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Stilbenes from common spruce (*Picea abies*) bark as natural antifungal agent against downy mildew (*Plasmopara viticola*)

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ABSTRACT

The antifungal activity of spruce bark (*Picea abies* (L.) Kars. Pinaceae) extract against downy mildew (*Plasmopara viticola*) was investigated. The analysis of the extract using UHPLC–MS allowed stilbenes to be targeted as the main components. The structure of stilbenes isolated from the hydro-alcoholic extract by preparative HPLC was determined by mass and NMR analysis. In addition to aglycone and glycosylated monomeric forms such as piceatannol, astringin, resveratrol, piceid, isorhapontigenin and isorhapontin, several stilbene glycoside dimers were also identified. Six new compounds (piceasides I-N) are reported for the first time in the literature and the plant kingdom. The extract as well as pure compounds showed significant antifungal activity with an inhibition of the zoospore mobility and mycelium growth of *P. viticola*. Our results show that spruce bark extract, which is considered as a wood industry co-product, could potentially be used as a natural fungicide against downy mildew.

1. Introduction

The oomycete *Plasmopara viticola* is the cause of downy mildew which is one of the most economically important diseases in cultivated grapevines worldwide (Gessler et al., 2011). Attacking flower clusters, leaves and grape berries, this endemic disease has generally been controlled by using several fungicide applications such as "Bordeaux mixture", which has been widely used since the late 19th century (Gessler et al., 2011; Leroux and Clerjeau, 1985). However, its extensive use causes a negative impact on the environment with the accumulation of copper in soil or metabolic deregulation in grape berries (La Torre et al., 2011; Martins et al., 2014) or the appearance of fungicide resistance (Corio-Costet, 2012). In addition, human health is concerned through the consumption of berries and wine especially with the possible presence of fungicide residues (Chen et al., 2016; Guo et al., 2016; Lagunas-Allué et al., 2012). As a consequence there is renewed interest in the search for effective ecofriendly and sustainable natural agents to control diseases and pests because of changes in environment and food safety regulations associated with consumer demand. The exploitation of plant extracts containing a wide variety of antimicrobial compounds that are less toxic and biodegradable might be a solution.

Stilbenes are phenolic compounds derived from secondary plant metabolism. Despite the chemical diversity of stilbenes from monomers to octamers, the distribution of natural stilbenoids in the plant kingdom is limited (Pawlus et al., 2012; Rivière et al., 2012). They are usually associated with resistance to plant diseases and their synthesis as phytoalexins is a response to an attack by plant pathogens. Some studies have focused on the involvement of these compounds in grapevine defense. It has been shown that resveratrol, pterostilbene (monomers) and ε- and δ-viniferins (dimers) from *Vitis vinifera* leaves and berries are able to inhibit *Plasmopara viticola* (downy mildew) or *Botrytis cinerea* (gray mold) (Adrian and Jeandet, 2012; Bavaresco et al., 1997; Dufour et al., 2013; Pezet et al., 2004). More complex stilbenes present in grapevine canes such as vitisin B and hopeaphenol (tetramers) have also shown significant antifungal activity against *P. viticola* (Schnee et al., 2013) and several fungi involved in wood diseases in vine (Lambert et al., 2012). In addition, grapevine cane extracts containing a wide spectrum of stilbenoid compounds have shown significant antifungal activity against *P. viticola* and are thus considered as a potential natural fungicide (Richard et al., 2016; Schnee et al., 2013).

Spruce (*Picea abies* (L.) Karst.), which is a member of the Pinaceae family, is one of the most abundant conifer species in Eurasian forests

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and the most promising in terms of added value. Often thrown away or burned, co-products generated by wood companies like bark are rich in stilbenes and potentially recoverable (Jyske et al., 2014; Latva-Mäenpää et al., 2014). Spruce bark is especially composed of glycosylated monomeric stilbenes such as astringin, piceid and isorhapontin. Their respective aglycone forms, *i.e.* piceatannol, resveratrol and isorhapontigenin, are in smaller amounts (Mulat et al., 2014). Dimeric stilbenes in spruce bark have also been identified with astringin-astringin and astringin-isorhapontin dimers named piceasides (A–H) (Li et al., 2008). Furthermore, the antifungal activity of stilbenoids from spruce bark has been investigated. Astringin, isorhapontin and isorhapontigenin have been assayed *in vitro* against conifer infecting fungi. The aglycone isorhapontigenin had the most toxic activity while the glycosylated stilbene exhibited a lower antifungal activity (Woodward and Pearce, 1988).

The aim of this study was to characterize a spruce bark extract with analytic tools (UHPLC–MS, 1D- and 2D-NMR) and to investigate its bioactivity against downy mildew (*P. viticola*). The antifungal activity of the main stilbene compounds present in the extract was also tested. The significant presence of stilbene in these co-products could allow spruce bark to be used as a new natural antifungal agent.

2. Materials and methods

2.1. Chemicals

For UHPLC–MS analysis, LC–MS-grade acetonitrile was purchased from VWR (Fontenay-sous-Bois, France) and formic acid from Fisher Scientific (Loughborough, U.K.). Water was purified by an Elga water purification system (Bucks, U.K.). HPLC-grade acetonitrile and trifluoroacetic acid (TFA) were obtained from Sigma-Aldrich (St. Louis, MO, USA) for preparative HPLC. HPLC-grade methanol was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Plant material

Grapevine plants (*Vitis vinifera* cv. Cabernet-Sauvignon) provided by UMR SAVE (INRA, Villenave d'Ornon, France) were propagated in a greenhouse at 25 °C with a relative humidity at 75% and 15 h day/9 h night photoperiod. To perform downy mildew assays, 60-day-old seedlings with 10–12 leaves were used and the third or fourth leaves below the apex were collected.

2.3. Pathogen material

Biological tests were conducted on *Plasmopara viticola* isolate (ANN-01) collected in 2015 on *Vitis vinifera* cv. Ugni blanc in commercial vineyards located in Charente (France). The isolate was multiplied and inoculated on grapevine leaves (*V. vinifera* cv. Cabernet Sauvignon) according to the method used by Corio-Costet et al. (2011). Sporangia were collected and suspended in sterile water at 4 °C and then inoculated on the abaxial face of fresh grapevine leaves with 20 droplets of 15 µL from an inoculum at 5000 sporangia per mL. After incubation at 22 °C in the dark for 24 h allowing stomata opening and zoospore penetration, the droplets were removed and Petri dishes were incubated for 7 days at 22 °C with a 16 h/8 h day/night photoperiod.

2.4. Spruce bark extract

Spruce bark (*Picea abies* (L.) Kars. Pinaceae) was kindly supplied by Actichem S.A (Montauban, France). Bark was harvested from common spruce trunks in the Eastern region of France, dried for one month in the dark and then ground in a crusher. Three kilograms of spruce bark powder were extracted with 30 L of ethanol-water mixture (85/15, v/v) at 60 °C for two hours. The filtrate was evaporated in a 150-L reactor/evaporator and the resulting extract was dried in a Guedu® dryer (De Dietrich SAS, Semur-en-Auxois, France) affording 200 g of spruce bark extract.

2.5. Isolation by preparative HPLC

The various stilbenes were isolated from spruce extract by preparative HPLC Varian Pro Star equipped by Agilent Zorbax SB-C18 PrepHT (250 mm × 21.2 mm, 7 µm) column. Elution was achieved with a solvent system consisting of water acidified with 0.025% TFA (solvent A) and acetonitrile acidified with 0.025% TFA (solvent B) with a flow rate at 18 mL/min. The purification was performed with the following gradient: 20% B (from 0 to 5 min), 20–30% B (from 5 to 30 min), 30–40% B (from 30 to 35 min), 40–100% B (from 35 to 36 min), 100% B (from 36 to 41 min), 100–20% B (from 41 to 45 min).

2.6. UPLC–DAD/ESI-IT analysis

The UPLC–DAD/ESI-IT apparatus was an Agilent 1290 Series (Agilent Technologies, Santa Clara, CA) equipped with an UV–VIS-DAD and an Esquire 6000 ion trap (IT) mass spectrometer using an ESI source (Bruker-Daltonics, Billerica, MA). Analysis was performed on an Agilent Zorbax SB-C18 (100 mm × 2.1 mm × 1.8 µm) column. Spruce extract was dissolved in methanol-water mixture (1/1; v/v) at 1 mg/mL, filtered with 0.45 µm PTFE, and injected using 1 µL injection volumes. Elution was carried out with a solvent system consisting of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). Separation was performed using a flow rate of 0.4 mL/min. The run was as follows: 10% B (from 0 to 1.7 min), 10–20% B (from 1.7 to 3.4 min), 20–30% B (from 3.4 to 5.1 min), 30% B (from 5.1 to 6.8 min), 30–35% B (from 6.8 to 8.5 min), 35–60% B (from 8.5 to 11.9 min), 60–100% B (from 11.9 to 15.3 min), 100% B (from 15.3 to 17 min), 100–10% B (from 17 to 17.3 min). Mass spectrometry analyses were performed in negative mode with a range of m/z 100–1200. The drying gas used was nitrogen at 10 L/min at 365 °C with nebulizer pressure at 40 psi. Capillary voltage was 3100 V. Bruker Data Analysis 3.2 software was used for data processing.

2.7. UPLC–DAD/ESI-Q-TOF analysis

The UPLC–DAD/ESI-Q-TOF system was an Agilent 1290 Infinity (Agilent Technologies, Santa Clara, CA) equipped with an UV–VIS-DAD and an ESI-Q-TOF mass spectrometer (Agilent 6530 Accurate Mass). Analysis was carried out on an Agilent Zorbax SB-C18 (100 mm × 2.1 mm × 1.8 µm) column. Pure compounds were dissolved at 250 µg/mL. Separation was performed with a solvent system consisting of solvent A (water with 0.1% formic acid) and solvent B (methanol with 0.1% formic acid). Separation was performed using a flow rate of 0.3 mL/min. The run was as follows: 1–100% B (from 0 to 5 min), 100% (from 5 to 6 min) and 100–1% (from 6 to 7 min). Mass spectrometry analyses were performed in negative mode. The drying gas used was nitrogen at 9 L/min at 300 °C with nebulizer pressure at 25 psi. The sheath gas flow and temperature were set at 11 L/min and 350 °C. Capillary voltage was 4000 V. The data was processed by Mass Hunter Qualitative Analysis software.

2.8. NMR analysis

All ^{1}H and 2D NMR (COSY, ROESY, HSQC, HMBC) analyses were performed on a Bruker Ultrashield 600 MHz NMR spectrometer equipped with a cryogenic TXI probe head. NMR spectra were obtained in 3-mm tubes with d_4-methanol as solvent. The data were analyzed with Bruker Topspin software version 3.2.

2.9. Antifungal assay against Plasmopara viticola

2.9.1. Spruce bark extract

After subculturing downy mildew sporangia on new grapevine leaves as described above and incubation for 7 days, the freshly inoculum was used for bioassays. For spruce extract bioassays, fresh leaves of *Vitis vinifera* cv. Cabernet Sauvignon were washed under water and dried between two sheets of filter paper. Leafs discs were generated with a 25-mm-wide pastry-cutter. Eight discs from eight different leaves were placed with the abaxial surface facing upward in Petri dishes containing Whatman paper soaked with 3.5 mL of sterile water. Finally, eight repetitions were carried out for each concentration. The bark extract range concentration (0, 0.05, 0.1, 0.2, 0.3, 0.5, 0.8, 1 g/L) was prepared in water with 1% of ethanol for better dissolution. Antifungal assays were performed by spraying the different dilutions with a hand-held sprayer having a pressure reserve (Ecospray®) on the several leaf discs. Control experiments were performed by applying sterile water with 1% of ethanol. After drying the surfaces for 24 h, three droplets of 15 µL per disc were inoculated from an inoculum at 15×10^3 sporangia per mL. The number of sporangia was previously counted with a Malassez cell under a light microscope at ×100 magnification. As described above, the Petri dishes were then incubated in a growth chamber at 22 °C with a 16 h light day (35 µm/m^2/s) for 7 days. Three independent experiments were done. Fungal mycelial growth and sporulation density were visually evaluated by scoring between 0 and 100. For each concentration, the average score was converted to a percentage of inhibition by comparison with the control disc. The dose response curve obtained by plotting inhibition scores against $\log_{10}$ of extract concentration made it possible to determine the concentration inhibiting growth at 50% (IC_{50}).

2.9.2. Stilbenoids

For stilbenoid experiments, target compounds were directly added to the sporangial suspensions as described by Schnee et al. (2013). Suspensions at 30×10^3 sporangia per ml were prepared while stilbenes were prepared with a range of eight concentrations (0, 100, 200, 400, 600, 1000, 1600, 2000 µM) in sterile water with 1% of ethanol. Thus, 1 vol of sporangial suspension (15×10^3 sporangia per ml final) and 1 vol of stilbene dilution (0, 50, 100, 200, 300, 500, 800, 1000 µM final) were mixed. Control experiments were performed by mixing sterile water with 1% of ethanol with sporangial suspension. As described above, three droplets of 15 µL per disc (eight discs total) of mixed suspension were inoculated and downy mildew inhibition was measured seven days after incubation.

In addition, 100 µL of mixed suspensions were deposited in triplicate in 96-well microtiter plates and the number of mobile zoospores was counted after two hours with reverse phase microscopy.

2.10. Statistical analyses

Three independent experiments were performed for the spruce bark extract as well as for the pure compounds. Data are shown as IC_{50} ± standard deviations. The statistical analysis was carried out with one-way ANOVA followed by Newman-Keuls multiple comparison post hoc tests by using GraphPad Prism software. Significant differences between IC_{50} values of stilbenes are represented by different letters.

3. Results and discussion

3.1. Characterization of spruce bark extract

Extract of spruce bark was analyzed by UHPLC–DAD/ESI–MS giving the chromatogram shown in Fig. 1. Nineteen compounds were recognized as stilbenes by maximum absorption at 280, 306 and 320 nm which are the UV absorption properties of stilbenes (Mulat et al., 2014). Each compound was isolated by preparative HPLC.

Compounds **1**, **4** and **5** exhibited quasi-molecular $[M-H]^-$ ions in negative mode at *m/z* 405, 389 and 419, respectively (Table 1). The fragmentation patterns of the three compounds showed the loss of hexose unit characteristic of glycosylated stilbenes. According to ^{1}H NMR spectra obtained and literature data (Mulat et al., 2014), compound **1** was identified as *trans*-astringin, compounds **4** as *trans*-piceid and compound **5** as *trans*-isorhapontin.

Compounds **8**, **12**, and **16** exhibited quasi-molecular $[M-H]^-$ ions at *m/z* 243, 227 and 257, respectively. These molecular ions corresponded to compounds **1**, **4** and **5** without the hexose unit, which means that it was probably their aglycone forms. Comparison of ^{1}H NMR data with the literature identified compounds **8**, **12** and **16** as *trans*-piceatannol, *trans*-resveratrol and *trans*-isorhapontigenin, respectively (Mulat et al., 2014).

Compounds **2**, **3**, **6** and **7** showed a quasi-molecular $[M-H]^-$ ion at *m/z* 809, whereas compounds **9** and **10** exhibited a quasi-molecular $[M-H]^-$ ion at *m/z* 823. These data combined with MS/MS fragmentation revealed the presence of glycosylated stilbene dimers. In addition, ^{1}H NMR and 2D NMR (COSY, ROESY, HSQC, HMBC) spectra matched with data reported by Li et al. (2008). Compounds **2**, **3**, **6** and **7** were identified as piceasides A, B, H and G, respectively. These molecules are astringin-astringin dimers. Compounds **9** and **10** were piceasides E and F, respectively, which are astringin-isorhapontin dimers.

Compound **19** with the molecular ion $[M-H]^-$ at *m/z* 485 was studied by ^{1}H and 2D NMR. It was identified as cassigarol E, a piceatannol-piceatannol dimer (Baba et al., 1994), and was previously isolated from the bark of *Picea jezoensis* (Wada et al., 2009).

Compound **11** was obtained as a pale brown powder. Its molecular formula was determined to be $C_{34}H_{32}O_{13}$ by HRESIMS. Its negative-mode HRESIMS spectrum exhibited a quasi-molecular $[M-H]^-$ ion at *m/z* 647.1796 (calculated: 647.1770). Its MS/MS fragmentation pattern exhibited three main fragments at *m/z* 485, 405 and 243 (Table 1), so compound **11** is an astringin-piceatannol dimer. Its structure was elucidated using ^{1}H NMR and 2D NMR (COSY, ROESY, HSQC, HMBC) spectra. NMR data are reported in Table S1. The NMR data indicated the presence of two ABC spin systems corresponding to the presence of 1,3,4-trisubstituted aromatic rings [δ_H 7.16 (H-2a), 6.95 (H-5a), 7.08 (H-6a) and 6.65 (H-2b), 6.64 (H-5b), 7.46 (H-6b)], two ABC spin systems associated to 1,3,5-trisubstituted aromatic rings [δ_H 6.81 (H-10a), 6.47 (H-12a), 6.64 (H-14a) and 6.11 (H-10/14b), 6.16 (H-12b)], two olefinic protons [δ_H 7.03 (H-7a) and 6.91 (H-8a)] with large coupling constant (J = 16.3 Hz) owing to a *trans* double bond system, a pair of AB doublets [δ_H 4.74 (H-7b) and 4.71 (H-8b)], and a glucose moiety. The presence of a dihydro-1,4-dioxin system was confirmed on the basis of the specific carbon chemical shifts of the AB system (δ_C 81.5 and 81.9) (Li et al., 2008). The connectivities between the different systems were established using HMBC and ROESY experiments (Table S1). Because HMBC correlations between the oxygen bridge are complicated to observe, the determination of the connections between the two piceatannol units was based on the ROESY correlation between H-2a and H-7b. This result is in agreement with literature data concerning stilbene dimers in the bark of *Picea* species (Li et al., 2008; Wada et al., 2009). The connection of the glucose unit to C-11a was based on the HMBC correlation between the anomeric proton H-1′ and C-11a and was confirmed by ROESY correlations H-1′/H-10a and H-1′/H-12a. The *trans* relative configuration of C-7b and C-8b was determined from the ROESY correlations between H-7b/H10/14b, H-8b/H-2b and H-8b/H-6b and confirmed by the coupling constant between H-7b and H-8b (J = 8.0 Hz) (Li et al., 2008). Compound **11** was accordingly characterized as a new astringin-piceatannol dimer, and named piceaside I (Fig. 2).

Compounds **13** and **14** were obtained as a brown powder. They exhibited a quasi-molecular $[M-H]^-$ ion at *m/z* 647.1793 and 647.1787 respectively. A molecular formula of $C_{34}H_{32}O_{13}$ was determined for these two compounds, which suggests that they could be

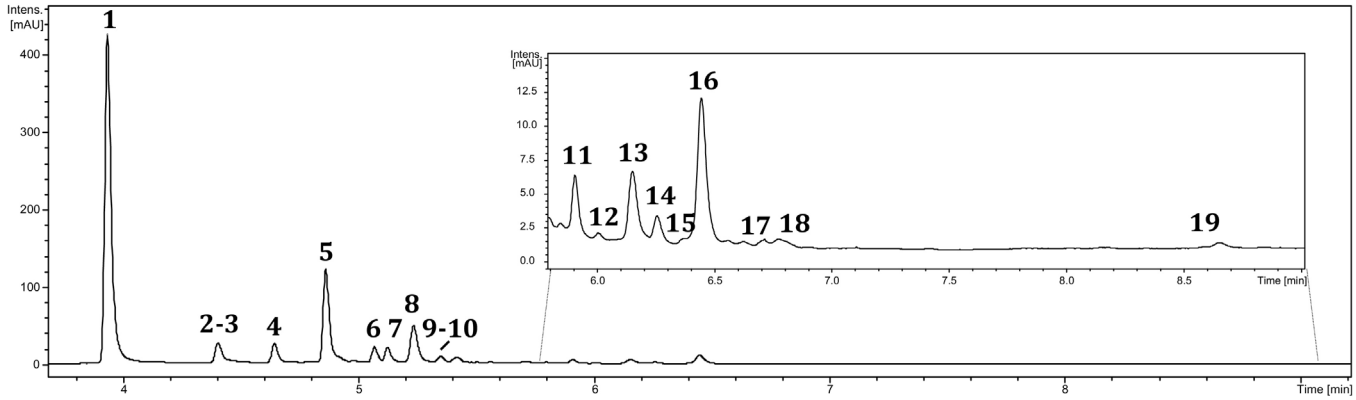

Fig. 1. UHPLC–DAD chromatogram of spruce bark extract at 320 nm.Footnote: (**1**) *trans*-astringin, (**2/3**) piceasides A/B, (**4**) *trans*-piceid, (**5**) *trans*-isorhapontin, (**6**) piceaside H, (**7**) piceaside G, (**8**) *trans*-piceatannol, (**9/10**) piceaside E/F, (**11**) piceaside I, (**12**) *trans*-resveratrol, (**13**) piceaside J, (**14**) piceaside K, (**15**) piceaside L, (**16**) *trans*-isorhapontigenin, (**17**) piceaside M, (**18**) piceaside N, (**19**) cassigarol E.

Table 1
Retention time, MS and MS/MS data of stilbenes identified in common spruce (*Picea abies*) bark by UHPLC/ESI–MS.

No.	Compound	R_t (min)	$[M-H]^-$ (*m/z*)	MS/MS fragments
1	*trans*-astringin	3.9	405	243
2–3	piceaside A/B	4.4	809	647; 485; 405; 243
4	*trans*-piceid	4.6	389	227
5	*trans*-isorhapontin	4.9	419	257
6	piceaside H	5.1	809	647; 485; 405; 243
7	piceaside G	5.1	809	647; 485; 405; 243
8	*trans*-piceatannol	5.2	243	–
9–10	piceaside E/F	5.3	823	661; 499; 405; 243
11	piceaside I	5.9	647	485; 405; 243
12	*trans*-resveratrol	6.1	227	–
13	piceaside J	6.2	647	485; 405; 243
14	piceaside K	6.3	647	485; 405; 243
15	piceaside L	6.4	661	499; 405; 243
16	*trans*-isorhapontigenin	6.5	257	–
17	piceaside M	6.7	661	499; 405; 243
18	piceaside N	6.7	661	499; 405; 243
19	cassigarol E	8.6	485	243

astringin-piceatannol dimers. Their structure was elucidated using ^1H NMR and 2D NMR experiments. Data are reported in Table S2. NMR data suggests that the aglycone part of compounds **13** and **14** is the same as that of **11**. The only difference between these compounds and piceaside I (**11**) is the connection of the glucose unit on carbon C-11b. This connection was determined by HMBC correlation between the anomeric proton H-1′ and C-11b and was confirmed by the ROESY correlations H-1′/H-10b and H-1′/H-12b. The specific connection of the two piceatannol units was based on the ROESY cross-peaks H-2a/H-7b and H-5a/H-8b. The coupling constant between H-7b and H-8b (J = 8.0 Hz) and the ROESY correlations between H-7b/H10b, H-7b/H-14b, H-8b/H-2b and H-8b/H-6b indicated the 7b,8b-*trans* relative configuration of the two compounds. Hence compounds **13** and **14** were identified as a pair of diastereomeric astringin-piceatannol dimers, and named piceasides J and K (Fig. 2). Biotransformation studies of astringin by a fungus named *Ceratocystis polonica* had already allowed the formation *in vitro* of compounds **13** and **14** (Hammerbacher et al., 2013). However, to our knowledge, these two astringin-piceatannol dimers are now reported for the first time in the plant kingdom.

Compound **15** was obtained as a brown powder with a quasi-molecular ion $[M-H]^-$ at *m/z* 661.1936 giving a molecular formula of $C_{35}H_{34}O_{13}$. The structure was identified by ^1H NMR and 2D NMR. NMR data are reported in Table S1. Comparison of their NMR data with those of piceaside I (**11**) indicated that the only difference between the two compounds was the presence of an *O*-methyl group on position C-3b. As for compound **11**, ROESY correlations indicated the *trans* relative configuration of C-7b and C-8b. These data are consistent with another type of stilbene dimer formed by the conjugation of an astringin unit and an isorhapontigenin unit. Hence compound **15** was accordingly characterized as a new stilbene dimer and named piceaside L (Fig. 2).

Compounds **17** and **18** were obtained as a brown powder and their molecular formula of $C_{35}H_{34}O_{13}$ was determined by the presence of a quasi-molecular ion $[M-H]^-$ at *m/z* 661.1961 and 661.1955, respectively. As for piceaside L (**15**), examination of their NMR data (Table S3) indicated that the basic skeleton of compounds **17** and **18** was the same as that of **13** and **14**. The only difference between the two pairs of compounds was the presence of an *O*-methyl group on position C-3b. Compounds **17** and **18** were identified as a new pair of diastereomeric isorhapontin-piceatannol dimers, named piceasides M and N, respectively (Fig. 2).

3.2. Quantification of stilbenes in spruce bark extract

Stilbenes were quantified in spruce trunk bark by UHPLC–DAD with standards isolated in laboratory from spruce bark extract. The calibration curves were carried out in the range of five concentrations (0–100 µg/mL) in quintuplicate according to Feinberg (2001). Each compound was quantified with its own calibration equation except piceaside H, which was quantified by piceaside G, piceaside J and K quantified by piceaside I and piceaside M and N quantified by piceaside L. The limits of detection (LOD) and quantification (LOQ) as well as the determination coefficient R^2 were determined for each compound (Table 2).

The main compounds were the glycosylated stilbenes astringin (11.57 g/kg spruce barks) and isorhapontin (2.59 g/kg spruce barks) (Table 2). Their aglycons form piceatannol (0.89 g/kg spruce barks) and isorhapontigenin (0.45 g/kg spruce barks) were respectively the third and sixth most present components. From dimers, the most abundant were the astringin-astringin dimers such as piceaside G (0.59 g/kg spruce barks), piceaside H (0.56 g/kg spruce barks) and the mixture piceasides A/B (0.41 g/kg spruce barks). Some dimeric stilbenes as isorhapontin-piceatannol dimers (piceasides M and N) were unable to be quantified as they are below to the LOQ score. Finally, the total content of stilbenes quantified amounted to 18.45 g/ kg of spruce barks.

The relative abundance of each stilbene in spruce trunk bark revealed the huge presence of monomeric stilbenes which represented 86.07% of stilbenes against 13.93% for dimeric stilbenes. Astringin alone accounted for 62.71% of stilbene total followed by isorhapontin with 14.04% of stilbene total (Table 2). Thus, only two stilbenes on nineteen identified represented three-quarters of the spruce bark stilbenes.

Literature data was in agreement with our observations. Angelis et al. (2016) reported that astringin was the major stilbene in spruce

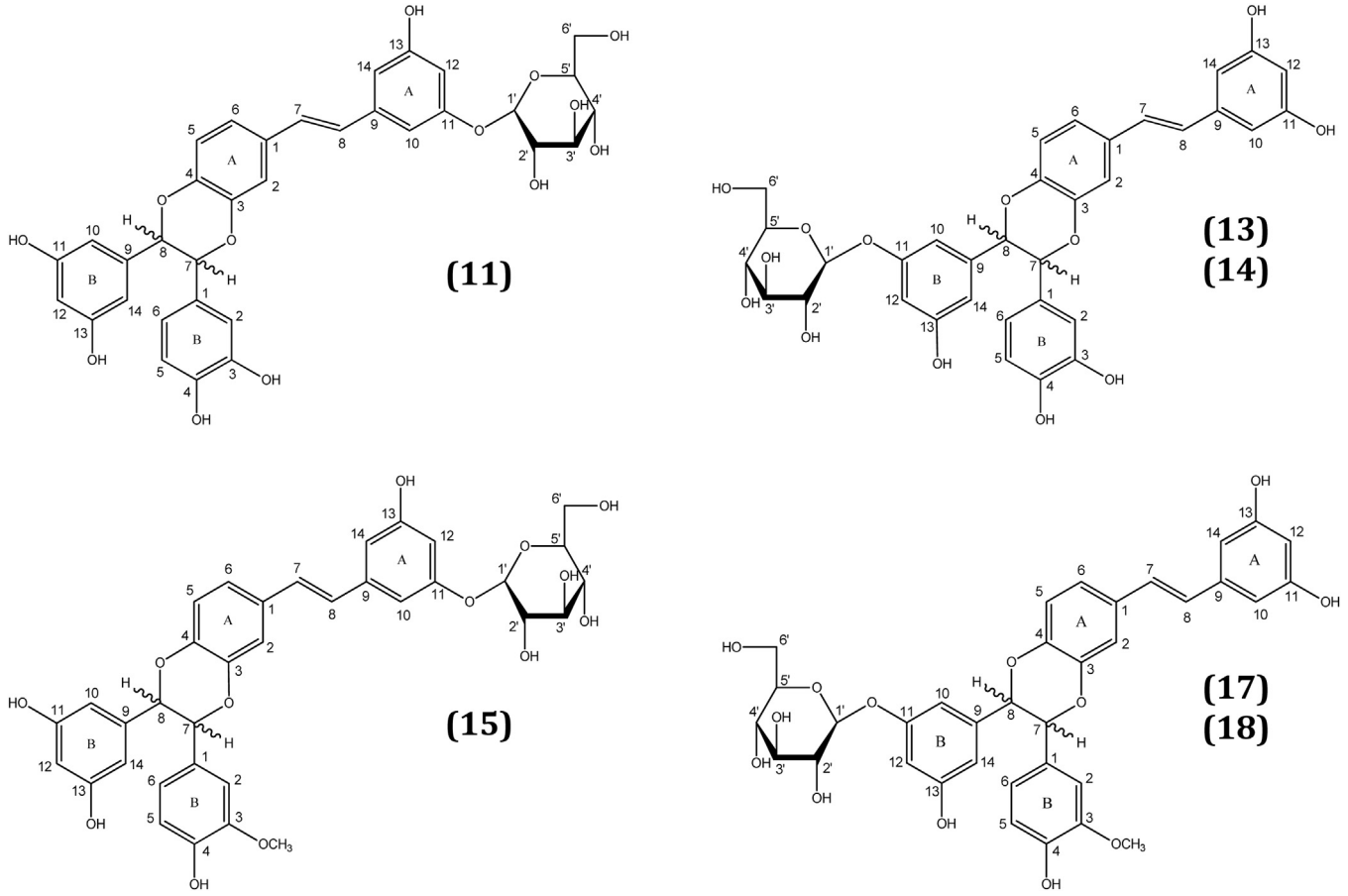

Fig. 2. Chemical structure of new dimers isolated from spruce bark extract.

Table 2
Quantification of stilbenoids in spruce bark (*Picea abies*).

No.	Compound	LOD (µg/mL)	LOQ (µg/mL)	Concentration (g/kg bark)	Proportion (%)
1	astringin	0.02	0.21	11.57	62.7
2/3	piceaside A/B	0.04	0.40	0.41	2.2
4	piceid	0.17	0.68	0.35	1.9
5	isorhapontin	0.03	0.31	2.59	14.0
6	piceaside H[a]	0.04	0.40	0.56	3.0
7	piceaside G	0.04	0.40	0.59	3.2
8	piceatannol	0.01	0.25	0.89	4.8
9/10	piceaside E/F	0.02	0.16	0.21	1.1
11	piceaside I	0.05	0.26	0.21	1.1
12	resveratrol	0.06	0.38	0.03	0.2
13	piceaside J[b]	0.05	0.26	0.35	1.9
14	piceaside K[b]	0.05	0.26	0.12	0.7
15	piceaside L	0.04	0.36	0.08	0.4
16	isorhapontigenin	0.04	0.31	0.45	2.4
17	piceaside M[c]	0.04	0.36	NQ	NQ
18	piceaside N[c]	0.04	0.36	NQ	NQ
19	cassigarol E	0.06	0.49	0.04	0.2
	Total			18.45	100

[a] Piceaside G (**7**) was used for quantification of Piceaside H (**6**).
[b] Piceaside I (**11**) was used for quantification of piceaside J (**13**) and K (**14**).
[c] Piceaside L (**15**) was used for quantification of piceaside M (**17**) et N (**18**). NQ means detected but not quantified because under LOQ score.

trunk bark followed by isorhapontin. In bark of spruce root, the opposite was revealed. Mulat et al. (2014) found respectively 29.5 g/kg dry mass and 10.7 g/kg dry mass for isorhapontin and astringin. Concerning dimer content in spruce bark, no data have been reported in the literature to our knowledge.

Table 3
A: Concentration inhibiting 50% of *P.viticola* development by spruce bark extract (g/L) ± standard deviation; B: Concentration inhibiting 50% of zoospore mobility and *P. viticola* development by stilbenes (µM) ± standard deviation.

A	Mildew development	
	IC_{50}	R^2
spruce bark extract	0.76 ± 0.12	1

B	Mildew development		Zoospore mobility	
compounds	IC_{50}	R^2	IC_{50}	R^2
astringin	705 ± 101[e]	1	659 ± 127[e]	0.985
isorhapontin	272 ± 34[c]	1	228 ± 38[c]	0.997
piceatannol	254 ± 35[c]	1	249 ± 95[c]	0.974
isorhapontigenin	116 ± 26[b]	0.994	76 ± 2[b]	0.992
piceaside G	677 ± 76[e]	0.982	799 ± 142[e]	0.977
piceaside H	551 ± 76[d]	1	524 ± 133[d]	1
piceaside I	147 ± 20[b]	1	225 ± 18[c]	1
piceaside J	96 ± 27[b]	1	109 ± 32[b]	0.957
piceaside L	145 ± 6[b]	1	142 ± 1[b]	1
cassigarol E	24 ± 2[a]	1	24 ± 2[a]	1

Letters indicate significant difference between IC_{50} values of stilbenes according to one-way ANOVA followed by Newman Keuls multiple comparison post hoc tests.

3.3. Downy mildew assays

After completing characterization, the antifungal activity of spruce bark extract was investigated. As winemakers need to use antifungal products upstream of infection, *i.e.* for the purpose of prevention, spruce bark extract was sprayed onto the leaves before inoculation of *P.*

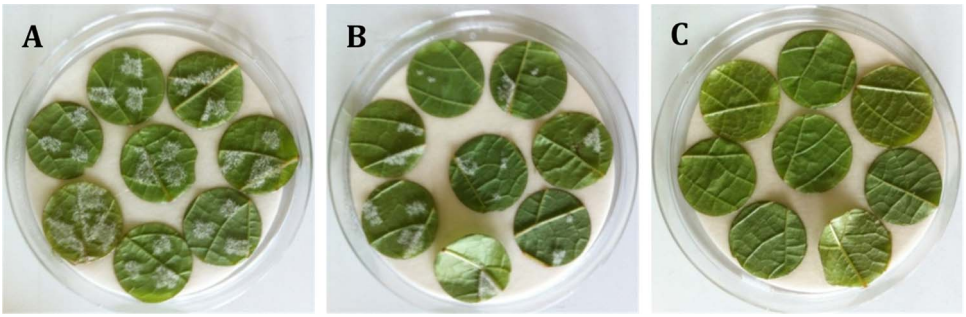

Fig. 3. Fungal mycelial development of *P. viticola* after addition of spruce bark extract. (A) control with aqueous solution at 1% of ethanol, (B) inhibition of downy mildew at 50% (IC_{50} = 0.76 g/L of spruce bark extract), (C) inhibition of downy mildew at 100% (total inhibition = 1 g/L of spruce bark extract).

viticola. Downy mildew development was evaluated by visual scoring seven days after infection to determine the inhibition potential of spruce bark extract.

A wide range of concentrations was performed (0.05–1 g/L) to observe the different levels of inhibition and obtain more precise information on IC_{50} evaluation (concentration which inhibits 50% of pathogen development). Spruce bark extract had an IC_{50} at 0.76 g/L (Table 3A) and totally inhibited the development of downy mildew approximately at 1 g/L (Fig. 3). *P. viticola* has already been the subject of antifungal tests by natural stilbene-enriched extracts with *Vitis vinifera* cane extracts (Schnee et al., 2013). Crude extract was tested at only 1 g/L and led to two classifications: "Fungitoxicity (+)/No fungitoxicity (−)". Stilbene-enriched extracts of cane were toxic on *P. viticola* at 1 g/L.

The main stilbenes of spruce bark extract were then isolated by preparative HPLC and tested on *P. viticola.* Ten major stilbenes were purified in sufficient quantity to allow bioassays (astringin, isorhapontin, piceatannol, isorhapontigenin, piceaside G, H, I, J, L and cassigarol E). To understand the mechanisms of action of stilbenes on downy mildew, zoospore mobility and mycelium development were investigated. Thus, stilbenes and zoospores were prepared in mixture and then added onto grapevine leaves or to 96-well microtiter plates.

Stilbenes with the highest activity against zoospore mobility and downy mildew development were the piceatannol-piceatannol dimer, also named cassigarol E (24 µM for both) (Table 3B). A slightly lower efficacy was found for the aglycone monomers isorhapontigenin (76 µM and 116 µM, respectively) as well as the new dimers piceaside J (109 µM and 96 µM, respectively), piceaside L (142 µM and 145 µM, respectively) and piceaside I (225 µM and 147 µM, respectively). To our knowledge, this is the first time that IC_{50} values are reported for these stilbenes. Interestingly, IC_{50} values of zoospore mobility and downy mildew development were similar, so they are correlated. As mentioned by Pezet et al. (2004); stilbenes were able to inhibit the movement of the zoospores, preventing their input in leaves via stomata and therefore blocking infection. At the cellular level, stilbenes may exert their antifungal activities via many modes of action such as the generation of ROS leading to apoptosis (Kim et al., 2013), and also a plasma membrane disruption effect with the leakage of the cellular content (Jian et al., 2015; Koh et al., 2016).

Among the lowest active compounds against *P. viticola*, the glycosylated monomers astringin and glycosylated diastereomeric dimers piceaside G and piceaside H were respectively three and five times less efficient than their aglycone forms piceatannol and piceaside I or piceaside J dimers. Our data are in agreement with those of Woodward and Pearce (1988) reporting that the antifungal activity against conifer infecting fungi of aglycone stilbenes was six times greater than that of glycosylated stilbenes. Piceid was also tested but showed no activity against downy mildew (data not shown).

The anti-mildew activities of stilbenes and their quantification in the spruce bark were different. Indeed, while the piceatannol-piceatannol dimer was the most active compound, it was the least present in bark. On the contrary, while the glycoside stilbenes astringin, and astringin-astringin dimers (piceasides G and H) were the major compounds, they had the lowest activities. Thus, enriching the extract in aglycone stilbenes by a chemical or enzymatic treatment to remove the sugars could increase its antifungal potential.

4. Conclusion

In summary, nineteen stilbenes were identified in spruce bark extract including six new compounds (piceasides I-N). In addition, spruce bark extract and pure compounds showed an antifungal activity against *P. viticola*, which is responsible for downy mildew. The aglycone forms (cassigarol E, isorhapontigenin, piceatannol, piceasides I, J and L) showed a better activity than their glucoside. Hence, these results suggest that spruce bark, which is a co-product from the wood industry, could potentially be used as a natural fungicide against downy mildew. Biodegradable and less toxic than fungicides, spruce bark waste could reduce the amount of chemical products used in the field and thus promote more sustainable agriculture.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2017.04.009.

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