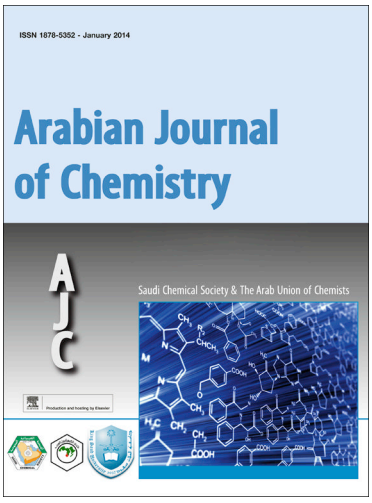

Original article

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Spencertoxin and spencer acid, new phytotoxic derivatives of diacrylic acid and dipyridinbutan-1,4-diol produced by *Spencermartinsia viticola*, a causal agent of grapevine Botryosphaeria dieback in Australia

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Abstract

Spencermartinsia viticola is one of the most widespread Botryosphaeriaceae species isolated from grapevines in South Australian and New South Wales vineyards in Australia. A new phytotoxic dispyridine-butane-1,4-diol and a new diacrylic acid derivatives, here named spencertoxin (**1**) and spencer acid (**2**), were isolated from the culture filtrates of *S. viticola* isolate DAR78870 together with *p*-hydroxybenzaldehyde (**3**) and 2-(4-hydroxyphenyl) acetic acid (**4**). Spencertoxin and spencer acid were characterized as 2,3-di(pyridin-3-yl)butane-1,4-diol and (2*Z*,2'*Z*)-3,3'-(carbonylbis(oxy))diacrylic acid, respectively, by spectroscopic methods (essentially NMR and HRESIMS). Spencertoxin (**1**), *p*-hydroxybenzaldehyde (**3**) and 2-(4-hydroxyphenyl) acetic acid (**4**) showed phytotoxicity when the pure compounds were assayed on grapevine leaves of *Vitis lambrusca* and *Vitis vinifera* cv. Shiraz.

Keywords: Grapevine, Botryosphaeria dieback, *Spencermartinsia viticola*, Phytotoxins, Spencertoxin and spencer acid

1. Introduction

Botryosphaeria dieback, caused by fungi belonging to the family Botryosphaeriaceae, is one of the major trunk diseases affecting grapevines. Over the past few decades, the incidence of symptoms of trunk diseases has increased considerably worldwide. Prevention of Botryosphaeria dieback currently relies on modifying the timing of grapevine pruning practices and protection of pruning wounds, while management of the disease relies on remedial surgery (Ayres et al., 2016; Ayres et al., 2017; Savocchia et al., 2017). Due to the lack of synthetic fungicides registered for the control of Botryosphaeria dieback an increasing number of chemists and plant pathologists are studying various aspects of this disease. Symptoms of the disease in infected vines include characteristic wedge-shaped wood necrosis of the trunk and cordons. Moreover, foliar symptoms associated with the disease have also been reported (Urbez-Torres, 2011). Leaf spots are associated with the production of phytotoxins by the pathogen which are translocated to the leaves from the wood (Tabacchi et al., 2000). Apart from the stunting of shoots, to date no foliar symptoms have been detected in Australian vineyards affected by Botryosphaeria dieback (Pitt et al., 2013). The absence of leaf spots raises questions about the capability of Botryosphaeriaceae isolated from grapevines in Australia to produce phytotoxins.

Different phytotoxins are produced by fungi involved in grapevine trunk diseases and these have been chemically characterised and tested for their toxicity on the leaves of various *Vitis* species and on non-host plants (Andolfi et al., 2011). These phytotoxins belong to different classes of organic compounds such as naphthalenone pentaketides like isosclerone and scytalone produced by fungi involved in the Esca complex. *Diplodia seriata* and *Neofusicoccum parvum*, two of the most widespread and virulent pathogens, are known to produce phytotoxic metabolites belonging to the isocoumarin family called melleins (Martos et al., 2008; Andolfi et al., 2011). *Neofusicoccum australe* haplotype H4, associated with grapevine cordon dieback, produced structurally different secondary metabolites such as a new phytotoxic cyclohexenone oxide, named cyclobotryoxide,

together with 3-methylcatechol and tyrosol (Andolfi et al., 2012) *in vitro*. *Lasiodiplodia* species were also investigated for their production of phytotoxic metabolites. *L. mediterranea*, recently isolated from grapevine and closely related to *L. pseudotheobromae*, produces *in vitro* three jasmonic acid esters, named lasiojasmonates A-C, and 16-*O*-acetylbotryosphaerilactones A and C, (1*R*,2*R*)-jasmonic acid, its methyl ester, botryosphaerilactone A, (3*S*,4*R*,5*R*)-4-hydroxymethyl-3,5-dimethyldihydro-2-furanone, and (3*R*,4*S*)-botryodiplodin (Andolfi et al., 2014). In a recent study, phytotoxic metabolites produced in liquid culture by six species of *Lasiodiplodia* isolated in Brazil and causing Botryosphaeria dieback of grapevine, were chemically identified. As ascertained by LC-MS, *L. brasiliense, L. crassispora, L. jatrophicola, L. pseudotheobromae* produced jasmonic acid, and *L. brasiliense*, synthesized jasmonic acid and (3*R*,4*S*)-4-hydroxymellein. *L. euphorbicola* produced (-)-mellein, (3*R*,4*R*)-(-)- and (3*R*,4S)-(-)-4-hydroxymellein and tyrosol, and *L. hormozganensis* synthesized tyrosol and *p*-hydroxybenzoic acid (Cimmino et al., 2017). Furthermore, fungi involved in Botryosphaeria dieback produced phytotoxic polysaccharides (EPSs), some of which were also recently chemically and biologically characterized (Cimmino et al., 2016).

A study on the identification, prevalence and distribution of Botryosphaeriaceae species in vineyards in winegrowing regions of eastern Australia resulted in eight species from four phylogenetic lineages being isolated from grapevines (Pitt et al., 2010). These species included *D. seriata*, *Diplodia mutila*, *L. theobromae*, *N. parvum*, *N. australe*, *Botryosphaeria dothidea*, *Dothiorella viticola* (syn. *Spencermartinsia viticola*) and *Dothiorella iberica*. *Neofusicoccum luteum* and *Neofusicoccum ribis* were also isolated in a separate surveys of vineyards in eastern Australia (Savocchia et al., 2007; Wunderlich et al., 2011)

This manuscript reports the isolation and chemical and biological characterization of new phytotoxic bispyridine-butane-1,4-diol and diacrylic acid derivatives, named spencertoxin and spencer acid, isolated from the culture filtrates of *S. viticola* (DAR78870) together with, *p*-hydroxybenzaldehyde and *p*-hydroxyphenyl acetic acid.

2. Materials and methods

2.1 General experimental procedure

IR spectra were recorded as a deposit glass film on a Thermo Electron Corporation Nicolet 5700 FT-IR spectrometer (Madison, WI, USA) and UV spectra were measured in MeCN on a Jasco V-530 spectrophotometer; ^{1}H and ^{13}C NMR spectra were recorded at 400 or 500 and 100 or 125 MHz in $CDCl_3$ on Bruker (Karlsruhe, Germany) and Varian (Palo Alto, CA, USA) instruments. The same solvent was used as an internal standard. The multiplicity were determined by DEPT spectra (Berger and Braun, 2004). The same solvent was also used as an internal standard. DEPT, COSY-45, HSQC and HMBC were performed using Bruker and Varian microprograms (Berger and Braun, 2004) HRESIMS and LC/MS analyses were performed using the LC/MS TOF system (AGILENT 6230B, HPLC 1260 Infinity, Milan, Italy), column Phenomenex LUNA (C18 (2) 5u 150x 4.6 mm). Analytical and preparative TLCs were carried out on silica gel (Kieselgel 60, F_{254}, 0.25 and 0.5 mm respectively) and on reverse phase (Kieselgel 60 RP-18, F_{254}, 0.20 mm) plates (Merck, Darmstadt, Germany). The spots were visualized by exposure to UV radiation, or by first spraying with 10% H_2SO_4 in MeOH, and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110°C for 10 min. Column chromatography was performed using silica gel (Kieselgel 60, 0.063-0.200 mm) (Merck).

2.2 Fungal strains and culture conditions

The isolate of *S. viticola* (DAR78870) used in this study was obtained from a grapevine showing symptoms of trunk diseases in a South Australia vineyard (Pitt et al., 2010) and was stored at the Australian Scientific Collections Unit (Orange, NSW, Australia). The isolate was grown in stationary conditions in four flasks containing 2 L of modified Czapeck–Dox medium with 0.5% yeast and 0.5% malt extract (pH 6.8). Each flask containing the medium was inoculated with 15

mycelium plugs of 1 week-old *S. viticola* cultured on potato dextrose agar. The cultures were incubated at 25°C in the dark for 14 hrs, after which the mycelial mats were removed by filtration through four layers of filter paper and kept at -20°C until further processing.

2.3 Extraction and purification of phytotoxins

The lyophilized residues of the culture filtrates of *S. viticola* (8 L) were dissolved in 500 ml of distilled water. The organic phase was extracted with EtOAc (3×60 ml) at pH 2. The organic extracts were combined, dried (Na_2SO_4), filtered and evaporated under low pressure, yielding a brown oily residue (1.12 g). This residue was purified by silica gel column chromatography, eluted with $CHCl_3$:*i*-PrOH (9:1), resulting in seven groups of different fractions. The chromatographic fractions were tested for their phytotoxicity on lemon fruits as described below. The residue (20.0 mg) of fraction two, was purified by preparative TLC on silica gel using $CHCl_3$:MeOH (95:5) as the eluent and yielded a yellow homogeneous solid which was identified as *p*-hydroxybenzaldehyde (**3** Fig. 1, 1.3 mg, 0.16 mg/L, R_f 0.5) as reported below. The residue (80.8 mg) of fraction three, purified on preparative TLC on silica gel, using $CHCl_3$:*i*-PrOH (9:1) as the eluent, resulted in a white solid substance. The latter was further purified by reverse phase TLC (Me_2CO:H_2O 1:1) yielding a homogeneous compound identified as 2-(4-hydroxyphenyl) acetic acid (**4,** Fig. 1, 1.6 mg, 0.2 mg/L, R_f 0.3) as reported below. The residue (7.0 mg) of the fifth fraction was purified by TLC eluted with EtOAc:MeOH:H_2O (90:8:2), yielding two amorphous solids, here named spencertoxin and spencer acid (**1** and **2**, Fig. 1, 4.7 mg, 0.6 mg/L, R_f 0.2, and 1.4 mg, 0.18 mg/L, R_f 0.4, respectively).

*2.4 Identification of compound **1-4***

Compounds **3** and **4** were identified by comparing their spectroscopic data (^{1}H-NMR and ESI/MS) with those already reported in the literature for *p*-hydroxybenzaldehyde (**3**) (Andolfi et al., 2015) and 2-(4-hydroxyphenyl)acetic acid (**4**) (Zhang et al., 2011).

*Spencertoxin (**1**).* UV λ_{max}: nm (log ε) 261 (3.1); IR ν_{max}: 3372, 2969, 2915, 2850, 1694, 1645, 1384 cm^{-1}; ^{1}H and ^{13}C NMR: Table 1; HRESIMS (+) *m/z*: 267.1100 $[M+Na]^+$, (calcd. for $C_{14}H_{15}N_2NaO_2$ 267.1109), 123 $[M/2+H]^+$.

*Spencer acid (**2**).* UV λ_{max}: (log ε) nm 357 (2.9), 258 (3.7); IR ν_{max}: 3347, 1721, 1664 cm^{-1}; ^{1}H and ^{13}C NMR: Table 2; HRESIMS (+) *m/z*: 225.0019 $[M+Na]^+$(calcd. for $C_7H_6NaO_7$ 225.0011).

2.5 Phytotoxicity Bioassays

The phytotoxic activity of crude extract chromatographic fractions was assayed on non-host lemon fruits. The samples were dissolved in dimethylsolfoxide (DMSO) and diluted in distilled water, up to a final concentration of 1 mg/ml and 4% DMSO. The lemon fruit surface was sterilized with sodium hypochlorite (50 µg/ml) and, subsequently, washed with three rinses of sterile distilled water (SDW). The fruit surface was treated with a 10 µl droplet of the test solution and the treated area wounded three times using a sterile needle. SDW and 4% DMSO solution were used as negative controls. The treated fruit was maintained at room temperature (16-22°C) and visually assessed after 72 h for necrotic spots. Compounds **1**-**4** were tested on grapevine leaves (*Vitis lambrusca* and *Vitis vinifera* cv. Shiraz). The compounds were dissolved in 100 µl methanol and the volume adjusted to 3 ml with distilled water (100 µg/ml, 10^{-3} M solution). Grapevine leaves were harvested from glasshouse grown grapevines and the petioles immersed in 1 ml of the phytotoxic solutions. SDW and SDW with 3% MeOH were used as negative controls. The petiole of each leaf was immersed in a vial containing 1 ml of the filtrate dilution for 20 h. Leaves were then transferred to a new vial with 2 ml SDW, placed in a growth chamber with 12 h light /12 h darkness period at 28 °C and maintained for an additional 28 h period . Lesions on the leaf surface were evaluated using a 0-3 scale: 0, no symptoms; 1, slight wilting of the leaf; 2, moderate wilting of the leaf; 3, severe wilting of the leaf (with occasional necrosis). Each treatment was conducted in triplicate.

3. Results and discussion

The organic extract obtained from the culture filtrates of *S. viticola* was purified following a bioguided fractionation and two new metabolites were isolated together with two already known ones (Fig. 2). The new metabolites were named spencertoxin and spencer acid (**1** and **2**, Fig. 1), and the other two were identified as *p*-hydroxybenzaldehyde and 2-(4-hydroxyphenyl)acetic acid (**3** and **4**, Fig. 1). Compounds **3** and **4** are already known metabolites and were identified by comparing their spectroscopic (^{1}H-NMR and HRESIMS) properties with those reported in earlier studies (Andolfi et al. 2015; Zhang et al 2011).

Spencertoxin (**1**) had a molecular formula $C_{14}H_{15}N_2O_2$ as deduced from its HRESIMS and consistent with eight hydrogen deficiencies. Its ^{1}H and COSY (Berger and Braun, 2004) spectra (Table 1) showed a broad singlet, a broad triplet (J = 7.7 Hz), a broad doublet (J = 7.7 Hz) and a broad singlet at δ 8.69 (H-6′), 7.55 (5’), 8.40 (4’) and 9.13 (H-2’) typical signal system of a monosubstituted pyridine ring. A to doudle doublets (J = 11.2 and 4.8 and J=11.2 and 5.8 Hz) due to the protons of a hydroxylated methyelene (H_2-1) was observed at δ 3.60 and 3.52, as also coupled with benzylic methine proton (H-2) appearing as multiplet at δ 3.58. (Pretsch et al., 2000). These systems and the molecular weight suggest a dimeric structure for **1**. Thus, the assignment of the proton is the same for those of the second half of the compound (Table 1). These signals were also in full agreement with the band for hydroxyl and aromatic groups observed in the IR spectrum (Nakanishi and Solomons 1977) as well as the absorption maximum recorded in the UV spectrum (Pretsch et al., 2000). The couplings observed in the HSQC spectrum (Table 2) (Berger and Braun, 2004) allowed the assigning of the carbons resonating in the ^{13}C NMR spectrum (Table 1) at δ 151.5, 150.1, 137.9, 123.7, 70.7 and 62.9 to the protonated carbons C-6,6”, C-2’2”, C-4’,4”, C-5’,5”, C-1,4 and C-2,3. Thus the residual signal at δ 148. 1 was assigned to the two quaternary carbons to C-3’,3” (Table 1).

On the basis of these findings spencertoxin (**1**) was formulated as 2,3-di(pyridin-3-yl)butane-1,4-diol. This structure was confirmed by the long-range couplings observed in the HMBC spectrum (Table 1) (Berger and Braun, 2004) and by the data of its HRESIMS spectrum. This latter spectrum showed the sodiated cluster $[M+Na]^+$ at *m/z* 225.0019 and a significant ion at *m/z* 123 corresponding to the protonated half molecule $[M/2+H]^+$.

Pyridine derivatives are well known as naturally occurring compounds such as nicotinic acid, which is formed in humans from the metabolism of dietary tryptophan involving the intermediate kynurenine. The substance is a precursor of the nicotinamide adenine dinucleotide and related metabolites and alkaloid present in foodstuff and tobacco products (Zwickenpflug et al., 2016). Fusaric acid is a well-known mycotoxin produced by several different *Fusarium* species reported as pathogens of cereals (Bacon et al., 1996). Furthermore, *Fusarium nygamai* which produces fusaric acid, was proposed for the biocontrol of *Striga hermontica* (Capasso et al., 1996) a devastating hemiparasitic plant. Several different *Fusarium* species have been proposed for the biocontrol of *Orobanche ramosa*, an holoparasitic plant responsible for major losses to vegetable, legume, and sunflower crops by interfering with water and mineral intake and by affecting photosynthate partitioning (Abouzeid et al., 2004).

Spencer acid (**2**) had a molecular formula of $C_7H_6O_7$ as deduced from its HRESIMS spectrum consistent with five hydrogen deficiencies. The ^{1}H-NMR and COSY spectra (Table 2) showed two doublets (J = 7.7 Hz) of a *cis*-disubstituted double bond at δ 7.39 and 5.62 (Pretsch et al., 2000). The ^{13}C NMR spectrum as well as the HSQC spectra (Table 2) showed the corresponding olefinic carbons at δ 142.1 and 102.5 in accordance with the oxygenate nature of C-2. Furthermore, the signals of two carboxylic carbonyls were observed at δ 174.9 and 165.9, respectively (Breitmaier and Voelter, 1987). These signals agreed with the bands for hydroxyl and carbonyls groups present in the IR spectrum (Nakanishi and Solomons, 1977) and the maxima recorded in the UV spectra (Pretsch et al., 2000).

Considering these finding and the molecular formula, **2** appeared to be a symmetrical compound and in particular a diacrylic acid derivative. Furthermore, the couplings observed in the HMBC spectrum (Table 1) between the signal at δ 165.9 with H-2,2' and H-3,3' allowed the identified the carbon 1 as the anhydride carbonyl, while the residual signal at δ 174.9 was assigned to the carbons of carboxylic acid groups.

On the basis of these findings spencer acid could be formulated as (2*Z*,2'*Z*)-3,3'-(carbonylbis(oxy))diacrylic acid (**2**).

The structure assigned to **2** was supported from the other couplings observed in the HMBC spectrum (Table 1) and from its HRESIMS. The last spectrum showed the sodium cluster at $[M+Na]^+$ at *m/z* 225.0019.

The isolation of low molecular weight mono- and di-carboxylic acids as phytotoxins has already been reported from bacteria and fungi. Phenylacetic acid was isolated from *Biscognauxia mediterranea* involved in the cork oak canker disease (Evidente et al., 2005), nitropropanoic acid was isolated from *Septoria cirsii* and *Melanconnis thelebola*, and more recently from *Diaporthe gulyae*. The latter three fungi were proposed as mycoherbicides for the control of Canada thistle (Hershernhorn et al, 1993), red alder (*Alus rubra* Bong), a forest infesting species (Evidente et al., 1992) and *Carthamus lanatus*, a widespread winter-growing annual weed of both pastures and crops throughout Australia (Andolfi et al., 2015). *p*-Hydroxy- and *p*-methyl benzoic acid has been also isolated from *C. lanatus*, and when grown in a bioreactor, succinic acid was isolated as the main metabolite (Andolfi et al., 2015). 3-acetoxy-2*S*-methylpropanoic, fumaric acid, methyl ester acid *p*-hydroxybenzoic acid, vanillic acid, (−)-sydonic acid, furan-2-carboxylic acid were produced from *Epichloe bromicola*, a fungus isolated from Wildrye grass (*Elymus tangutorum*) and some of these showed antifungal activity (Song et al., 2015).

Compounds **1-4**, were assayed on grapevine leaves at concentrations of 100 μl (10^{-3} M). The most phytotoxic compounds on grapevine leaves were *p*-hydroxybenzaldehyde (**3**) and 2-(4-

hydroxyphenil)acetic acid (**4**) followed by spencertoxin (**1**), while spencer acid (**2**) produced no symptoms of phytotoxicity under the test conditions (Table 3).

Considering the appearance of necrosis and wilting on grapevine leaves when **2**, **3** and **4** were assayed *in vitro* (Fig. 3) and the absence of foliar symptoms on naturally infected grapevine in Australia, further investigations are required to clarify the role of phytotoxic metabolites in the pathogenicity and symptom expression of Botryosphaeria dieback pathogens in Australian vineyards.

4. Conclusion

To our knowledge this is the first report of the isolation of secondary metabolites from *S. viticola*, a pathogen involved in grapevine trunk diseases. A new dispyridine-butane-1,4-diol and a new diacrylic acid derivatives, named spencertoxin and spencer acid, were isolated from the culture filtrates of *S. viticola* together with, *p*-hydroxybenzaldehyde and 2-(4-hydroxyphenyl) acetic acid. The phytotoxicity of *S. viticola* was also discussed in relation to the symptoms caused *in vivo* on grapevine leaves.

This achievement provides new knowledge on the ability of *S. viticola* to produce phytotoxins *in vitro* however further investigations are required to clarify the role of these phytotoxins in the disease cycle of Botryosphaeria dieback.

Contributors

Pierluigi Reveglia, Sandra Savocchia, Regina Billones-Baaijens, Marco Masi and Antonio Evidente designed the study, analyzed the data and wrote the manuscript. Pierluigi Reveglia performed the experiments.

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Appendix A

Supplementary material Supplementary data associated with this article can be found, in the online version, at…

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Table 1. ^{1}H and ^{13}C NMR data of spencertoxin (**1**) [a,b]

Position	**1**		
	δ_C [c]	δ_H (*J* in Hz)	HMBC
1,4	70.7 *t*	3.60 (2H) *dd* (11.2, 4.8) 3.52 (2H) *dd* (11.2, 5.8)	H-2,3
2,3	62.9 *d*	3.65 (2H) *m*	H-1,4
2',2"	150.1 *d*	9.13 (2H) *br s*	
3',3''	148.1 *s*		
4',4''	137.9 *d*	8.40 (2H) *br d* (7.7)	
5',5''	123.7 *d*	7.55 (2H) *br t* (7.7)	
6',6''	151.5 *d*	8.69 (2H) *br s*	H-4',4"

[a]The chemical shifts are in δ values (ppm) from TMS. [b]2D $^{1}H,^{1}H$ (COSY) and 2D $^{13}C,^{1}H$ (HSQC) NMR experiments delineated the correlations of all protons and the corresponding carbons. [c]Multiplicities were assigned by DEPT spectra.

Table 2. ^{1}H and ^{13}C NMR data of spencer acid (**2**)[a,b]

Position	**2**		
	δ_C [c]	δ_H (*J* in Hz)	HMBC
1	165.9 *s*		H-2,2', H-3,3'
2,2'	142.1 *d*	7.39 (2H) *d* (7.7)	H-3,3'
3,3'	102.5 *d*	5.62 (2H) *d* (7.7)	H-2,2'
4,4'	174.9 *s*		

[a]The chemical shifts are in δ values (ppm) from TMS. [b]2D $^{1}H,^{1}H$ (COSY) and 2D $^{13}C,^{1}H$ (HSQC) NMR experiments delineated the correlations of all protons and the corresponding carbons. [c]Multiplicities were assigned by DEPT spectra.

Table 3. Level of toxicity induced 28 h after treatment on *Vitis lambrusca* and *Vitis vinifera* cv. Shiraz leaves by compounds produced by *Spencermartinsia viticola*.

Treatment	**Level of toxicity**[a]	
	Vitis lambrusca	***Vitis vinifera* (cv. shiraz)**
spencertoxin (**1**)	2	2
spencer acid (**2**)	0	0
p-hydroxybenzaldehyde (**3**)	2.5	3
2-(4-hydroxyphenil)acetic acid (**4**)	3	2.5
SDW	0	0
SDW 3% MeOH	0	0

[a]Severity scale: (0) no symptoms; (1) slight wilting; (2) moderate wilting, necrotic spots; (3) severe necrosis and shrivelling.

Figure legend

Figure 1. Structures of spencertoxin, spencer acid, *p*-hydroxybenzaldehyde, *p*-hydroxyphenilacet acid (**1**-**4**).

Figure 2. Flow chart of the entire experiment process.

Figure 3. Symptoms caused by compounds **1** and **3** on leaves of *Vitis vinifera* cv. Shiraz, after *in vitro* bio-assaying at 10^{-3} M: **a** severe necrosis and shrivelling caused by **3** ; **b** moderate wilting and necrotic spots caused by **1**; **c** symptomless leaf (negative control SDW).

Figure 1

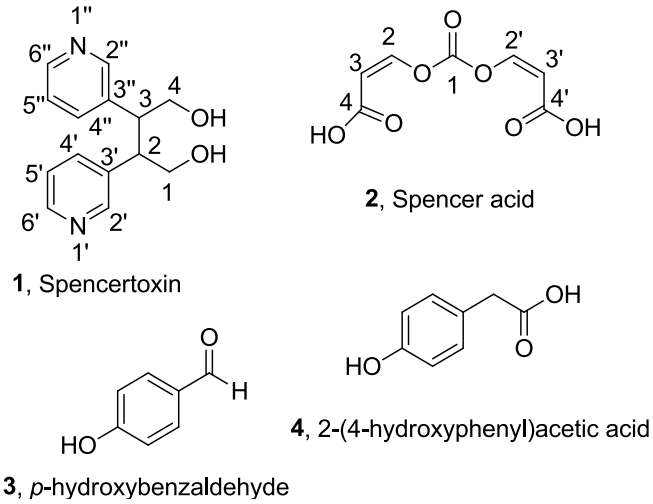

Figure 2

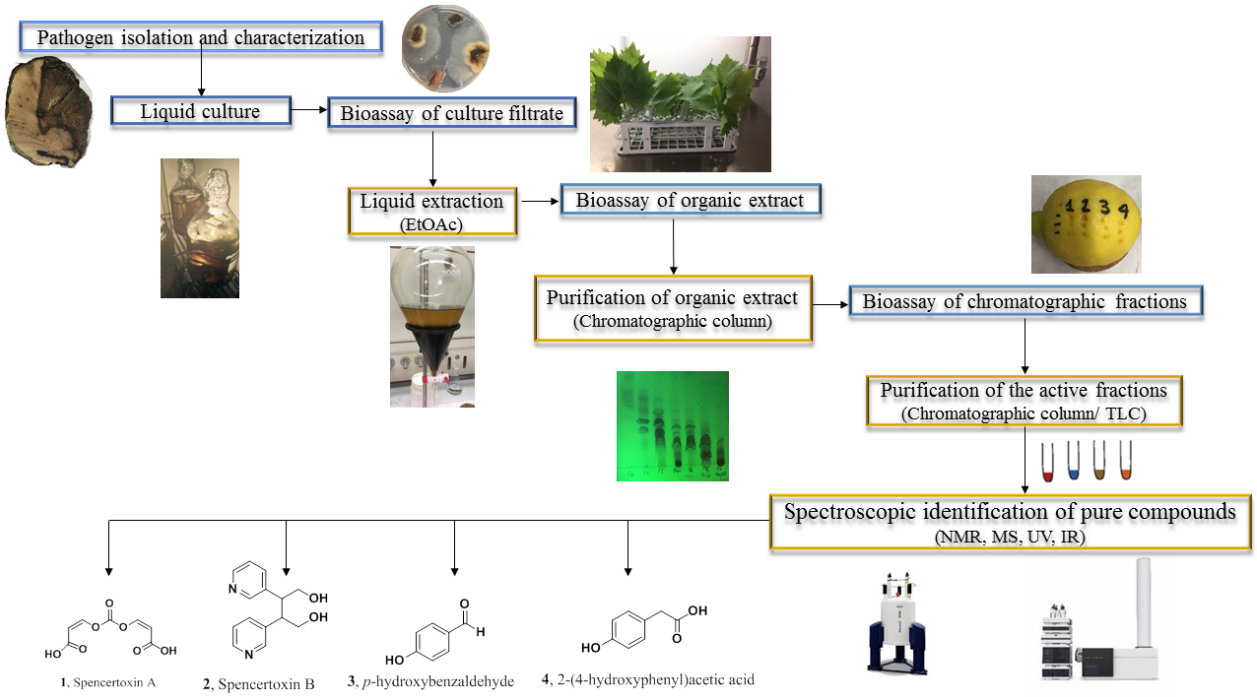

Figure 3