 (1H, dd, *J* = 8.0, 2.0 Hz, H-6'), 3.98 (2H, d, *J* = 8.0 Hz, H-9'), 3.81 (3H, s, OCH_3), 3.78 (3H, s, OCH_3), 3.12 (1H, d, *J* = 13.6 Hz, H-7), 2.84 (1H, d, *J* = 13.6 Hz, H-7), 2.78 (1H, dd, *J* = 13.6, 5.1 Hz, H-7'), 2.47 (1H, m, H-7'), 2.42 (1H, m, H-8')

DOI: 10.1021/acs.jafc.7b04129
J. Agric. Food Chem. 2017, 65, 8884−8891

Table 1. continued

Compounds	t_R (min)	λ_{max}	(m/z)	δ_H
pinoresinol **8** 	7.5	281	357	6.94 (2H, d, J = 2.0 Hz, H-2, 2'), 6.81 (2H, dd, *J* = 8.2, 2.0 Hz, H-6, 6'), 6.76 (2H, d, *J* = 8.2 Hz, H-5, 5'), 4.70 (2H, d, *J* = 4.4 Hz, H-7, 7'), 4.23 (2H, m, H-9b, 9b'), 3.85 (6H, s, 3, 3'-O CH_3), 3.83 (2H, dd, J = 9.2, 3.6 Hz, H-9a, 9'a), 3.14 (2H, m, H-8, 8')
matairesinol **9** 	8.8	282	357	6.70 (1H, d, *J* = 8.0 Hz, H-5), 6.68 (1H, d, *J* = 8.0 Hz, H-5'), 6.67 (1H, d, *J* = 2.0 Hz, H-2), 6.58 (1H, dd, *J* = 8.0, 2.0 Hz, H-6), 6.56 (1H, d, *J* = 2.0 Hz, H-2'), 6.51 (1H, dd, *J* = 8.0, 2.0 Hz, H-6'), 4.16 (1H, dd, *J* = 9.1, 7.3 Hz, H-9'b), 3.93 (1H, dd, *J* = 9.1, 7.8 Hz, H-9'a), 3.79 (3H, s, OCH_3), 3.78 (3H, s, OCH_3), 2.88 (1H, dd, *J* = 14.1, 5.5 Hz, H-7b), 2.82 (1H, dd, *J* = 14.1, 7.0 Hz, H-7a), 2.66 (1H, m, H-8), 2.53 (2H, m, H-7'), 2.49 (1H, m, H-8')
pinobanksin **10** 	9.0	289	271	7.54 (2H, d, *J* = 8.0 Hz, H-2', 6'), 7.43 (2H, t, *J* = 6.9 Hz, H-3', 5'), 7.39 (1H, t, *J* = 6.9 Hz, H-4'), 5.94 (1H, d, *J* = 2.1 Hz, H-8), 5.91 (1H, d, *J* = 2.1 Hz, H-6), 5.08 (1H,d, *J* = 11.5 Hz, H-2), 4.55 (1H, d, *J* = 11.5 Hz, H-3)
pinosylvin **11** 	10.6	306	211	7.51 (2H, d, *J* = 8.0 Hz, H-10, 14), 7.33 (2H, t, *J* = 7.6 Hz, H-11, 13), 7.22 (1H, t, *J* = 7.4 Hz, H-12), 7.04 (1H, d, *J* = 16.3 Hz, H-8), 6.99 (1H, d, *J* = 16.3 Hz, H-7), 6.48 (2H, d, *J* = 2.0 Hz, H-2, 6), 6.19 (1H, t, J = 2.2 Hz, H-4)
pinocembrin **12** 	11.7	289	255	7.50 (2H, d, *J* = 8.0 Hz, H-2', 6'), 7.42 (2H, t, *J* = 7.7 Hz, H-3', 5'), 7.36 (1H, t, *J* = 7.4 Hz, H-4'), 5.93 (1H, d, *J* = 2.2 Hz, H-8), 5.90 (1H, d, *J* = 2.2 Hz, H-6), 5.46 (1H, dd, *J* = 12.8, 3.1 Hz, H-2), 3.10 (1H, dd, *J* = 17.1, 12.8 Hz, H-3a), 2.77 (1H, dd, *J* = 17.1, 3.2 Hz, H-3b)
pterostilbene **13** 	12.4	320	255	7.38 (2H, d, *J* = 8.5 Hz, H-10, 14), 7.05 (1H, d, *J* = 16.4 Hz, H-8), 6.89 (1H, d, *J* = 16.4 Hz, H-7), 6.77 (2H, d, *J* = 8.5 Hz, H-11, 13), 6.66 (2H, d, *J* = 2.2 Hz, H-2, 6), 6.35 (1H, t, *J* = 2.2 Hz, H-4), 3.79 (6H, s, 3, 5-OCH_3)
pinosylvin monomethyl ether **14** 	12.6	306	225	7.52 (2H, d, *J* = 8.0 Hz, H-10, 14), 7.33 (2H, t, *J* = 7.6 Hz, H-11, 13), 7.23 (1H, t, *J* = 7.4 Hz, H-12), 7.09 (1H, d, *J* = 16.3 Hz, H-8), 7.04 (1H, d, *J* = 16.3 Hz, H-7), 6.61 (1H, t, *J* = 2.1 Hz, H-6), 6.59 (1H, t, J = 1.8 Hz, H-4), 6.28 (1H, t, *J* = 1.8 Hz, H-2), 3.79 (3H, s, 3-OCH_3)

100, 200, 300, 500, 800, 1000 mg/L) in water with 1% of ethanol for better dissolution. In parallel, leaves of *Vitis vinifera* (cv. Cabernet Sauvignon) were collected in greenhouse, washed under water, and dried with filter paper. Foliar disks were obtained with a 25 mm wide pastry cutter, and eight heterogenic disks were randomly deposited in Petri dishes containing Whatman paper humidified with 3.5 mL of sterile water. Dilutions of pine knot extract were sprayed on the foliar disks. Negative controls were prepared by applying sterile water with

DOI: 10.1021/acs.jafc.7b04129
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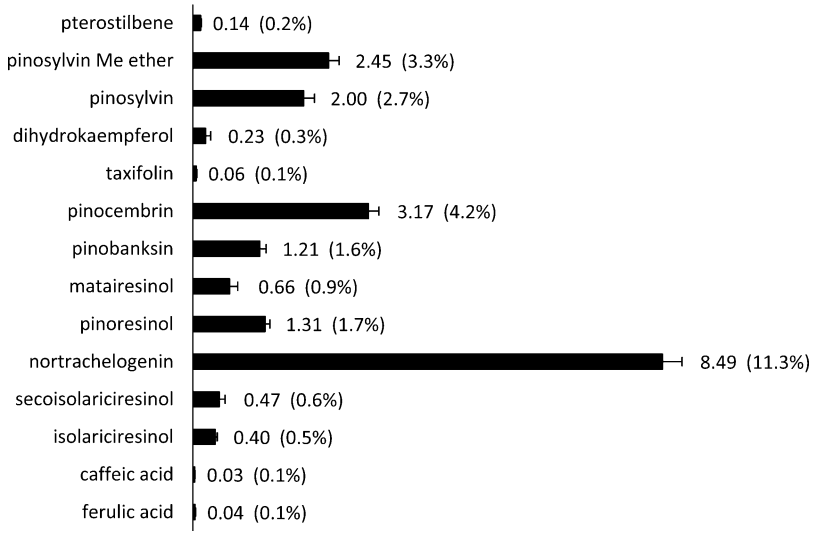

Figure 2. Polyphenol concentrations in pine knot (g/kg knot). Contents in pine knot extract (% m/m) in parentheses; pinosylvin Me ether = pinosylvin monomethyl ether.

1% of ethanol while Dithane NeoTec 75 WG (Dow AgroSciences, Sophia Antipolis, France) containing mancozeb at 75% was used as positive control with a range of eight concentrations (0, 10, 20, 30, 50, 100, 200, 500 mg/L). Inoculation of *Plasmopara viticola* isolate was performed 1 d later with 3 droplets of 15 μL/disk from an inoculum at 15000 sporangia/mL. Petri dishes were incubated for 7 d in a growth chamber at 22 °C with a photoperiod of 16 h light day (35 $\mu m/m^2/s$). Three independent experiments were performed. Mildew development, i.e., mycelial growth and sporulation density, was evaluated by visual scoring and converted to a percentage of inhibition by comparison with the control disk. Dose−response curves were obtained by plotting inhibition scores and $\log_{10}$ of extract concentration. The regression equation generated on the linear part of the sigmoid curve expressed the concentration inhibiting growth at 50% (IC_{50}).[20,22]

Pure Compounds. As previously described in the literature, pure compounds were directly added to the inoculums.[23] A final concentration of sporangial suspension at 15000 sporangia/mL final was coupled to different polyphenol dilutions (0, 50, 100, 200, 300, 500, 800, 1000 μM). Positive control (Dithane NeoTec 75 WG) and negative control (water with 1% of ethanol) experiments were carried out by mixing preparations with sporangial solution. As described above, 3 droplets of 15 μL/disk of mixed solution were deposited on foliar disks. After 7 d incubation, percentage of inhibition of *P. viticola* was determined. Furthermore, zoospore mobility was investigated by disposing 100 μL of mixed suspensions in 96-well microtiter plates. Mobile zoospores were counted after 2 h with reverse phase microscopy (magnification ×20) on five representative fields in triplicate.

Statistical Analyses. Three independent experiments were carried out for the pine knot extract and pure compounds. Statistical analysis was performed with one-way ANOVA followed by Newman−Keuls multiple comparison post hoc tests. GraphPad Prism software version 5.03 (La Jolla, CA) was used. Significant differences between IC_{50} values of polyphenols are represented by different letters (Figure 3).

RESULTS AND DISCUSSION

Characterization of Pine Knot Extract. Analysis of pine knot extract by UHPLC-DAD-MS revealed 14 polyphenols (Figure 1). Five lignans (isolariciresinol, secoisolariciresinol, nortrachelogenin, pinoresinol, matairesinol), four flavonoids (pinocembrin, pinobanksin, taxifolin, dihydrokaempferol), three stilbenes (pinosylvin, pinosylvin monomethyl ether, pterostilbene), and two phenolic acids (ferulic acid, caffeic acid) were determined by comparison to standards, UV data (λ_{max}), and mass spectrometric data (Table 1). Pinosylvin, pinosylvin monomethyl ether, pterostilbene, ferulic acid, and caffeic acid showing λ_{max} between 306 and 325 nm were correlated with the presence in their structure of one double bond in *E* configuration. In contrast, isolariciresinol, secoisolariciresinol, nortrachelogenin, pinoresinol, matairesinol, pinocembrin, pinobanksin, taxifolin, and dihydrokaempferol showed λ_{max} between 281 and 290 nm, suggesting the absence of conjugation between aromatic rings.[24] In addition, the structural discriminating properties of NMR were necessary for the unambiguous identification of the polyphenols. All compounds were isolated by preparative HPLC and subjected to NMR analysis. Structures of polyphenols in pine knot extract as well as ^{1}H NMR data presented in Table 1 were in agreement with those described by other authors.[25−31] This is the first time that pterostilbene has been clearly identified in the *Pinus* genus. A previous study revealed the presence of hydroxymonomethylpinosylvin which could be pterostilbene, but the absence of NMR data did not make it possible to confirm the correspondence.[1] In addition, the lignan matairesinol present in the *Pinus* genus is now reported for the first time in maritime pine (*P. pinaster*).

Quantitation of Polyphenols from Pine Knot Extract. To determine the polyphenol concentrations with reliability, calibration curves were plotted with purchased standard and isolated pure compounds. A range of five concentrations (0−100 μg/mL) was used in quintuplicate for each compound to obtain regression equation and assess response linearity (R^2), limits of detection (LOD), and limits of quantification (LOQ).[32] Each compound was quantitated by UV/vis diode-array detector (DAD) at its maximum wavelength for better accuracy.

In pine knot extract, the main compounds were the lignan nortrachelogenin (11.3%, w/w), followed by the flavonoid pinocembrin (4.2%, w/w), and then the stilbenes pinosylvin monomethyl ether and pinosylvin (3.3% and 2.7%, w/w respectively) (Figure 2). Among the minority compounds, the phenolic acids (caffeic acid and ferulic acid) and the flavonoid taxifolin represented 0.1% (w/w). By class of polyphenols, lignans were the major compounds with 15%, followed by

DOI: 10.1021/acs.jafc.7b04129
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stilbenes and flavonoids, which both exhibited 6.2%. The minor class was the phenol acids with 0.2%. Thus, polyphenols represented 27.6% (w/w) of pine knot extract. These data are in agreement with the literature. A previous study reported the lignan content in acetone/water extract to be between 11.1 and 21.7%, stilbene concentration up to 3.3%, and flavonoid content between 1.1 and 26.6%.[1] The low level of phenolic acids in our study (0.2%) is also in agreement with the data found in that study (0.09−0.29%).

The data concerning the contents of these polyphenols in pine knot are fundamental for industrial applications (Figure 2). Six of the 14 polyphenols, nortrachelogenin, pinocembrin, pinosylvin monomethyl ether, pinosylvin, pinoresinol, and pinobanksin with 8.49, 3.17, 2.45, 2.00, 1.31, and 1.21 g/kg of pine knot, respectively, together represented 90% of the total polyphenols isolated (20.66 g/kg of pine knot). The total polyphenol content for each class is consistent with the existing findings.[1] Indeed, by calculating their reported average yields of extract per kg of pine knot, their lignan levels ranged from 6.32 to 12.40 g/kg of pine knot while our content was 11.3 g/kg of knot. They reported flavonoid concentrations in a wide range between 0.62 and 15.2 g/kg of knot while we found 4.67 g/kg of knot. In addition, phenolic acids ranged from 0.05 to 0.16 g/kg of knot in their study while the concentration was 0.07 g/kg of knot in ours. The content of stilbenes was 4.59 g/kg of knot in our study, a value higher than theirs, which ranged from 0.22 to 1.88 g/kg of knot. The different methods of extraction together with different samples of pine knot could explain this discrepancy.

The quantitative and qualitative heterogeneity of the polyphenols we found in pine knot extract, especially the high stilbene content, strongly suggest that it can potentially be used for the biological control against *Plasmopara viticola*. The antimildew activities of the main compounds were also tested.

Antimildew Bioassays. Extract of *Pinus pinaster* knot was applied before inoculation of *Plasmopara viticola* in order to study its preventive effect. The development of mildew, i.e. mycelium density and sporulation site, was measured by visual scoring to define the inhibitory properties of pine knot extract.[20] Several concentrations were tested (0.05−1 g/L) to visualize its inhibitory potential as a sigmoid curve. At 50 and 100 mg/L, pine knot extract had an inhibition percentage of 21.3 and 69.6% respectively (data not shown). The regression equation of the linear part of the sigmoid curve allowed us to measure the IC_{50} values (concentration that inhibits 50% of mildew development). The extract had an IC_{50} value of 76 mg/L and provided total inhibition at 500 mg/L (Table 2). Previous studies have demonstrated the antifungal activities of natural plant extracts against *P. viticola*. For example, an experiment on *Picea abies* bark, another conifer, revealed an IC_{50} value of 760 mg/L, so pine extract seems to be 10-fold more powerful.[23] A study in which 3000 extracts from 800 plants and 100 fungi were screened against downy mildew showed a strong antifungal activity of *Juncus effusus* extract with an IC_{50} value of 123 mg/L, which is still less active than pine extract.[33] Furthermore, extracts of vine byproducts such as grapevine cane, wood, and root showed IC_{50} values of 210, 60, and 120 mg/L respectively.[34] Therefore, like vine wood extract, pine knot extract could become a very important natural antifungal agent. In addition, a comparison with a commercial fungicide (Dithane NeoTec 75 WG) that provides total inhibition at 200 mg/L suggests that the antimildew activity of pine knot extract is comparable (Table 2). Thus, to reduce chemical inputs, the extract of pine knot could constitute a complementary strategy to the use of pesticides against downy mildew.

Table 2. Inhibition (IC_{50} and IC_{100}) of Downy Mildew (*Plasmopara viticola*) Development by Pine Knot Extract (*Pinus pinaster*) (mg/L)

	mildew development		
	IC_{50}	IC_{100}	R^2
negative control[a]	–[c]	–	–
positive control[b]	15	200	0.985
pine knot extract	76	500	1

[a]Negative control = water with 1% of ethanol. [b]Positive control = Dithane NeoTec 75 WG (mancozeb at 75%). [c]Absence of inhibition.

To explain its antifungal activity, the main compounds of pine knot extract were tested against downy mildew. Four lignans (nortrachelogenin, pinoresinol, matairesinol, isolariciresinol), two flavonoids (pinocembrin, pinobanksin), and two stilbenes (pinosylvin, pinosylvin monomethyl ether) were prepared with mildew zoospores to investigate their impact on zoospore mobility and mildew development. The compounds with the greatest ability to block zoospore mobility and inhibit mildew development (IC_{50} values) were pinosylvin monomethyl ether (23 and 18 μM respectively), pinosylvin (34 and 23 μM respectively), and pinocembrin (22 and 19 μM respectively) (Figure 3). These results are in agreement with those described by other authors. Indeed, the pinosylvins from *Pinus* trees have already shown significant antifungal activities against white rot and brown rot fungi.[14] The flavonoid pinocembrin also possesses antifungal properties against *Penicillium italicum* (green mold) and *Candida albicans*.[11,35] Moreover, these low IC_{50} values, meaning high antifungal activity, are similar in efficacy against downy mildew to the most active natural compounds reported in the literature such as pterostilbene, δ-viniferin, and vitisin B with IC_{50} which have values of 12, 14, and 13 μM respectively.[36,37] Among the least effective compounds, lignans such as nortrachelogenin, pinoresinol, and isolariciresinol showed IC_{50} values greater than 220 μM. These results are consistent with the literature in which the antimicrobial activities (*Aspergillus fumigatus* and *Penicillium brevicompactum*) of pine extracts were correlated with the presence of stilbenes while the activity of lignans was shown to be more marginal.[12]

In conclusion, we report the strong antifungal activity of *Pinus pinaster* knot extract against *Plasmopara viticola*. The high concentrations of pinosylvin, pinosylvin monomethyl ether, and pinocembrin that it contains could contribute to this effect. Indeed, bioassays with pure compounds showed the very promising antimildew properties of these three molecules. The present findings therefore show that *Pinus pinaster* byproducts such as knot could be used in viticulture to control *Plasmopara viticola*. As a source of biodegradable, less toxic, and bioactive compounds, pine knot represents a valuable biomass with a very promising economic potential. The search for alternative strategies to the use of synthetic pesticides by developing natural plant extracts such as pine knot extract is now paving the way for major steps to be made in sustainable viticulture.

DOI: 10.1021/acs.jafc.7b04129
J. Agric. Food Chem. 2017, 65, 8884−8891

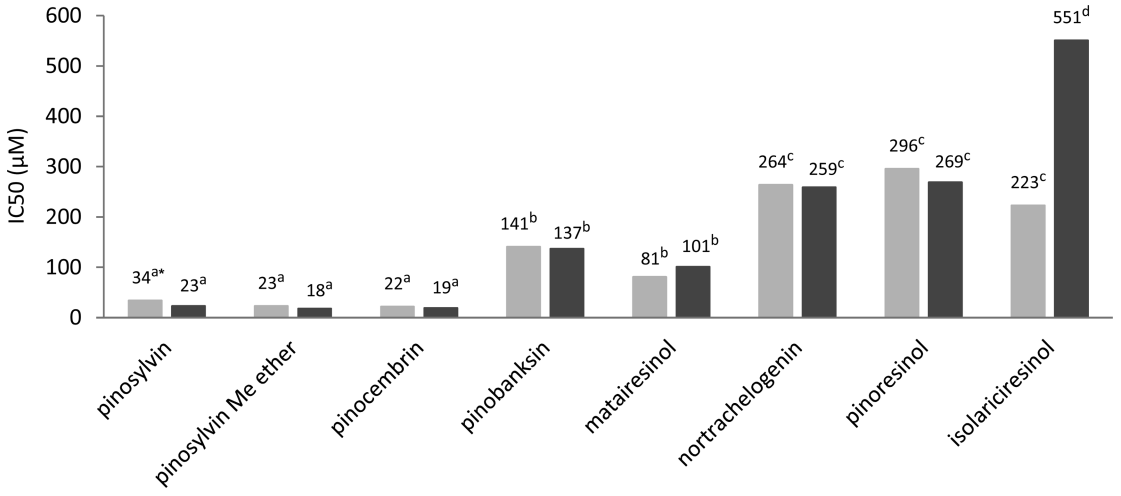

Figure 3. IC_{50} values of zoospore mobility and downy mildew development for pure compounds (μM). Light gray bar = zoospore mobility; dark gray bar = mildew development; pinosylvin Me ether = pinosylvin monomethyl ether. *Different letters indicate significant difference between IC_{50} values of polyphenols according to one-way ANOVA followed by Newman−Keuls multiple comparison post hoc tests.

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Funding

The work was supported by the Bordeaux Metabolome Facility and MetaboHUB (ANR-11-INBS-0010 project).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We kindly thank Gérard Fondeville and Eric Pedrot for their technical assistance. We are also grateful to S. Gambier for providing the plants (INRA, UMR SAVE).

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