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ORIGINAL ARTICLE

Secondary metabolites of *Phlebopus* species from Northern Thailand

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Abstract

Submerged cultures of the edible mushrooms *Phlebopus portentosus* and *Phlebopus spongiosus* were screened for their secondary metabolites by HPLC-UV/Vis and HR-LC-ESI-MS. Two new compounds, 9′-hydroxyphenyl pulvinone (**1**), containing an unusual pulvinone structure, and phlebopyron (**2**), together with the seven known pigments, atromentic acid (**3**), xerocomic acid (**4**), variegatic acid (**5**), methyl atromentate (**6**), methyl isoxerocomate (**7**), methyl variegatate (**8**), and variegatorubin (**9**) were isolated from the cultures. Their structures were assigned on the basis of extensive 1D/2D NMR spectroscopic analyses, as well as HR-ESI-MS, and HR-ESI-MS/MS measurements. Furthermore, the isolated compounds were evaluated for their antimicrobial and cytotoxic properties. 9′-hydroxyphenyl pulvinone (**1**), xerocomic acid (**4**), and methyl variegatate (**8**) exhibited weak to moderate cytotoxic activities against several tumor cell lines. The present paper provides a comprehensive characterization of pigments from the class of pulvinic acids that are present in the basidiomes of many edible bolete species.

Keywords Boletales · Basidiomycota · Edible mushrooms · Pulvinic acid derivatives · Secondary metabolites · Structure elucidation

Introduction

Since the early days of civilization, edible mushrooms have been appreciated for their delicious taste, their nutritional properties, i.e., the high protein and mineral content, and their health benefit effects for humans (Hyde et al. 2019). Although their medicinal use in health care is as old as their use as food source, it was only in the 1950s that scientists have focused on the potential of bioactive compounds from mushrooms and the development of medicines (Wani et al. 2010; Sandargo et al. 2019). The genus *Phlebopus* (R. Heim) Singer, classified in the Boletinellaceae (Boletales), contains about 12 species and is widely distributed in tropical and subtropical areas (He et al. 2019). Some of them are highly important as local food source. For example, *Phlebopus portentosus* (Berk. and Broome) Boedijn is one of the most popular edible wild mushrooms in Northern Thailand and China. The species can be easily recognized by its dark boletoid basidiomata with a yellow context, which turns blue when injured. Because of their prized flavor, large size, and nutritional value, the basidiomes are harvested and sold at high prices on local markets in Yunnan, China and Thailand (Ji et al. 2011). Recently, *Phlebopus spongiosus* Pham and Har. Takah. was described from Vietnam as an edible species with medium to large, dark yellowish-brown basidiomata and a sponge-like tissue reminiscent to *P. portentosus*, but differing in its dark brown spore print and distinct hymenial cystidia (Pham et al. 2012). It has also been reported from Northern Thailand (Kumla et al. 2020).

Despite the fact that *P. portentosus* and *P. spongiosus* grow both around wild trees and in plantations of, e.g., *Artocarpus heterophyllus*, *Citrus maxima*, *Coffea arabica*, and *Eucalyptus* sp., these species can live a saprotrophic life style and are both suitable for mushroom cultivation (Ji et al. 2011; Zhang et al. 2017). Cultivation trials on *P. portentosus* and

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P. spongiosus resulted in the production of basidiomes without a host plant (Ji et al. 2011; Kumla et al. 2015; Kumla et al. 2020). In 2016, industrial scale cultivation of *P. portentosus* in Yunnan (China) with a yield of 400 kg of mushrooms per day was achieved (Ji 2016). Also, since 2018 cultivation of *P. spongiosus* started in Vietnam (Le Thi et al. 2018). Both *Phlebopus* species are the only species from the order Boletales that can be successfully cultivated under artificial conditions for commercial purpose (Zhang et al. 2017). So far, only a few secondary metabolites have been reported from the genus of *Phlebopus*. For instance, phlebopines A-C (**10–12**) and other pyrrole alkaloids (**13–16**) were isolated from dried basidiomes of *P. portentosus* (Fig. 1, Sun et al. 2018).

The present report deals with the isolation and characterization of secondary metabolites from cultures of *P. portentosus* and *P. spongiosus*.

Materials and methods

Fungal material

Basidiomes of *Phlebopus portentosus* were bought at Pa Daet Market (Chiang Rai Province, Thailand) (22/05/2016, leg./det. B. Chuankid). A voucher specimen was deposited in the Herbarium of Mae Fah Luang University (MFLU16-2208). Basidiomes of *Phlebopus spongiosus* were collected around Mae Fah Luang University campus (Chiang Rai Province, Thailand) (30/04/2018, leg./det. B. Chuankid). A voucher specimen was deposited in the Herbarium of Mae Fah Luang University (MFLU18-1760).

Tissue cultures of *P. portentosus* and *P. spongiosus*

Small tissue explants (about 3 × 3 × 3 mm) were cut with a scalpel from fracture planes made by band cleavage of clean basidiomes of *P. portentosus* and *P. spongiosus* and transferred to potato dextrose (PDA) and malt extract agar (MEA), respectively (for media compositions see Supporting Information, SI). The living strains of *P. portentosus* (MFLUCC-T19–0088) and *P. spongiosus* (MFLUCC-T19-0091) were deposited in the Mae Fah Luang culture collection (MFLUCC) and periodically transferred.

Molecular phylogenetic analyses

Three DNA regions, ATP synthase subunit 6 (*ATP6*), DNA-directed RNA polymerase II largest subunit (*RPB2*) and translation elongation factor 1-alpha (*TEF1*), were amplified for both isolates by polymerase chain reaction (PCR) following published protocols (Raspé et al. 2016; Chuankid et al. 2019). For the amplification of *RPB2* and *TEF1*, the protocol was slightly modified with the use of JumpStart Taq ReadyMix (Sigma-Aldrich). Amplification of *RPB2* was performed in 25-μL reactions containing 12.5-μL JumpStart Taq ReadyMix, 0.3 μM of forward and reverse primers, and 0.75 M betaine (B0300 Sigma-Aldrich, Germany). For *TEF1*, amplification was performed in 40-μL reactions containing 20-μL JumpStart Taq ReadyMix, 0.3 μM of forward and reverse primers. The PCR products were purified by EZ-10 spin column purification kit (BIO BASIC) and sequenced by HZI in house sequencing service. The sequences were submitted to GenBank. A list of fungal strains studied and species descriptions are provided in the SI.

Investigation of the secondary metabolite production

General experimental procedures

UV/Vis spectra were recorded using a Shimadzu UV−2450 UV/Vis spectrophotometer. NMR spectra were recorded on a Bruker Avance III 700 spectrometer with a 5-mm TXI cryoprobe ($^{1}H/^{13}C$ NMR: 700 MHz/176 MHz) and a Bruker Avance III 500 ($^{1}H/^{13}C$ NMR: 500 MHz/126 MHz) spectrometer. Chemical shifts are reported in ppm using the solvent as an internal standard [$(CD_3)_2CO$ (^{1}H at 2.05 ppm; ^{13}C at 29.84 ppm), CD_3OD (^{1}H at 3.31 ppm; ^{13}C at 49.0 ppm); CD_3CN (^{1}H at 1.94 ppm; ^{13}C at 1.32 ppm)] (Gottlieb et al. 1997).

HPLC-MS analyses (in both, the positive and negative ESI mode) were performed on an Agilent 1260 Infinity Systems instrument with a diode array detector and a Waters C18 Acquity UPLC BEH column (2.1 mm × 50 mm, 1.7 μm) using the following gradient system. Gradient A: Deionized water (Milli-Q, Millipore, Schwalbach, Germany) with 0.1% formic acid (FA; solvent A); acetonitrile with 0.1% FA (solvent B), gradient: 5% B for 0.5 min increasing to 100% in 19.5 min and then maintaining 100% B for 5 min, flow rate 0.6 mL/min. UV/Vis detection was conducted at λ = 200–600 nm combined with an ion trap MS (Amazon Speed, Bruker).

HR-LC-ESI-MS and HR-ESI-MS/MS spectra were recorded with an Agilent 1200 series HPLC-UV system with a Waters C18 Acquity UPLC BEH column (2.1 mm × 50 mm, 1.7 μm) using gradient A. UV/Vis detection was conducted at λ = 200–600 nm combined with ESI-TOF-MS (Maxis, Bruker), scan range 100–2500 m/z, capillary voltage 4500 V, and dry temperature 200 °C.

Preparative RP HPLC was performed on a PLC 2020 from Gilson equipped with a Macherey-Nagel VP Nucleodur 100-5 C18 ec column (250 mm × 40 mm, 7 μm) used as a stationary phase with the following gradient programs. Gradient B: Deionized water (Milli-Q, Millipore, Schwalbach, Germany) with 0.1% TFA (Trifluoroacetic acid; solvent A) and acetonitrile with 0.1% TFA (solvent B) were used as mobile phase. Elution gradient: 3% solvent B increased to 60% in

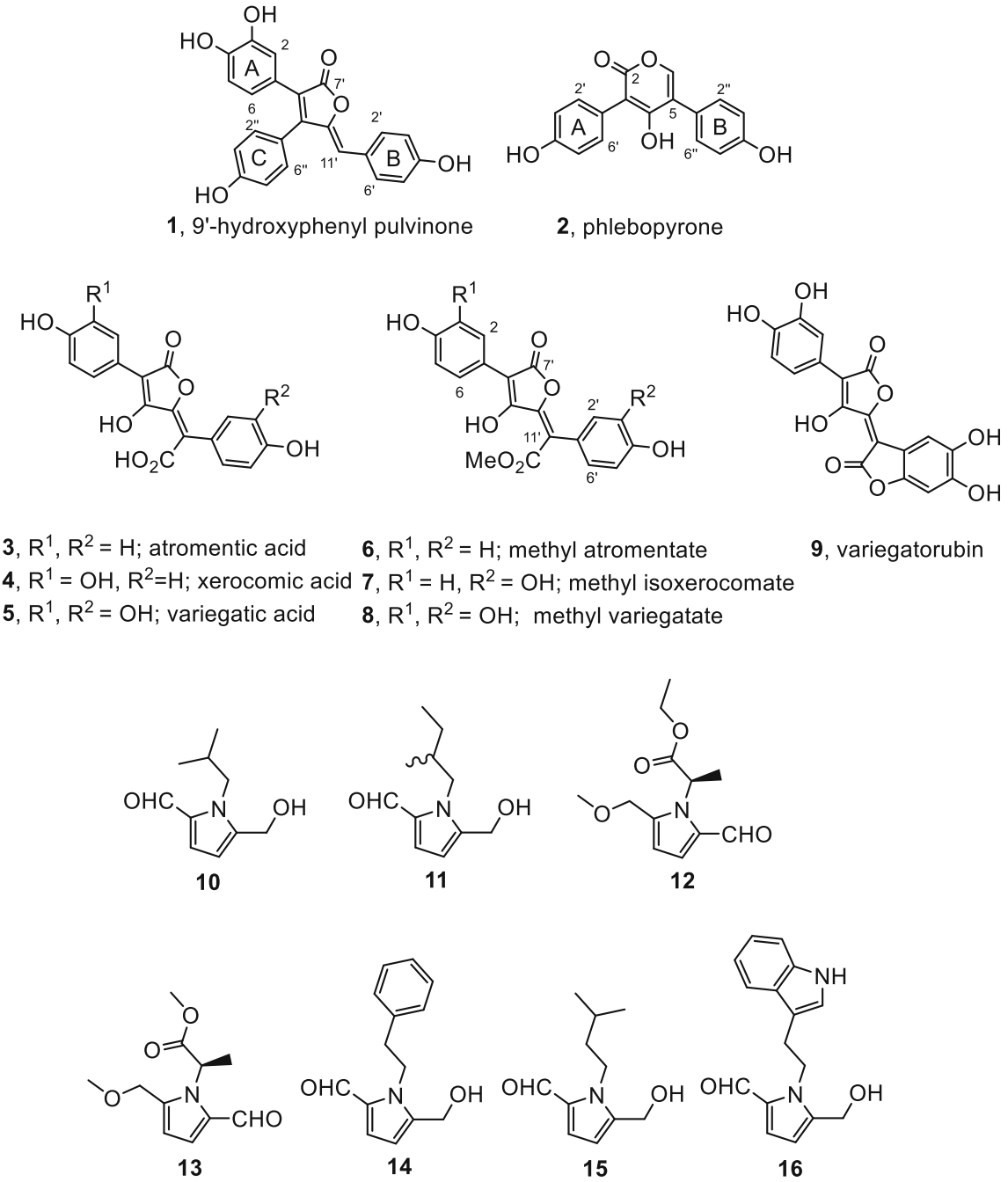

Fig. 1 Chemical structures of compounds **1**–**9** isolated from *P. portentosus* and *P. spongiosus* (liquid cultivation) and known compounds (**10**–**16**) isolated from air-dried basidiomes from *P. portentosus* (Sun et al. 2018)

55 min, then 60 to 100% solvent B in 15 min, and finally isocratic conditions at 100% solvent B for 10 min. The flow rate was 40 mL/min, and UV detection was carried out at λ = 210 and 380 nm. Gradient C: Deionized water (Milli-Q, Millipore) with 0.1% formic acid (FA; solvent A) and acetonitrile with 0.1% FA (solvent B) were used as mobile phase. Elution gradient: 3% solvent B increased to 50% in 30 min, then 50 to 100% solvent B in 10 min, and finally isocratic conditions at 100% solvent B for 10 min. The flow rate was 40 mL/min and UV detection was carried out at λ = 250 and 300 nm. Gradient D: Deionized water (Milli-Q, Millipore) with 0.1% FA (solvent A) and acetonitrile with 0.1% FA (solvent B) were used as mobile phase. Elution gradient: 3% solvent B increased to 60% in 50 min and finally isocratic conditions at 100% solvent B for 10 min. The flow rate was 20 mL/min, and UV detection was carried out at λ = 250, 300, and 350 nm. Moreover, the separation was performed on a MZ Analysentechnik Kromasil 100–5 C18 column (20 × 250 mm, 7 μm) using the following

gradient. Gradient E: Deionized water (Milli-Q, Millipore) with 0.1% FA (solvent A) and acetonitrile with 0.1% FA (solvent B) were used as mobile phase. Elution gradient: 3 to 45% solvent B in 60 min, then 45 to 100% solvent B in 5 min, and isocratic conditions at 100% solvent B for 5 min. The flow rate was 30 mL/min and UV detection was carried out at $\lambda = 210$ and 280 nm.

Analytical TLC was performed on silica gel 60 F_{254} aluminum foils (Merck). Chemicals and solvents were obtained from AppliChem GmbH, Avantor Performance Materials, Carl Roth GmbH & Co. KG, and Merck KGaA in analytical and HPLC grades. Samples of atromentic acid (**3**), variegatic acid (**5**), and variegatorubin (**9**) originating from previous work in Steglich's group (LMU Munich), were kindly provided by the Department of Bioorganic Chemistry, Halle, and used as reference compounds for HR-LC-ESI-MS measurements and co-injection.

Small-scale liquid culture

Both *Phlebopus* species were cultivated in five different liquid media: biotin medium (BAF), maltose medium (MGP), mannitol salt medium (MMK), yeast-malt medium (YM 5.5), and cotton seed medium (ZM 1/2); for the media compositions see SI, Table S1). For establishing the liquid cultivation of *P. portentosus* and *P. spongiosus*, a well-grown culture from a PDA was cut into small pieces using a cork borer (7 mm), and five pieces were transferred to 250-mL Erlenmeyer flasks containing 100-mL media. The cultures were incubated on a rotary shaker (140 rpm) at 24 °C for 23–40 days (for growth conditions see SI, Table S2 and Table S3). The growth of the mycelia was monitored by constantly checking the amount of free glucose using Medi-test Glucose (Macherey-Nagel). The liquid cultivations were terminated three days after glucose depletion. HR-LC-ESI-MS dereplication was carried out by comparing the masses of the detected peaks and their molecular formulas obtained from HR-ESI-MS measurements with those reported in the Dictionary of Natural Products (http://dnp.chemnetbase.com).

Scale-up of liquid culture

HR-LC-ESI-MS results indicated that similar metabolites were produced in all media since YM 5.5 medium produced the highest amounts of interesting secondary metabolites (compounds **1** and **2**), it was used in the scale-up process. A well-grown PDA plate of mycelial culture from *P. spongiosus* was cut into small pieces using 7-mm cork borer and five pieces inoculated into 25 sterile flasks (500 mL) containing YM 5.5 medium (200 mL). The cultures were incubated on a rotary shaker (140 rpm) at 24 °C for 30 days.

Preparation of the extracts

Liquid media and biomass from small-scale liquid cultivation on different media were separated by filtration. Each liquid medium was partitioned with ethyl acetate (1 × 100 mL). The light-yellow organic layers were dried over anhydrous Na_2SO_4 and evaporated to dryness in vacuo (for the rate of yield see SI, Table S2 and Table S3). Each mycelium was extracted with acetone (200 mL) in an ultrasonic bath at room temperature for 30 min, filtered, redissolved in deionized water (100 mL), and partitioned with ethyl acetate (1 × 100 mL). The ethyl acetate layers were dried over anhydrous Na_2SO_4 and evaporated to dryness (for the rate of yield see SI, Table S2 and Table S3).

Liquid media and biomass from the large-scale liquid cultivation of *P. spongiosus* on YM 5.5 medium were separated by vacuum filtration. To the liquid medium 10% Amberlite XAD™ 16 N absorbent (Rohm & Haas Deutschland GmbH, Frankfurt am Main, Germany) was added and shaken for 4 h. The Amberlite resin was recovered by filtration and eluted with acetone (4 × 400 mL). The resulting extract was evaporated to dryness and redissolved in deionized water (100 mL) and partitioned with ethyl acetate (3 × 100 mL). The organic layer was dried over anhydrous Na_2SO_4, evaporated to dryness, and afforded 1.8-g crude extract. The mycelium was ultrasonicated for 30 min with acetone (4 × 400 mL). The extracts were combined, evaporated to dryness, and redissolved in deionized water (200 mL). The water phase was partitioned with ethyl acetate (3 × 400 ml). The combined organic layers were filtered, dried over anhydrous Na_2SO_4, evaporated to dryness, and afforded 0.9 g of a brown crude extract.

Isolation of compounds 1–4 and 6

The lipophilic components of the extract from large-scale liquid cultivation of *P. spongiosus* mycelium in YM 5.5 were removed by MeOH/*n*-heptane (1:1) partition. The methanol layer (0.6 g) was fractionated on Sephadex LH-20 (Pharmacia) (solvent MeOH: dichloromethane 1:1; 33 × 820 mm; flow rate 3.4 mL/min). Sixty-seven fractions were collected according to the observed bands (F1–F67). Fractions F41–67 were combined and purified by preparative RP HPLC (gradient B) and provided compounds **1** (t_R 48.5 min, 2.7 mg), **3** (t_R 42.5 min, 20.3 mg), and **4** (t_R 38.5 min, 40.1 mg). Fractions F4–25 were combined and purified by preparative RP HPLC (gradient C) resulting in compound **2** (4.0 mg, t_R 28.5 min). Compound **6** (0.4 mg, t_R 43.5 min) was isolated from fractions F26–37 by preparative RP HPLC (gradient D).

Isolation of compounds 5, 7, and 8

HR-LC-ESI-MS measurements (in both positive and negative modes) of the crude extracts from small-scale cultivation indicated that *P. portentosus* in MMK medium produced the highest amounts of compounds **5**, **7**, and **8** (Table 2). Therefore, crude extract (0.09 g) from small-scale liquid cultivation of *P. portentosus* mycelium in MMK medium was dissolved in MeOH and purified with a SPME Strata-X 33 u Polymeric RP cartridge (Phenomenex, Aschaffenburg, Germany) by using H_2O (6 mL), H_2O/acetonitrile (1:1, v/v, 6 mL) and acetonitril (6 mL) as eluents. After combining the three fractions and evaporation of the solvents, the residue (0.05 g) was redissolved and separated by preparative RP HPLC (gradient E) and afforded compounds **5** (1.0 mg, t_R 25.5 min), **7** (7.5 mg, t_R 38.5 min), and **8** (3.6 mg, t_R 45.5 min), respectively.

9′-Hydroxyphenyl pulvinone (1) Yield 2.7 mg, orange-yellow solid, $R_f = 0.44$ (CH_2Cl_2: MeOH, 6: 4); UV/Vis (MeOH): λ_{max} (log ε) = 245 (4.09), 386 (4.22) nm; analytical HPLC (t_R) = 7.66 min; HR-ESI(+)-MS: *m/z* (%) = 799.1789 (6) $[2\ M + Na]^+$ ($C_{46}H_{32}NaO_{12}$ cald. for 799.1786), 777.1973 (0.6) $[2\ M + H]^+$ ($C_{46}H_{33}O_{12}$ cald. for 777.1967), 411.0841 (19) [M + Na] + ($C_{23}H_{16}NaO_6$, cald. for 411.0839), 389.1020 (100) $[M + H]^+$ ($C_{23}H_{17}O_6$, cald. for 389.1020); HR-ESI(+)-MS/MS (precursor ion *m/z* 389.1024, 34.6 eV): *m/z* (%) = 371.0921 (45) $[M + H–H_2O)]^+$ ($C_{23}H_{15}O_5$, cald. for 371.0921), 325.0864 (100) $[M + H–H_2O\text{-}CO]^+$ ($C_{22}H_{13}O_3$, cald. for 325.0859), 297.0915 (79) ($C_{21}H_{13}O_2$, cald. for 297.0910); ^1H and ^{13}C NMR data see Table 1.

Phlebopyrone (2) Yield 4.0 mg, colorless solid, $R_f = 0.53$ (CH_2Cl_2: MeOH, 6: 4); UV/Vis (MeOH): λ_{max} (log ε) = 245 (4.15), 328 (3.47) nm; analytical HPLC (t_R) = 5.03 min; HR-ESI(+)-MS: *m/z* (%) = 615.1264 (5) $[2\ M + Na]^+$ ($C_{34}H_{24}NaO_{10}$, cald. for 615.1262), 593.1440 (1) $[2\ M + H]^+$ ($C_{34}H_{25}O_{10}$, cald. for 593.1442), 319.0576 (20) $[M + Na]^+$ ($C_{17}H_{12}NaO_5$, cald. for 319.0577), 297.0754 (100) $[M + H]^+$ ($C_{17}H_{13}O_5$, cald. for 297.0758); HR-ESI(+)-MS/MS (precursor ion *m/z* 297.0775, 35.0 eV): *m/z* (%) = 251.0718 (12) $[M + H–H_2O–CO]^+$ ($C_{16}H_{11}O_3$, cald. for 251.0703), 223.0766 (100) $[M + H–CO–CO\text{-}H_2O]^+$ ($C_{15}H_{11}O_2$, cald. for 223.0754), 175.0399 (45) ($C_{10}H_7O_3$, cald. for 175.0390), 147.0446 (83) ($C_9H_7O_2$, cald. for 175.0390); ^1H and ^{13}C NMR data see Table 1.

Atromentic acid (3) Yield 20.3 mg; orange-yellow solid (for detailed data see SI). Identical in all aspects (NMR, MS, TLC, HPLC-coinjection) with an authentic sample.

Xerocomic acid (4) Yield 40.1 mg; orange-yellow solid (for detailed data see SI). NMR chemical shifts are comparable with those reported (Steglich et al. 1974; Ahmed and Langer 2005).

Table 1 ^1H NMR and ^{13}C NMR Data (δ) for compounds **1** and **2** (700 MHz, 300 K) (δ in ppm, *J* in Hz)

	1[a]		**2**[b]	
No.	δ_C	δ_H (mult., *J*)	δ_C	δ_H (mult., *J*)
1	122.55, C	–	–	–
2	117.2, CH	6.91 (d, 2.2)	164.5, C	–
3	148.3, C	–	106.4, C	–
4	146.7, C	–	164.1, C	–
5	116.2, CH	6.64 (d, 8.3)	118.4, C	–
6	122.6, CH	6.77 (dd, 8.3; 2.2)[c]	149.2, CH	7.48 (s)
1’	125.6, C	–	123.6, C	–
2’	133.7, CH	7.62 (d, 8.7)	133.4, CH	7.25 (d, 8.7)
3’	117.8, CH	6.77 (d, 8.7) [c]	116.5, CH	6.91 (d, 8.7)
4’	162.4, C	–	157.7, C	–
5’	117.8, CH	6.77 (d, 8.7) [c]	116.5, CH	6.91 (d, 8.7)
6’	133.7, CH	7.62 (d, 8.7)	133.4, CH	7.25 (d, 8.7)
7’	171.8, C	–	–	–
8’	123.2, C	–	–	–
9’	151.0, C	–	–	–
10’	147.8, C	–	–	–
11’	114.7, CH	5.97 (s)	–	–
1”	122.4, C	–	123.6, C	–
2”	132.0, CH	7.16 (d, 8.5)	131.9, CH	7.29 (d, 8.6)
3”	117.5, CH	6.87 (d, 8.5)	116.4, CH	6.89 (d, 8.6)
4”	161.6, C	–	158.2, C	–
5”	117.5, CH	6.87 (d, 8.5)	116.4, CH	6.89 (d, 8.6)
6”	132.0, CH	7.16 (d, 8.5)	131.9, CH	7.29 (d, 8.6)

[a] CD_3OD. [b] CD_3CN, the carbon chemical shift values were obtained from the ^1H,^{13}C HSQC and ^1H,^{13}C HMBC spectra. [c] Overlapping signals

Variegatic acid (5) Yield 1 mg; orange-yellow solid (for detailed data see SI). Identical in all aspects (NMR, MS, TLC, HPLC-coinjection) with an authentic sample.

Methyl atromentate (6) Yield 0.4 mg, orange-yellow solid. (for detailed data see SI). NMR chemical shift values are comparable with those reported (Murr et al. 2006).

Methyl isoxerocomate (7) Yield 7.5 mg, orange-yellow solid (for detailed data see SI). NMR chemical shifts are comparable with those reported (Gruber et al. 2014).

Methyl variegatate (8) Yield 3.6 mg, orange-yellow solid (for detailed data see SI). NMR chemical shifts are comparable with those reported (Edwards 1977).

Variegatorubin (9) Identified by HR-LC-MS and HPLC-coinjection with an authentic sample (for detailed data see SI).

Antimicrobial activity and cytotoxicity assays

Minimum inhibitory concentrations (MIC)

Compounds **1**–**4** and **6**–**8** were tested against several bacterial and fungal strains by using a 96-well serial dilution technique in Mueller-Hinton broth (MHB) media for bacteria and YMG media for filamentous fungi and yeasts as previously described (Becker et al. 2020). The selected organisms represent a broad spectrum of pathogens of clinical interest, as well as sensitive indicator strains, to discover new antiinfectives (bacteria: *Bacillus subtilis*, *Chromobacterium violaceum*, *Escherichia coli*, *Micrococcus luteus*, *Mycolicibacterium smegmatis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*; fungi: *Mucor hiemalis*, *Pichia anomala*, *Rhodotorula glutinis*, *Candida albicans*, and *Schizosaccharomyces pombe*). The compounds were redissolved in MeOH (1 mg/mL), diluted to a final concentration range of 66.7 to 0.52 μg/mL and incubated with the test organisms overnight. MeOH was used as negative control. Kanamycin (1.0 mg/mL; 2 μL), gentamicin (1.0 mg/mL; 2 μL), nystatin (1.0 mg/mL; 20 μL), and oxytetracycline (1.0 mg/mL; 2 μL [*C. violaceum*, *E. coli*, *M. luteus*, *S. aureus*] and 20 μL [*B. subtilis*]) were used as positive controls against tested organisms. Compound **5** was not tested because of the low yield.

In vitro cytotoxicity (IC_{50}) assays

The evaluation of in vitro cytotoxicity (IC_{50}) was performed with mouse fibroblast cell line L929 and mammalian HeLa KB3.1 cancer cells for compounds **1**–**4**, and **6**–**8**, as previously described (Becker et al. 2020). If an inhibition of cell viability with an $IC_{50} < 50$ μM was observed, further cell lines were tested: human ovarian carcinoma (SKOV-3 [compounds **4**, **8**]), human prostate cancer (PC-3 [compounds **4**, **8**]), human lung carcinoma tissue (A549 [compounds **4**, **8**]), human skin squamous cell carcinoma (A431 [compounds **4**, **8**]), and human breast adenocarcinoma (MCF-7 [compounds **1**, **4**, **8**]). The compounds were dissolved in MeOH (1 mg/mL) which was used as negative control in this study; epothilone B (1 mg/mL) was used as positive control. After incubating the cell lines with a serial dilution of the test compounds (final range from 37 to 0.6×10^{-3} μg/mL) for five days, the cells were dyed using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), which is only converted to its purple formazan derivative by living cells. The absorption at 595 nm was measured using a microplate reader to calculate the percentage of cell viability. Results were expressed as IC_{50}, the half maximal inhibitory concentration (μM). Compound **5** was not tested because of the low yield.

Nematicidal assay

Caenorhabditis elegans AB1 was used for nematicidal activity of compounds **3** and **4**. The nematodes were cultured on nematode agar [soypeptone 2 g, NaCl 1 g, agar 20 g, 1000 mL of deionized water; adding 0.5 mL of cholesterol (1 mg/mL EtOH), 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$, and 12.5 mL of 40 mM potassium phosphate buffer after autoclaving; pH adjusted to 6.8] with living *E. coli* DSM 498, at 20 °C for a week, was assessed according to our previously reported protocol (Phukhamsakda et al. 2019). The compounds were dissolved in MeOH (1 mg/mL) of five different concentration of each compound as 100, 50, 25, 12.5, and 6.25 μg/mL. Ivermectin (1.0 μg/mL) and methanol were used as positive and negative control, respectively. The plates were incubated at 20 °C in the dark, and nematicidal activity was recorded after 18 h of incubation and expressed as an LD_{50}.

Biofilm inhibition

Compounds **3** and **4** were evaluated in a biofilm inhibition assay against *Staphylococcus aureus* DSM 1104. *S. aureus* from − 20 °C stock was incubated in 20 mL CASO (casein-peptone soymeal-peptone medium) at 37 °C on a rotary shaker (100 rpm) for 16 h. The cell concentration was adjusted to match the turbidity of 0.5 McFarland standard. The assay was performed in 96-well flat bottom plates (Falcon™ Microplates, USA) as previously described (Chepkirui et al. 2018). The tested compounds were dissolved in MeOH (5 mg/mL), diluted to a final range of 250 to 15.6 μg/mL and incubated with the *S. aureus* at 37 °C for 24 h. All experiments were carried out in duplicates and microporenic acid A (5 mg/mL, 15 μL) was used as standard, MeOH was used as negative control (15 μL). The cells were dyed with crystal violet and the absorption was measured at $\lambda = 550$ nm using a microplate reader to calculate the percentage of inhibition.

Results and discussion

Analyses of the secondary metabolite profiles of extracts from small-scale liquid cultivation of *P. portentosus* and *P. spongiosus* by HPLC-UV/Vis and HR-LC-ESI-MS indicated the presence of interesting pigments with absorption maxima between $\lambda = 300$–550 nm. Therefore, extensive chromatographic studies of the crude extracts were conducted. These finally led to the isolation of two new compounds, named 9′-hydroxyphenyl pulvinone (**1**) and phlebopyrone (**2**).

Compound **1** was isolated as a yellow amorphous solid and exhibited absorption maxima at $\lambda = 245$ and 386 nm suggesting the existence of an extended conjugated π system in the molecule. The molecular formula was determined to be $C_{23}H_{16}O_6$, based on HR-(+)-ESI-MS measurements of the

molecular ion *m/z* 389.1020 ([M + H]$^+$, cald. for 389.1020), corresponding to 16 degrees of unsaturation. The ^{1}H NMR spectrum, recorded at 300 K in CD_3OD, exhibited 12 nonexchangeable protons (Table 1). Consequently, **1** contains four exchangeable protons. Furthermore, the ^{13}C NMR spectrum revealed the presence of 23 carbons (Table 1). According to the molecular formula and the ^{1}H,^{13}C HSQC spectrum, the carbon signals could further be classified into 12 methine groups showing eight signals, four of which are assigned to a pair of equivalent carbons each in two paradisubstituted aromatic rings, and 11-nonprotonated carbons including six oxygenated ones. Analysis of the NMR spectra for **1** indicates the presence of a 1,3,4-trisubstituted benzene ring [benzene ring A: H-2 (δ_H 6.91 ppm, d, J = 2.2 Hz), H-5 (δ_H 6.64 ppm, d, J = 8.3 Hz), H-6 (δ_H 6.77 ppm, dd, J = 8.3, 2.2 Hz)], and two 1,4-disubstituted benzene rings [benzene ring B: H-2′/6′ (δ_H 7.62 ppm, d, J = 8.7 Hz), H-3′/5’(δ_H 6.77 ppm, d, J = 8.7 Hz); benzene ring C: H-2″/6″ (δ_H 7.16 ppm, d, J = 8.5 Hz), H-3″/5″ (δ_H 6.87 ppm, d, J = 8.5 Hz)], as well as an isolated methine proton C-11′ (δ_H 5.97 ppm, s). Moreover, the 1D NMR data of pigment **1** resembled those of pulvinone analogues (Gao et al. 2013). The 1,3,4-trisubstituted benzene ring exhibits deshielded chemical shift values for C-3 (δ_C 148.3 ppm) and C-4 (δ_C 146.7 ppm) requiring substitution by oxygen. According to a strong ^3J(H,C) HMBC correlation of H-2 (δ_H 6.91 ppm) and H-6 (δ_H 6.77 ppm) with C-8′ (δ_C 123.2 ppm), the trisubstituted benzene group is connected to the butenolide unit at C-8′ through C-1 (δ_C 122.55 ppm) (Fig. 2). Furthermore, the strong ^3J(H,C) HMBC correlation of H-2’/H-6′ (δ_H 7.62 ppm) to C-11′ (δ_C 114.7 ppm) indicates that the substituted benzene ring B is connected via C-1′ (δ_C 125.6 ppm) to the methine carbon C-11′. On account of its chemical shift value C-4′ (δ_C 162.4 ppm) is substituted by a phenol group. Due to the ^{1}H,^{13}C HMBC correlation from H-2″/6″ (δ_H 7.16 ppm) to C-9′ (δ_C 151.0 ppm), benzene ring C has to be connected at C-9′ in the butenolide unit via C-1″ (δ_C 122.4 ppm) forming the pulvinone derivative **1** (Fig. 2), named 9′-hydroxyphenyl pulvinone. The configuration of the double bond between C-10′ and C-11′ was assigned by NOE experiments as *Z*-configured according to a NOE correlation between H-11′ (δ_H 5.97 ppm) and H-2”/H-6″ (δ_H 7.16 ppm).

Pulvinones, characterized by a 4-hydroxy-2(5*H*)-furanone ring, are known as a key structural element in many natural products (Georgiadis 2013). Prominent representatives are the trihydroxypulvinones (Edwards and Gill 1973) isolated from *Suillus grevillei* or the group of aspulvinones from *Aspergillus* spp. (Ojima et al. 1973; Golding and Rickards 1975; Pang et al. 2017). However, substitution of the butenolide unit by a third phenyl group at C-9′ has not been found in nature so far and provides a new subclass of pulvinones. A synthetic derivative was only described in the one-step synthesis of α-phenyl-γ-benzylidene-α,β-unsaturated-butenolide in 1975 (Rao 1975).

The UV/Vis spectrum of compound **2** exhibits absorption maxima at λ = 245 and 328 nm suggesting the presence of a conjugated π system in the molecule. The HR(+)-ESI-MS spectra of **2** shows an [M + H]$^+$ ion at *m/z* 297.0754 (cald. for 297.0758) consistent with the molecular formula $C_{17}H_{12}O_5$ and 12 degrees of unsaturation. The ^{1}H NMR spectrum of **2**, recorded at 300 K in CD_3CN, exhibits nine nonexchangeable protons (Table 1) which can further be classified as one isolated methine C-5 (δ_H 7.48 ppm) and four pairs of aromatic methines. According to the molecular formula, compound **2** contains three exchangeable protons. Furthermore, the NMR spectra suggested two para-substituted benzene rings [benzene ring A: H-2′/6′ (δ_H 7.25 ppm, d, J = 8.7 Hz), H-3′/5′ (δ_H 6.91 ppm, d, J = 8.7 Hz); benzene ring B: H-2″/6″ (δ_H 7.29 ppm, d, J = 8.6 Hz), H-3″/5″ (δ_H 6.89 ppm, d, J = 8.6 Hz)] with deshielded resonances for C-4′ (δ_C 157.7 ppm) and C-4″ (δ_C 158.2 ppm) requiring substitution by oxygen. The ^{1}H,^{13}C HMBC spectrum exhibits correlations of methine proton H-6 (δ_H 7.48 ppm) with C-2 (δ_C 164.5 ppm), C-3 (δ_C 106.4 ppm), C-4 (δ_C 164.1 ppm), and C-5 (δ_C 118.4 ppm) (Fig. 2). Consideration of the number of oxygen atoms and the characteristic chemical shifts of C-3-C-6 and H-6 indicates compound **2** contains a 4-hydroxy-α-pyrone moiety. The substituted benzene ring A is connected to the α-pyrone moiety at C-3 (δ_C 106.4 ppm) through C-1′ (δ_C 123.6 ppm), since ^{1}H,^{13}C HMBC correlations of the aromatic protons H-2′/6′ (δ_H 7.25 ppm) to C-3 (δ_C 106.4 ppm) can be observed.

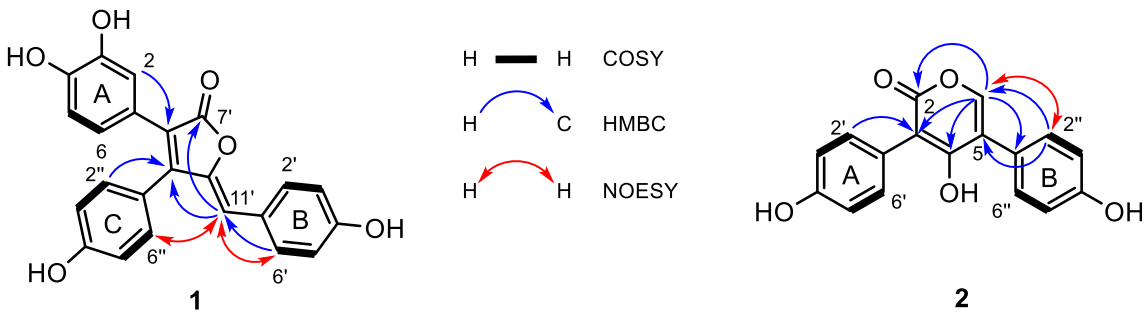

Fig. 2 ^{1}H,^{1}H COSY, ^{1}H,^{13}C HMBC, and key ^{1}H,^{1}H NOESY correlations for compounds **1** and **2**

Furthermore, protons H-2″/6″ (δ_H 7.29 ppm) show a strong ^3J(H,C) HMBC correlation to C-5 (δ_C 118.4 ppm) of the α-pyrone moiety. Therefore, substituted benzene ring B has to be connected to C-5 through C-1″ (δ_C 123.6 ppm) forming the bis(hydroxyphenyl)-α-pyrone derivative **2**, named phlebopyrone.

α-Pyrones are widely distributed in nature, represented by a huge number of biological active metabolites with antimicrobial, antitumor, and cytotoxic effects (Schäberle 2016). Their structural variety reach from monocyclic-α-pyrones, like gibepyrones (Barrero et al. 1993), corallopyronins (Irschik et al. 1985), or trichopyrone (Abdel-Lateff et al. 2009), to complex monobenzo- and dibenzo-α-pyrone ring systems, such as coumarins, aflatoxins, or alternariol derivatives (Schäberle 2016). Known representatives which resemble phlebopyrone (**2**) to some degree are for example the monocyclic-α-pyrones penicipyran A and B from *Penicillium raistrickii* (Ma et al. 2017).

Furthermore, seven known pigments were isolated from the crude extracts [large-scale cultivation (YM 5.5 medium) from *P. spongiosus* and small-scale (MMK medium) from *P. portentosus*]. The structures were identified based on their spectral data [UV/Vis, 1D/2D NMR, MS (**3**–**8**)] as atromentic acid (**3**), xerocomic acid (**4**), variegatic acid (**5**), methyl atromentate (**6**), methyl isoxerocomate (**7**), and methyl variegatate (**8**) and by HPLC comparison with authentic samples (**3** and **5**). Variegatorubin (**9**) was identified by UV/Vis spectra, HR-LC-ESI(+)-MS, HR-ESI(+)-MS/MS, and HPLC comparison with an authentic sample. The red pigment variegatorubin (**9**) could only be detected in traces in the crude extracts of *P. portentosus* and was probably formed during isolation process by oxidation of variegatic acid (**5**) (Steglich et al. 1970).

HR-LC-ESI(+)-MS comparison of the crude extracts of *P. portentosus* and *P. spongiosus* growing on different media indicated that atromentic acid (**3**), xerocomic acid (**4**), and variegatic acid (**5**) are the major pigments in both species (Table 2). The compounds belong to the pulvinic acids, a group of impressive pigments which are responsible for the yellow and red colors of most boletes. They have been detected in over 100 species of the Boletales (Gill and Steglich 1987; Nelsen 2010). Mainly variegatic acid is responsible for the bluing reaction of most boletoid basidiomata due to the oxidation to the corresponding blue quinone methide anion (Velíšek and Cejpek 2011). Pulvinic acids are chemotaxonomic marker compounds of Boletales sensu lato and successfully used to reorder the systematics of this order (Gill and Steglich 1987; Bresinsky 2014). Therefore, the isolation of pulvinic acids **3**–**5** and **9** from *P. portentosus* and *P. spongiosus* underlines the taxonomic position of *Phlebopus* in the Boletales and even the Boletaceae. Remarkably, the new compounds 9′-hydroxyphenyl pulvinone (**1**) and phlebopyrone (**2**) could be isolated for the first time. Both compounds may act as chemotaxonomic marker compounds for the genus *Phlebopus*. HR-LC-ESI(+)-MS comparison of the crude extracts from different media indicated that compounds **1**–**9** appear in both species (Table 2). Furthermore, depending of the liquid media, differences could be observed in the occurrence of compounds **1**–**9** between mycelium and liquid media (Table 2). A metabolite profile of the crude extracts of dried basidiomata of

Table 2 Occurrence of compounds **1**–**9** in *P. spongiosus* and *P. portentosus* cultivated in different media

			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
P. portentosus	BAF	m	++	+	+++	+++	+++	-	++	-	+
		l	++	++	+++	+++	+++	-	+	-	+
	MGP	m	+	++	+++	+++	++	+	+++	++	+
		l	+	++	+++	+++	++	-	+	-	+
	MMK	m	+	+	++	+++	+++	-	+++	+++	-
		l	-	++	-	+++	-	-	+++	-	-
	YM 5.5	m	+	++	+++	-	-	+	+	-	-
		l	-	+	+++	-	-	+	+	-	-
P. spongiosus	BAF	m	+	++	+++	+++	+++	-	+	-	+
		l	+	++	++	++	++	-	+	-	+
	MGP	m	+	++	+++	+++	+++	+	++	-	+
		l	+	++	+++	+++	+++	-	+	-	-
	MMK	m	-	++	-	+++	+++	-	-	-	-
		l	-	++	-	+++	++	-	-	-	-
	YM 5.5	m	++	++	+++	+++	-	+	-	-	-
		l	++	++	+++	++	-	-	++	-	-

m = mycelium, l = liquid media. (−) = not observed, (+) = observed in traces, (++) = observed in small amounts, (+++) = observed in high amounts

Table 3 Antimicrobial activity of compounds **1**, **7**, and **8**

Tested organisms	Strain No.	Minimum inhibitory concentration (MIC) [μg/mL]			
		1	**7**	**8**	Reference
Bacteria					
Bacillus subtilis	DSM 10	–	–	–	8.3[a]
Chromobacterium violaceum	DSM 30191	–	–	–	0.8[a]
Escherichia coli	DSM 1116	–	–	–	6.7[a]
Micrococcus luteus	DSM 1790	–	–	33.3	0.8[a]
Mycobacterium smegmatis	ATCC 700084	–	–	–	1.7[b]
Pseudomonas aeruginosa	PA14	–	–	–	0.4[c]
Staphylococcus aureus	DSM 346	66.7	66.7	66.7	0.8[a]
Fungi					
Mucor hiemalis	DSM 2656	–	66.7	66.7	33.3[d]
Pichia anomala	DSM 6766	–	66.7	66.7	16.7[d]
Rhodoturula glutinis	DSM 10134	–	–	–	8.3[d]
Yeast					
Candida albicans	DSM 1665	–	–	–	66.7[d]
Schizosaccharomyces pombe	DSM 70572	–	66.7	66.7	8.3[d]

[a] Oxytetracycline hydrochloride, [b] kanamycin, [c] gentamicin, [d] nystatin

P. portentosus and *P. spongiosus* indicated dimer forms of pulvinic acids as the major compounds of the fruiting bodies. HR-LC-ESI(+)-MS measurements of *P. portentosus* revealed the presence of ions at *m*/*z*: 651.0775, 679.0722, and 695.0671, consistent with the molecular formulae of bisnorbadiochinone A (Steffan and Steglich 1984), norbadione A (Steffan and Steglich 1984), and pisoquinone (Gill and Kiefel 1994) (for further information see SI). In *P. spongiosus*, HR-LC-ESI(+)-MS comparison indicated small amounts of atromentic acid (**3**) and xerocomic acid (**4**) together with large amounts of tentatively identified bisnorbadiochinone A (Steffan and Steglich 1984) (*m*/*z*: 651.0774) and norbadione A (*m*/*z*: 679.0726) (Steffan and Steglich 1984). Norbadione A, which was isolated from the cap skin of *Boletus badius*, is biosynthetically derived from two molecules of xerocomic acid (**4**) via [4 + 2]cycloaddition (Winner et al. 2004). The orange-brown pigment can complex K or Cs ions (Aumann et al. 1989).

Mushrooms that contain this type of pigment can strongly concentrate radioactive ^{137}Cs in their fruiting bodies, which is absorbed from the environment by their mycelia and transported to the basidiomata (Aumann et al. 1989). To investigate whether the basidiomata of *Phlebopus* species show high values for radionuclides, further investigations are needed. Interestingly, phlebopyrone (**2**) could be detected as a minor compound in the fruiting bodies of both *Phlebopus* species.

Pulvinic acids and the pulvinones exhibit an impressive structural variety, but less is known about their biological activities. Xerocomic acid (**4**) showed weak antiviral effects

Table 4 Cytotoxic activity of compounds **1**, **4**, and **8**

Cell lines	IC_{50} [μM]			
	1	**4**	**8**	Epothilone B
Epidermoid carcinoma A431	nt	9.0	16.8	0.000028
Adenocarcinomic human alveolar basal epithelial A549	nt	8.7	16.0	0.000019
HeLa KB3.1	18.5	17.4	17.6	0.000024
Fibroblasts L929	–	16.5	17.8	0.00061
MCF-7	46.4	3.9	6.5	0.000025
Human prostate cancer PC-3	nt	–	–	0.000028
Ovarian cancer SKOV-3	nt	25.6	49.2	0.00009

(−) = no inhibition, (nt) = not tested

Table 5 Nematicidal activitiy of compounds **3** and **4**

Tested organisms	Strain No.	LD_{50} [μg/mL]		
		3	**4**	Ivermectin
Caenorhabditis elegans	AB1	100.0	100.0	1.0

and inhibited swarming and colony biofilm spreading of *Bacillus subtilis* (Gordon et al. 2007; Tauber et al. 2018). Moreover, xerocomic acid (**4**) and variegatic acid (**5**) are inhibitors of cytochrome P450 enzymes (Huang et al. 2009). Significant antibacterial activities were observed for synthetic biphenyl-substituted pulvinones against Gram-positive bacteria (Antane et al. 2006).

To investigate the antimicrobial, cytotoxic, biofilm inhibition, and nematicidal effects, the isolated compounds **1**–**4** and **6**–**8** were tested against selected microorganisms (Tables 3 and 4). Thus, xerocomic acid (**4**) and methyl variegatate (**8**) showed weak to moderate cytotoxic activities against several tumor mammalian cell lines (Table 4) except for human prostate cancer PC-3. Weak cytotoxic activity against Hela KB3.1 cells and MCF-7 cells could be observed for 9′-hydroxypulvinone (**1**). Moreover, methyl isoxerocomate (**7**) and methyl variegatate (**8**) showed weak effects against several microorganisms, e.g. *S. aureus*, *M. hiemalis*, *S. pombe* (Table 3). In former studies, antibacterial activities were already observed from the acetone extracts of *P. sulphureus* and *P. lignicola* cultures against *B. subtilis* and *E. coli* (Madhosingh 1966). In addition, atromentic acid (**3**) and xerocomic acid (**4**) showed weak activity against *C. elegans* (Table 5). Phlebopyrone (**2**), atromentic acid (**3**), and methyl atromentate (**6**) showed neither antimicrobial nor cytotoxic activities, and no inhibition could be observed for compounds **3** and **4** for biofilm formation (see SI, Table S6).

In conclusion, from the liquid cultivation of the edible mushrooms *P. portentosus* and *P. spongiosus*, a series of pulvinic acid derivatives (**3**–**9**) could be recognized, some of them with significant antimicrobial and cytotoxic activities. Interestingly, alkaloids (**10**–**16**) as reported from the basidiomes could not be observed from liquid cultures (Sun et al. 2018).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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