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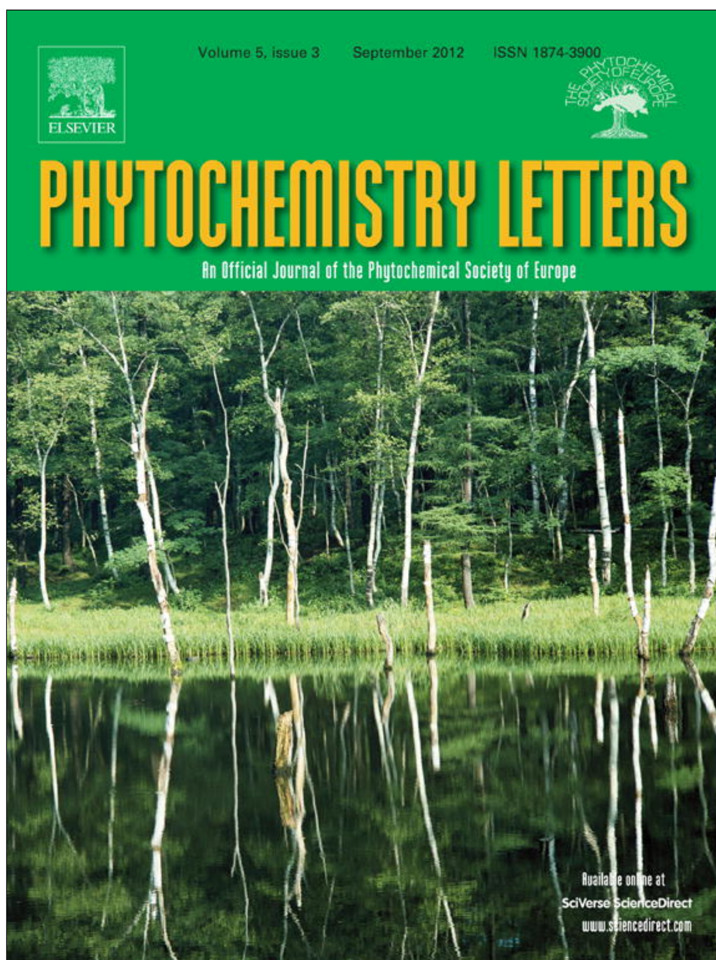

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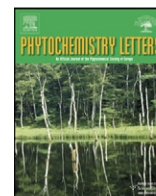

Depsidones and other constituents from *Phomopsis* sp. CAFT69 and its host plant *Endodesmia calophylloides* with potent inhibitory effect on motility of zoospores of grapevine pathogen *Plasmopara viticola*

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ABSTRACT

In our search for secondary metabolites regulating the motility behavior of zoospores of the grapevine downy mildew pathogen *Plasmopara viticola*, we found that extracts from an endophytic fungus *Phomopsis* sp. CAFT69 and its host plant *Endodesmia calophylloides* remarkably impaired motility of zoospores followed by lysis. The active principles in the extracts were isolated and identified as two new compounds, namely excelsional (**1a**) and 9-hydroxyphomopsidin (**2a**), together with excelsione (**1b**), phomopsidin (**2b**), alternariol (**3a**), alternariol-5-*O*-methyl ether (**3b**), the hitherto undescribed 5′-hydroxyalternariol (**3c**), altenusin (**4**) from the fungus, xanthochymol (**5**) and 1,5-dihydroxy-3-methoxyxanthone (mesuaxanthone, **6**) from the plant. Bioassays revealed that compounds **1a**/**b**, **2a**/**b**, and **3a**–**6** displayed motility inhibition and lytic activities against zoospores of the grapevine downy mildew pathogen *P. viticola* in a dose- and time-dependent manner from 1 to 10 μg/mL. Their structures were elucidated by extensive spectroscopic analyses including 2D NMR techniques. This is the first report of an endophyte and its natural products from *E. calophylloides* and the first isolation of compounds **5** and **6** from this plant.
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1. Introduction

The obligate biotrophic downy mildew pathogen *Plasmopara viticola* is a serious pathogen of grapevine worldwide. It belongs to the class Peronosporomycetes (Oomycetes in the old classification) in the kingdom Chromista. Under favorable environmental conditions, this fungus-like organism infects grapevine leaves through characteristic biflagellate motile zoospores released from airborne sporangia coming from infected plants. The zoospores aggregate near stomata of moist grapevine leaves by swimming through the water film and then rapidly encysted to form round cystospores by shedding their flagella. The cystospores then rapidly germinate to form germ tubes and penetrate host tissue through the stomata. Disruption of any of these asexual stages eliminates the potential for pathogenesis (Islam et al., 2005). The success of any zoosporic pathogen can be attributed in part to the speed of asexual differentiation to generate biflagellate motile zoospores. The motility of wall-less zoospores is a critical factor in the disease cycle as well as virulence of the peronosporomycete phytopathogens (Latijnhouwers et al., 2004). As *P. viticola* is an obligate biotrophic pathogen, it is recalcitrant to cultivate in host-free system. Because of their physiology, which differs from that of fungi, many fungicides are ineffective against peronosporomycetes (Islam and Tahara, 2001; West et al., 2003). Therefore, new approaches are needed to find novel targets and to develop effective strategies for a sustainable and biorational management of these phytopathogens (West et al., 2003).

Endodesmia calophylloides (Clusiaceae) is a small tree widely distributed in Nigeria, Cameroon, Gabon and Angola. Traditionally, this plant has been used for the treatment of a wide range of disorders such as for eye-instillation and against filariae (Hutchinson and Dalziel, 1954). Flavanoids, xanthones and triterpenoids with antiplasmodial potential were reported as chemical constituents of this plant (Tambou et al., 2008). To the best of our knowledge, endophytes of this plant have not been investigated so far.

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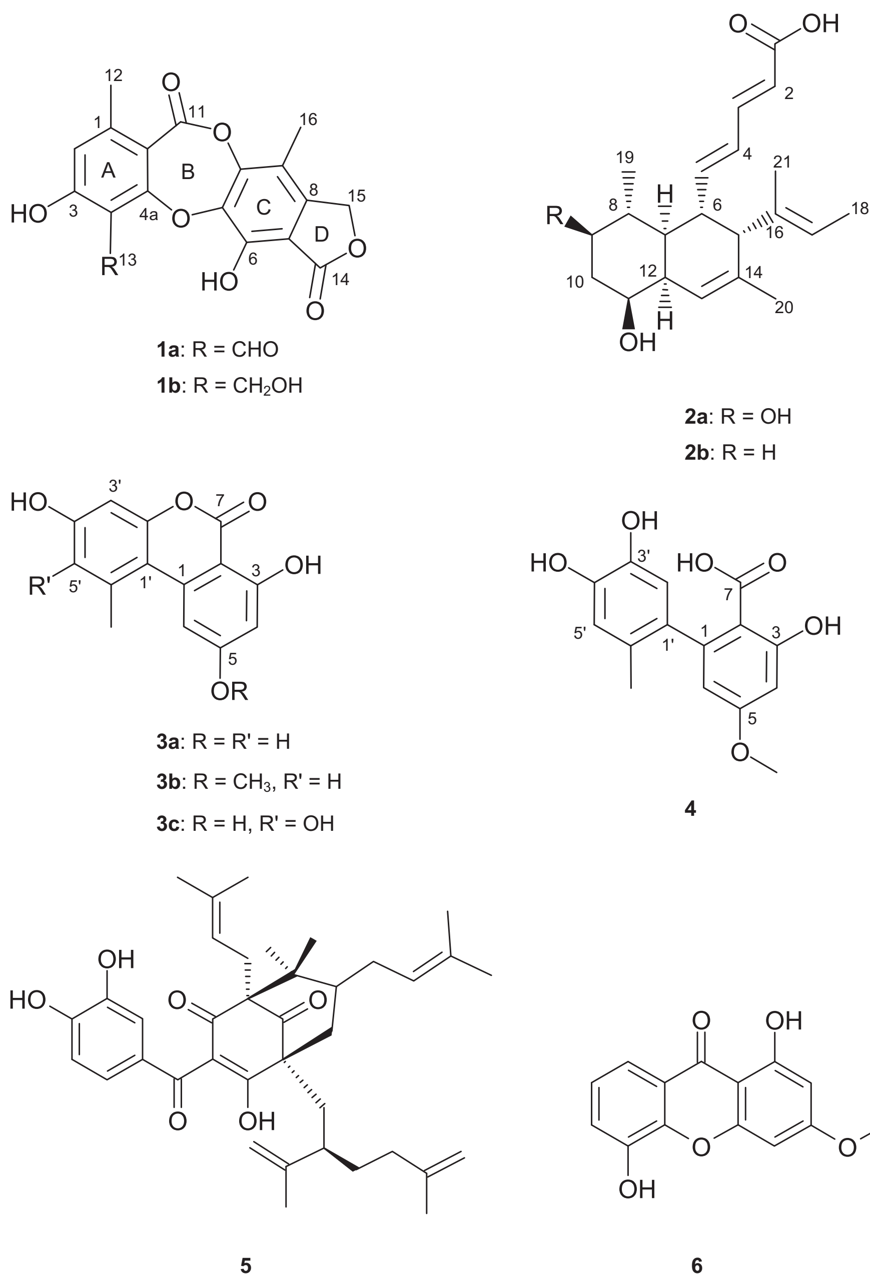

Fig. 1. Chemical structures of isolated compounds from *Phomopsis* sp. CAFT69.

In the course of our chemical screening program of endophytes from Cameroonian medicinal plants (Talontsi, 2011), we found that crude extracts from *Phomopsis* sp. CAFT69 remarkably inhibited motility of *P. viticola* zoospores at a concentration of 30 μg/mL (Table 3). This observation prompted us to isolate and characterize the motility-inhibitory and lytic factors (Fig. 1). In this paper, we describe the bioassay-guided isolation and structure elucidation of the new depsidone excelsional (**1a**), the new decaline derivative 9-hydroxyphomopsidin (**2a**), and further known compounds **1b**, **2b**, **3a**, **3b**, **3c**, **4**, **5**, and **6**. This paper is also the first description of **3c** as a natural compound from *Phomopsis* sp. CAFT69 and its host plant. The inhibitory and lytic potential of the isolated compounds towards zoospores of *P. viticola* were investigated. The potential role of these naturally occurring zoosporicidal compounds in microbe–microbe and plant–microbe interactions is also discussed in relation to the biorational control of phytopathogenic peronosporomycetes.

2. Results and discussion

Bioassay-guided fractionation of extracts from the fungus and its host plant *E. calophylloides* led to the isolation and characterization of ten zoospore inhibitors (**1a**–**6**), of which two were new (**1a**–**2a**) and **3c** isolated for the first time as natural product. The structure of excelsional (**1a**), 9-hydroxyphomopsidin (**2a**), and 5′-hydroxyalternariol (**3c**) were elucidated by spectroscopic and spectrometric methods, while known compounds were identified by comparing their spectroscopic data with previously reported data.

Excelsional (**1a**) was isolated as white amorphous powder with a molecular formula $C_{18}H_{12}O_8$ obtained by HRESIMS. Its IR spectrum showed a strong absorption band at 3418 cm^{-1} indicating a hydroxyl group, three bands at 1766, 1738 and 1659 cm^{-1} assigned to the depsidone ring, butyrolactone and the aldehyde group (Papadopoulou et al., 2007), respectively. The ^{1}H

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Table 1
^{1}H (300 MHz) and ^{13}C (125 MHz) NMR data (δ values) of excelsional (**1a**) and excelsione (**1b**) in DMSO-d_6.

Position	**1a**		**1b**
	δ_H (mult.)	δ_C	δ_C
1		152.2	144.5
2	6.90 (s)	117.5	115.7
3		164.7	161.8
4		111.0	115.3
4a		162.6	159.7
5a		138.4	138.7
6		147.6	147.2w
7		109.1	109.2
8		144.6	144.4
9		114.3	113.2[a]
9a		145.9	148.0
11		160.2	161.0
11a		112.3	110.8
12	2.41 (s)	21.3	20.8
13	10.61 (s)	193.1	52.1
14		168.1	168.8
15	5.30 (s)	68.2	67.9
16	2.18 (s)	10.8	10.8

w = weak.
[a] Signal only in HMBC spectrum visible.

NMR spectrum was very similar to that of excelsione (=phomopsidone, **1b**),[1] the major difference being the absence of the methylene singlet found at δ_H 5.27 in **1b** and the presence of a singlet at δ_H 10.57 in **1a**, characteristic of an aldehyde group. Analysis of its ^{13}C NMR/APT and HSQC data displayed 18 carbon signals, consisting of two methyl units, one oxymethylene and one aromatic methine. In addition, the signal at δ_C 193.1 (C-13) together with the strong IR absorption at 1659 cm^{-1} confirmed the aldehyde group.

The aldehyde proton H-13 showed strong HMBC correlations to the oxygenated quaternary carbon atoms at δ_C 164.7 (C-3), 111.0 (C-4), and 162.6 (C-4a). The methyl group at δ_H 2.44 (C-12) correlated with the carbon atoms at δ_C 152.2 (C-1), 117.5 (C-2), and 112.3 (C-11a), while the aromatic methine at δ_H 6.86 (H-2; δ_C 117.5) showed strong HMBC cross signals with C-1, C-3, C-4, C-11a, and 12-Me, supporting the assignment of the first polysubstituted aromatic ring A. Moreover, the oxymethylene at δ_H 5.27 (C-15) correlated with the ester carbonyl at δ_C 168.1 (C-14) and showed long range couplings with the C-6, C-7, C-8 and C-9 carbon atoms of the benzene ring C. The above information suggested the same partial structure as in excelsione (**1b**), but with an aldehyde replacing the hydroxymethylene function in the first aromatic ring A. Thus, the structure of **1a** was elucidated as shown.

Out of the 12 possible isomers with substituent permutations in rings C and D, only two types have been found in nature: the four angular depsidones with the skeleton of deoxystictic acid (Papadopoulou et al., 2007) were easily excluded on the basis of the HMBC signal pattern and chemical shifts. Our data for rings C and D agreed, however, very well with excelsione (**1b**), re-isolated from the same strain (see Table 1). It should be mentioned, that two different shift assignments for carbon atoms C-5a and C-6 in **1b** have been published (Meister et al., 2007; Lang et al., 2007): The second one is in better agreement with our own measurements and predicted values. Our assignment (Table 1) in parallel to the interpretation of Meister et al. (2007) was proved by a relatively strong ^4J coupling of CH$_2$-15 with C-6 and a very weak ^5J coupling with C-5a. 1D NOE experiments on **1a** showed enhancement on the methyl (H$_3$-16) by irradiating the methylene protons H$_2$-15; enhancement of H$_3$-12 upon irradiation H-2 were also seen. Full analysis of the NOE data and the HMBC confirmed the position of substituents on the two aromatic rings (A and C) and supported that H$_2$-15 and H$_3$-16 are in the same side. Thus, the new compound was elucidated as **1a**, which we named excelsional. Metabolites with a structural similarity to **1a/1b**, such as salazinic acid (Eifler-Lima et al., 2000), deoxystictic acid (Papadopoulou et al., 2007), and stictic acid (Huneck and Tabacchi, 1987), or the phomopsides (Tao et al., 2008) have been isolated previously from lichens and fungi.

9-Hydroxyphomopsidin (**2a**) was isolated as colorless oil with the molecular formula $C_{21}H_{30}O_4$ from HRESIMS. Its IR spectrum showed absorption bands due to a conjugated carboxylic acid (3422, 1637, and 1560 cm^{-1}). The ^{1}H NMR spectrum revealed the presence of six olefinic protons, one tertiary methyl, three vinylic methyls, and one methylene, which was confirmed by ^{13}C NMR data (Table 2). The NMR data were similar to those of phomopsidin (**2b**) (Namikoshi et al.), also isolated from this species here, the only difference being the additional oxygenated methine at δ_C 71.7 (C-9) in **2a** and one missing methylene signal instead. It could be inferred therefore, that one additional hydroxyl group was present in **2a**.

The further elucidation is best started at CH$_2$-10: the signal at δ_H 1.21 appeared as quartet with J 11.6 Hz, which indicated a geminal and two vicinal couplings with H-9 and H-11 in the same magnitude; all three protons must be therefore in axial orientations, and the hydroxy groups OH-9 and OH-11 must adopt β-orientations. H-11 appears as doublet (11.9 Hz) of triplets (4.6 Hz), which indicated equatorial orientations of both H-12 and H-10$_{eq}$ (δ_H 1.76). H-12 showed a broad multiplet, which was partially hidden under the solvent signal. The olefinic methine singlet of H-13 showed allylic couplings with H-15 and H$_3$-20. H-9 correlated with the methine multiplet of H-8, which itself gave rise to the doublet splitting of H$_3$-19. COSY correlations of H-8 with H-7, of H-7 with H-6, and of H-6 with H-15 closed the octahydronaphthalene system. The pentadienoic acid part structure was also identified and was found to be attached at C-6 followed from COSY and HMBC correlations (Fig. 2). The structure was further established by a high number of resolved HMBC correlations (see Fig. 2): the butenyl side chain did not show H,H couplings, but displayed clear HMBC signals with CH-15. The methyl doublet of H$_3$-19 (δ_H 0.96) showed ^2J and ^3J couplings with the methines at δ_C 45.2 (C-7), 36.4 (C-8), and 71.7 (C-9), respectively, while the methylene protons at δ_H 1.21 (H-10$_{ax}$) and 1.76 (H-10$_{eq}$) displayed ^2J correlations with the oxygenated methine carbon at δ_C 71.7.

There were NOE and COSY signals of H-12 with H-7 and H-13, but as in **2b**, the coupling constant with the latter was <1 Hz, confirming the pseudoequatorial orientation of H-12 and a dihedral angle $\alpha_{12,13}$ near 90°. These assignments were also confirmed by HMBC spectra, where all expected ^3J correlations were seen. It follows that the new compound is 9-hydroxyphomopsidin (**2a**) or the respective derivative of the stereoisomeric carneic acid A, previously isolated from the stromata of *Hypoxylon carneum* (Quang et al., 2006).

The two double bonds of the pentadienoic acid unit are having *E*-configuration, according to their H,H coupling constants of 15.0 and 15.3 Hz, respectively. Additionally, H-4 showed NOE signals with H-2 and H-6, as expected for the all-*E* configuration. The double bond at the but-2-enyl side chain also has an *E* configuration, as indicated by the NOE signal between H-15/17. H-15 appeared as a broadened singlet, indicating that the dihedral angle between H-6 and H-15 was close to 90° as reported for **2b** (Namikoshi et al., 2000). NOESY contacts of H-4/5 with H-7, H-12, H-17, and H$_3$-21 indicated that all these protons were on the same face of the molecule. The additional correlation of H-4 with H-15 seems therefore contradictory, but is explained by a change between the two pseudo-chair conformations of the right-hand

[1] To avoid confusion, we derived our name for **1a** not from phomopsidone, a second name for **1b**, as **1b** and **2b** (phomopsidin) are belonging to completely different chemical groups.

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Table 2
^{1}H (300 MHz) and ^{13}C (125 MHz) NMR data (δ values) of 9-hydroxy-phomopsidin (**2a**) and phomopsidin (**2b**) in DMSO-d_6.

Nr.	δ_H (mult. *J* Hz)	NOESY	HMBC	δ_C	
				2a	**2b**[a]
1				169.8	171.2
2	5.74 (d, 15.3)	4	1, 3	126.4	128.4
3	6.85 (dd, 15.3, 9.8)	5	1, 2, 4	141.0	138.0
4	6.05 (m, overlapped)	6, 15	2, 3, 5	128.2	128.4
5	6.03 (m, overlapped)	3, 6, 7, 19	3, 4, 6	142.5	141.0
6	2.63 (t br, 6.7)	5, 7, 17, 19	4, 5, 7, 12, 15	43.0	43.4
7	1.12 (m)	5, 6, 11, 15, 19	9, 13, 15	45.2	40.5
8	1.13 (m)		7, 9	36.4	34.4
9	2.88 (m)	7, 11	7, 19	71.7	28.3
10	1.21 (*ax*, q, 11.6)	11	8, 9, 11, 12	40.2	30.4
	1.76 (*eq*, dt, 11.6, 3.4)		8, 9, 11, 12		
11	3.46 (dt, 11.9, 4.6)	7, 9, 10, 12	10, 12, 13	68.1	70.5
12	2.49 (s, + solvent)	8, 11, 13	8, 11	37.4	37.4
13	5.70 (s)	12, 20	7, 11, 12, 14, 15, 20	123.3	123.5
14				133.3	133.3
15	2.68 (s br)	5, 17, 21	6, 13, 14, 16, 21	49.3	49.5
16				135.2	135.3
17	5.18 (q, 6.4)	15, 20	15, 16	121.3	121.1
18	1.51 (d, 6.4)	17	16, 17	13.2	13.2
19	0.96 (d, 5.2)	6, 7	7, 8, 9	14.5	18.9
20	1.55 (s)	13, 15, 17	13, 14, 15	21.9	21.9
21	1.42 (s)	5, 15, 18	15, 16, 17	16.2	16.2

[a] The ^{13}C NMR literature values for phomopsidin in $CDCl_3$ (Namikoshi et al., 2000) and in CD_3OD (Namikoshi et al., 1997) are completely identical, although the solvents are different.

ring. Further NOE key correlations of H-4 with H-7, H-12, H-17, and H_3-21, and between H-6 and H-8 assigned the remaining stereo centers: accordingly, 9-hydroxyphomopsidin (**2a**) is having the configuration of phomopsidin (**2b**), with an additional (9*R*)-hydroxy group (Fig. 1).

In parallel to alternariol (**3a**) (Freeman, 1965), a compound $C_{14}H_{10}O_6$ (HRMS) was isolated, which resembled **3a** closely in its ^{13}C NMR data; it had, however, one aromatic proton signal and the respective carbon signal less and showed instead a downfield signal of a further oxygenated quaternary carbon. As the C/H signals of the atoms C-1–C-7 were nearly unchanged with respect to **3a**, 3′-hydroxy- or 5′-hydroxy-alternariol (**3c**) was expected. HMBC correlations confirmed indeed the same connectivity in the eastern aromatic ring as in **3a**/**3b**. Cross signals of H-6 and the methyl signal with C-1′ confirmed the expected position of the methyl group at C-6′ (Fig. 3). There was, however, no interaction between the methine proton at δ_H 6.70 and the methyl carbon, and *vice versa.* The methyl group coupled instead with an oxygenated quaternary carbon, and the proton coupled with three phenolic carbons, which confirmed structure **3c** unequivocally (see Fig. 3 and Supplementary information). The hydroxyalternariol **3c** had been obtained by oxidation of **3a** (Pfeiffer et al., 2007), but was not spectroscopically characterized and is described here for the first time as natural product.

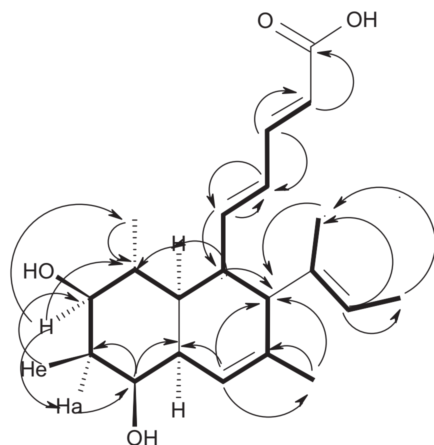

Fig. 2. H,H COSY (—) and selected HMBC (→) correlations of 9-hydroxyphomopsidin (**2a**).

The known fungal metabolites alternariol (**3a**) (Freeman, 1965), alternariol-5-*O*-methyl ether (**3b**) (Freeman, 1965), and altenusin (**4**) (Nakanishi et al., 1995) were also isolated from the fungal extracts and characterized, while fractionation of the extract from stems of the host plant delivered xanthochymol (**5**) (Gustafson et al., 1992) and 1,5-dihydroxy-3-methoxyxanthone (mesuaxanthone, **6**) (Wolfender et al., 1991). The structures of these compounds were identified on the basis of their spectroscopic data (1D, 2D NMR and HRMS).

The motility inhibitory and zoosporicidal potential of the isolated compounds toward zoospores of *P. viticola* was evaluated using the homogeneous solution bioassay method (Abdalla et al., 2011; Islam et al., 2005, 2011). The time-course motility inhibition and lytic activities of the isolated compounds are presented in Table 4 and Fig. 4. Motility of zoospores of *P. viticola* was inhibited by all the metabolites at different doses. Excelsional (**1a**), alternariol (**3a**), and 9-hydroxyphomopsidin (**2a**) exhibited the strongest motility inhibition and subsequent lysis of zoospores, followed by alternariol-5-*O*-methyl ether (**3b**), xanthochymol (**5**), phomopsidin (**2b**), excelsione (**1b**) and altenusin (**6**). The estrogenic and clastogenic potential of alternariol (**3a**), its methyl ether **3b** and altenusin (**4**) as well as their inhibitory effect on the cell proliferation by interference with the cell cycle have already

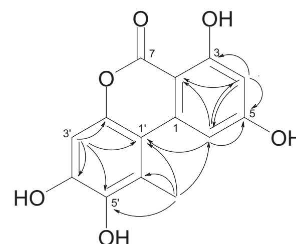

Fig. 3. HMBC correlations of 5′-hydroxyalternariol (**3c**).

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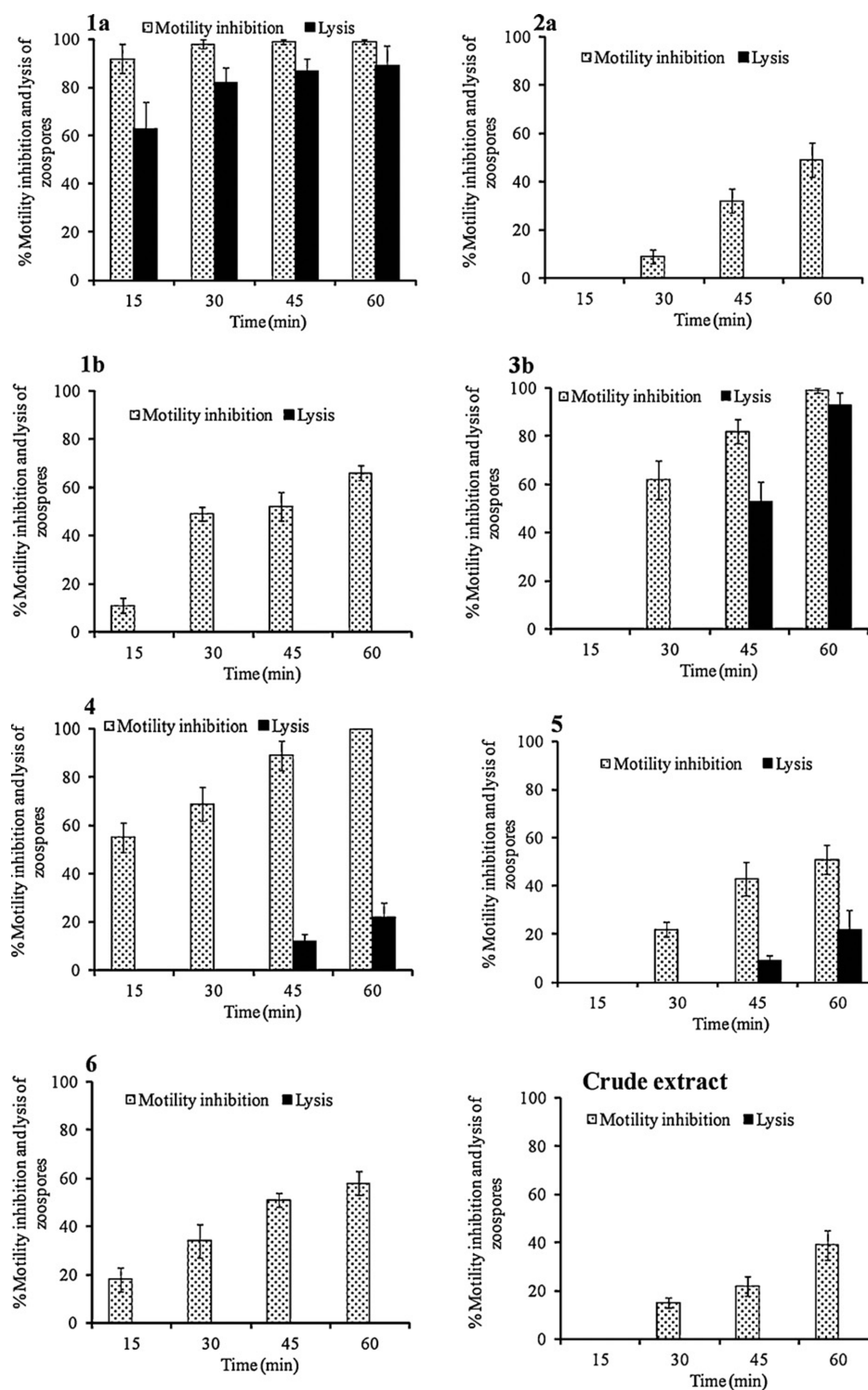

Fig. 4. Comparative motility inhibitory and lytic activities of compounds isolated from *Phomopsis* sp. CAFT69 and its host plant *E. calophylloides* against zoospores of the grapevine downy mildew pathogen *Plasmopara viticola* at 10 μg/ml. Crude extract means EtOAc extracts (30 ppm) of *Phomopsis* sp. grown on solid rice substrate.

been reported recently (Lehmann et al., 2006; Tiemann et al., 2009); in this study they were shown to be excellent zoosporicides (see Tables 3 and 4, and Fig. 3). Generally, zoospores were halted initially and the cellular material rapidly fragmented and dispersed into the surrounding water medium by bursting cell membranes. Phomopsidin (**2b**) also exhibited potent anti-microtubule activity (Namikoshi et al., 1997). The new metabolite 9-hydroxyphomopsidin (**2a**) exhibited approximately 5-fold higher potency than phomopsidin (**2b**). The dynamic assembly and disassembly of microtubules in the zoospore flagella are required for motility. Further studies are needed to understand the modes of actions of motility inhibitory and lytic effects of these metabolites against phytopathogenic peronosporomycete zoospores.

The motility inhibition and subsequent lysis of zoospores shown by secondary metabolites from *Phomopsis* sp. CAFT69 suggest that these metabolites might play a vital role to ward-off zoosporic phytopathogens and protect the host plants in concert with the host's own defence system. Endophytes and bacteria harboured by many plants raise questions on occurrence of this phenomenon particularly during plant–parasite interactions.

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Table 3
Motility halting and zoosporicidal activity of isolated compounds against zoospores of the grapevine downy mildew pathogen *Plasmopara viticola*.

Name of extract	Dose (μg/mL)	Motility halting and zoosporicidal activity (% ± SE)[a]							
		15 min		30 min		45 min		60 min	
		Halted	Burst	Halted	Burst	Halted	Burst	Halted	Burst
c.e.r.	10	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	30	37 ± 9	0 ± 0	34 ± 7	0 ± 0	51 ± 3	0 ± 0	58 ± 5	0 ± 0
	50		0 ± 0	81 ± 3	0 ± 0	88 ± 4	0 ± 0	93 ± 8	0 ± 0
c.e.l.	10	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	30	9 ± 3	0 ± 0	13 ± 7	0 ± 0	27 ± 5	0 ± 0	39 ± 3	0 ± 0
	50	11 ± 5	0 ± 0	18 ± 6	0 ± 0	33 ± 6	0 ± 0	48 ± 6	0 ± 0

c.e.r.: crude extract from rice solid substrate culture; c.e.l.: crude extract from liquid culture.
[a] Data presented here are average value ± SE of at least three replications in each dose of extract.

The indolocarbazol alkaloid, staurosporine from *Streptomyces* spp. and some specific inhibitors of protein kinase C (such as chelerythrine) have recently been found to show motility inhibitory activity of *P. viticola* zoospores without causing lysis of the cells (Islam et al., 2011). This is the first report on motility inhibition of zoospores of a downy mildew pathogen *P. viticola* by secondary metabolites from a non-host plant and its endophytic fungus. Isolation of motility inhibitory secondary metabolites from the non-host plant *E. calophylloides* and its endophyte *Phomopsis* sp. CAFT69 suggest that non-host plants and their endophytes might form interesting compounds, which may result in leads with new mode of actions for developing effective strategies to control these economically important phytopathogenic peronosporomycetes.

In summary, we have isolated and characterized two new compounds, excelsional (**1a**)[2] and 9-hydroxyphomopsidin (**2a**), which showed strong motility inhibitory and lytic effects against the zoospores of an obligate biotrophic downy mildew pathogen of grapevine, *P. viticola*. Therefore, the plant benzophenone xanthochymol (**5**) and the new fungal metabolites **1a** and **2a** could be useful for protecting the host plant against pathogens or grapevine plants from the attack of the downy mildew pathogen. Further studies on the motility inhibitory and zoosporicidal mode of action of these zoospore regulating metabolites and their effects on other phytopathogenic peronomycetes are needed for considering their practical use. Moreover, the hitherto undescribed 5′-hydroxyalternariol (**3c**) is reported here for the first time as a natural product. The zoosporicidal compounds **1a** and **2a** merit further study as potential antiperonosporomycetal agents or as lead compounds, and the synthesis of analogues or derivatives of compounds **1a**, **2a** and **5** for further studies of structure–activity relationships are desirable. The phenolic compounds **5** and **6** were isolated from the host plant for the first time.

3. Experimental

3.1. General experimental procedures

The NMR spectra were recorded on Bruker AMX 300 (300.135 MHz), Varian Unity 300 (300.145 MHz) and Varian Inova 600 (599.740 MHz) spectrometers. Optical rotation was measured on a Perkin-Elmer polarimeter, model 241. UV/VIS spectra were recorded on a Perkin-Elmer Lambda 15 UV/VIS spectrometer. IR spectra were recorded on Perkin-Elmer (Model 1600) FTIR spectrometer. Electrospray-ionization mass spectrometry (ESIMS) and high-resolution mass spectra (HRESIMS) were recorded on a micrOTOF time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany) and on an Apex IV 7 T Fourier-Transform Ion Cyclotron Resonance Mass Spectrometer (Bruker Daltonics, Billerica, MA). Column chromatography was carried out on silica gel (0.05–0.2 mm, 230 mesh, Macherey-Nagel and Co., Düren, Germany). TLC was carried out on pre-coated silica gel sheets of Polygram SIL G/UV254 (Macherey-Nagel and Co., Düren, Germany) with visualization under UV (254 and 365 nm) and with anisaldehyde/sulfuric acid as spray reagent. Size exclusion chromatography was done on Sephadex LH-20 (Lipophilic Sephadex; Amersham Biosciences, Ltd., purchased from Sigma-Aldrich Chemie, Steinheim, Germany). All solvents used were spectral grade or distilled prior to use.

3.2. Plant material

Leaves and stems bark of *E. calophylloides* were collected at Balmayo, in the Centre Province of the Republic of Cameroon, in August 2007, and identified by Mr. Nana Victor, botanist at the National Herbarium of Cameroon, where a voucher specimen (No. 2952 SRFCam) is deposited.

3.3. Fungal material

The plant samples were washed with tap water to remove dust and debris, and then air-dried on sterile filter paper and cut into small pieces using a blade. Sterile conditions were maintained for the isolation of endophytes and all the work was performed in a laminar flow hood to avoid contamination. Surface sterilization of the samples was achieved with 95% EtOH for 30 s, 10% sodium hypochlorite for 10 min, 70% EtOH for 2 min, and then dried aseptically. The inner tissues were placed on isolation media (water agar; WA) in Petri dishes supplemented with 100 mg/L chloramphenicol to suppress bacterial growth, and incubated at 25 °C until the outgrowth of endophytes was discerned. Individual fungal colonies were picked and transferred onto sterile potato dextrose agar (PDA: 200 g potato/L, glucose 40 g/L, and agar-agar 20 g/L) and potato sucrose agar (PSA: boiling water of 200 g potato/L, sucrose 40 g/L, agar-agar 20 g/L) or M_2 agar (malt extract 10 g/L, yeast extract 4 g/L, glucose 4 g/L, and agar-agar 15 g/L) and periodically checked for purity. Each isolate was kept on slant agar for future investigations. A total of 14 strains were isolated and each strain was inoculated on YMG (4 g yeast, 10 g malt extract, 4 g glucose, 1 L water, pH 7.2), PDB (cooking water from 200 g/L potatoes, 20 g dextrose, water ad 1 L), rice (100 g, 100 mL of distilled water), and ML1589 (2 g yeast extract, 20 g malt extract, 10 g glucose, 0.5 g $(NH_4)_2HPO_4$, 1 L water, pH 6.0) media for pre-screening. A *Phomopsis* sp. isolate CAFT69 was selected for further studies due to its higher potency for producing zoospores regulatory compounds. The fresh mycelium of the fungus grown on PDA medium for 5 days was inoculated into 10 P-flasks containing ML1589 liquid medium and on solid rice medium at room temperature under static conditions for 21 and 30 days,

[2] During proof-reading, we came to know that the same compound has been published recently by Varughese et al., 2012.

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Table 4
Motility halting and zoosporicidal activity of isolated compounds **1a**/**b**, **2a**/**b**, and **3a**–**6** against zoospores of the grapevine downy mildew pathogen *Plasmopara viticola*.

	Dose (μg/mL)	Motility inhibitory and lytic activity (% ± SE)[a]							
		15 min		30 min		45 min		60 min	
		Halted	Lysis	Halted	Lysis	Halted	Lysis	Halted	Lysis
1a	1.0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	5 ± 1	0 ± 0	8 ± 0	0 ± 0
	5.0	0 ± 0	0 ± 0	19 ± 4	0 ± 0	33 ± 3	0 ± 0	43 ± 6	0 ± 0
	10.0	92 ± 6	63 ± 1	98 ± 2	82 ± 6	99 ± 1	87 ± 5	99 ± 2	89 ± 5
	20.0	98 ± 2	78 ± 1	100 ± 0	91 ± 8	100 ± 0	100 ± 0	nt	nt
	30.0	100 ± 0	100 ± 0	nt	nt	nt	nt	nt	nt
2a	1.0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	10.0	0 ± 0	0 ± 0	9 ± 3	0 ± 0	32 ± 5	0 ± 0	49 ± 7	0 ± 0
	20.0	0 ± 0	0 ± 0	19 ± 2	0 ± 0	45 ± 7	10 ± 0	62 ± 4	21 ± 9
	30.0	10 ± 2	0 ± 0	51 ± 3	19 ± 5	72 ± 6	39 ± 6	81 ± 6	68 ± 3
	50.0	82 ± 6	12 ± 0	92 ± 6	73 ± 0	100 ± 0	82 ± 4	100 ± 0	89 ± 7
	100.0	98 ± 2	62 ± 1	100 ± 0	92 ± 5	100 ± 0	100 ± 0	nt	nt
1b	5.0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	15 ± 1	0 ± 0	17 ± 3	0 ± 0
	10.0	11 ± 3	0 ± 0	49 ± 3	0 ± 0	52 ± 6	0 ± 0	66 ± 3	0 ± 0
	50.0	31 ± 4	0 ± 0	57 ± 6	0 ± 0	64 ± 8	0 ± 0	72 ± 1	0 ± 0
	1000	60 ± 5	0 ± 0	76 ± 7	0 ± 0	82 ± 6	0 ± 0	89 ± 6	71 ± 0
2b	10.0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	30.0	17 ± 4	0 ± 0	22 ± 6	0 ± 0	26 ± 7	0 ± 0	31 ± 3	0 ± 0
	50.0	28 ± 7	0 ± 0	36 ± 7	0 ± 0	44 ± 6	0 ± 0	47 ± 8	0 ± 0
3a	10.0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	30.0	0 ± 0	0 ± 0	12 ± 4	0 ± 0	18 ± 5	0 ± 0	28 ± 4	0 ± 0
	50.0	0 ± 0	0 ± 0	21 ± 2	0 ± 0	29 ± 0	6 ± 2	38 ± 5	11 ± 2
3b	10.0	0 ± 0	0 ± 0	62 ± 8	0 ± 0	82 ± 5	53 ± 8	99 ± 1	100 ± 0
	25.0	38 ± 6	0 ± 0	82 ± 5	0 ± 0	96 ± 3	58 ± 6	100 ± 0	nt
	50.0	80 ± 6	40 ± 7	99 ± 1	97 ± 3	100 ± 0	100 ± 0	nt	nt
4	10.0	55 ± 6	0 ± 0	69 ± 7	0 ± 0	89 ± 6	12 ± 3	100 ± 0	22 ± 6
	25.0	89 ± 5	0 ± 0	90 ± 5	0 ± 0	100 ± 0	85 ± 5	100 ± 0	100 ± 0
	50.0	96 ± 8	0 ± 0	98 ± 3	82 ± 7	100 ± 0	100 ± 0	nt	nt
5	10.0	0 ± 0	0 ± 0	22 ± 3	0 ± 0	43 ± 7	9 ±± 2	51 ± 6	22 ± 8
	20.0	80 ± 6	71 ± 9	91 ± 5	83 ± 7	98 ± 2	89 ± 6	99 ± 0	96 ± 3
	25.0	88 ± 7	71 ± 3	98 ± 2	87 ± 5	100 ± 0	100 ± 0	nt	nt
	30.0	100 ± 0	97 ± 4	100 ± 0	100 ± 0	nt	nt	nt	nt
6	10.0	0 ± 0	0 ± 0	15 ± 2	0 ± 0	22 ± 4	0 ± 0	39 ± 6	0 ± 0
	30.0	10 ± 1	0 ± 0	17 ± 7	0 ± 0	29 ± 8	0 ± 0	42 ± 1	0 ± 0
	50.0	14 ± 2	0 ± 0	21 ± 6	0 ± 0	33 ± 2	0 ± 0	47 ± 6	0 ± 0

[a] Data presented here are average value ± SE of at least three replications in each dose of compound.

respectively. The endophytic fungus isolate CAFT69 was identified as *Phomopsis* sp. by one of the authors (CMD) and deposited in our microbial collection at the Institute of Organic and Biomolecular Chemistry, Georg-August University of Göttingen, Germany.

3.4. *Extraction and isolation of compounds from* Phomopsis sp. *CAFT69*

The fermented rice substrate culture was extracted repeatedly with EtOAc (3 × 5 L), and the filtrate was evaporated to dryness under vacuum to afford the crude extract (10.0 g), which was fractionated by silica gel vacuum liquid chromatography (VLC) using CH_2Cl_2–MeOH gradient elution with increasing polarity (v/v: 100–0 to 0–100) to delivered 20 fractions (Fr_1–Fr_{20}) after TLC. The motility inhibitory and zoosporicidal activity was located in fractions Fr_{11}–Fr_{13}. Fractions Fr_{11}–Fr_{13} were combined (2.4 g) and separated again by Sephadex LH-20 column chromatography eluting with MeOH to afforded ten subfractions (A_1–A_{10}) after TLC. The activity was located in subfraction A_7 (870 mg), which was further purified by Sephadex LH-20 with CH_2Cl_2–MeOH (v/v, 1:1) to yield excelsional (**1a**, 70 mg), excelsione (**1b**, 4.5 mg) (Lang et al., 2007; Meister et al., 2007), alternariol (**3a**, 80 mg) (Freeman, 1965) its methyl ether (**3b**, 30 mg) (Freeman, 1965) and 5′-hydroxyalternariol (**3c**, 7 mg). Subfraction A_9 (56 mg) was also chromatographed over Sephadex LH-20 eluting with MeOH and later by PTLC (v/v, 90–10, CH_2Cl_2–MeOH) to yield 9-hydroxyphomopsidin (**2a**, 1.4 mg) and phomopsidin (**2b**, 11 mg) (Namikoshi et al., 1997).

The mycelium from liquid culture was extracted exhaustively with EtOAc (3 × 4 L) and MeOH (5 L). The EtOAc extract (23 g), which showed motility inhibitory and lytic activity was defatted with *n*-hexane to yield 10 g of an inactive fraction. The residue was extracted with methanol and concentrated to dryness *in vacuo* to afford 5 g of active methanol soluble fraction. The motility inhibitory and lytic activity was more pronounced in the MeOH fraction than *n*-hexane fraction. The methanol extract was then purified by silica gel column chromatography and eluted first with dichloromethane followed by a stepwise gradient from 5% to 100% methanol to afford excelsione (**1b**, 8 mg) and altenusin (**4**, 7.5 mg) (Nakanishi et al., 1995).

3.5. *Extraction and isolation of compounds from* E. calophylloides

The dried powdered stem bark of *E. calophylloides* (4 kg) was macerated with MeOH (6 L) at room temperature for 72 h and the filtrate was evaporated to dryness under vacuum to afford the crude extract (103 g). The extract was extracted with *n*-hexane (12 g soluble material), CH_2Cl_2 (28 g), and EtOAc (21 g). The motility inhibitory and lytic activity was observed in the CH_2Cl_2-

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soluble fraction, which was then purified by silica gel column chromatography eluted first with dichloromethane followed by a stepwise CH_2Cl_2–MeOH gradient from 5% to 100% methanol to give five fractions (B_1–B_5). The highest activity was observed in fraction A_3, which was further purified using preparative TLC eluting with CH_2Cl_2/MeOH (85:15) and finally Sephadex LH-20 column chromatography eluting with MeOH to afford the halting factors xanthochymol (**5**, 30 mg) (Gustafson et al., 1992) and **6** (1.3 mg) (Wolfender et al., 1991) as the active components.

3.6. Motility inhibitory and zoosporicidal assay

The test was performed according to our previous descriptions (Abdalla et al., 2011).

Excelsional (**1a**): White powder; mp 268–270 °C; R_f 0.41, CH_2Cl_2/MeOH (95:5). IR (KBr) ν_{max} 3418, 1766, 1738, 1659, 1427, 880 cm^{-1}; UV (MeOH) λ_{max} (log ε) 313 (4.42); ^1H and ^{13}C NMR (DMSO-d_6, 300 MHz) see Table 1; CIMS (pos. ion mode) m/z (%) 374 $[M+NH_4]^+$ (100), 298 (32), 274 (8), 198 (45). HRESIMS (pos. ion mode) m/z 357.06052 $[M+H]^+$ (calcd. 357.06050 for $C_{18}H_{13}O_8$).

9-Hydroxyphomopsidin (**2a**): Colourless oil; R_f 0.51, silica gel 60 F_{254}, CH_2Cl_2/MeOH (95:5); $[\alpha]_D^{20} = -64°$ (c 0.14; MeOH). UV (MeOH) λ_{max} (log ε) 280 (3.12), 321 (4.14); IR (KBr) ν_{max} 3422, 1636, 1560 cm^{-1}; ^1H and ^{13}C NMR (DMSO-d_6, 300 MHz) see Table 2; ESIMS (pos. ion mode) m/z (%) 369 $[M+Na]^+$; HRESIMS (pos. ion mode) m/z 369.20370 $[M+H]^+$ (calcd. 369.20364 $C_{21}H_{30}O_4Na$).

5′-Hydroxyalternariol (**3c**): Yellowish powder. ^1H NMR (DMSO-d_6, 300 MHz) δ 11.87 (s br, 1H, 3-OH), 6.35 (d, J = 1.9 Hz, 1H, 4-H), 7.27 (d, J = 1.9 Hz, 1H, 6-H), 6.70 (s, 1H, 3′-H), 10.00 (s br, 2H, 4′,5′-OH), 2.60 (s, 3H, 6′-Me); ^{13}C NMR (DMSO-d_6, 500 MHz) δ 138.4 (C_q-1), 97.6 (C_q-2), 163.8 (C_q-3), 100.6 (CH-4), 164.9 (C_q-5), 104.1 (CH-6), 164.7 (C_q-7), 108.9 (C_q-1′), 144.6 (C_q-2′), 100.7 (CH-3′), 147.5 (C_q-4′), 141.4 (C_q-5′), 121.7 (C_q-6′), 15.9 (6′-Me). HRESIMS (neg. ion mode) m/z 273.04048 $[M-H]^-$ (calcd. 273.04046 $C_{14}H_9O_6$).

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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.phytol.2012.06.017.

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