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Antifungal compounds from turmeric and nutmeg with activity against plant pathogens

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ABSTRACT

The antifungal activity of twenty-two common spices was evaluated against plant pathogens using direct-bioautography coupled *Colletotrichum* bioassays. Turmeric, nutmeg, ginger, clove, oregano, cinnamon, anise, fennel, basil, black cumin, and black pepper showed antifungal activity against the plant pathogens *Colletotrichum acutatum*, *Colletotrichum fragariae*, and *Colletotrichum gloeosporioides*. Among the active extracts, turmeric and nutmeg were the most active and were chosen for further investigation. The bioassay-guided fractionation led to the isolation of three compounds from turmeric (**1**–**3**) and three compounds from nutmeg (**4**–**6**). Their chemical structures were elucidated by spectroscopic analysis including HR-MS, 1D, and 2D NMR as curcumin (**1**), demethoxycurcumin (**2**) and bisdemethoxy-curcumin (**3**), *erythro*-(7R,8R)-$\Delta^{8'}$-4,7-dihydroxy-3,3′,5′-trimethoxy-8-*O*-4′-neolignan (**4**), *erythro*-(7R,8R)-Δ8′-7-acetoxy-3,4,3′,5′-tetra-methoxy-8-*O*-4′-neolignan (**5**), and 5-hydroxy-eugenol (**6**). The isolated compounds were subsequently evaluated using a 96-well microbioassay against plant pathogens. At 30 μM, compounds **2** and **3** possessed the most antifungal activity against *Phomopsis obscurans* and *Phomopsis viticola*, respectively.

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1. Introduction

Spices have been used since ancient times not only as flavoring agents, but also as folk medicines and food preservatives [1]. In addition, some spices are used to prolong the storage life of foods by preventing rancidity through their antioxidant activity or through antifungal or bactericidal activity [2]. Spices are generally recognized as safe (GRAS) due to their daily use over centuries in our food supply without any reports of deleterious effects [3].

Numerous studies have been published on the antifungal activity of plant extracts against different types of plant fungi. The methanol extracts of some commonly used Lamiaceae species were tested against four mycotoxigenic fungal species, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus ochraceus*, and *Fusarium proliferatum*. Of which, *Origanum vulgare*, *Origanum minutiflorum*, and *Tilia spicata* extracts showed significant antifungal activity [4].

The in vitro antifungal activity of water extracts of seven spices from cardamom, chili, coriander, onion, garlic, ginger and galangal was evaluated against three Roselle (*Hibiscus sabdariffa*) pathogens, *Phoma exigua*, *Fusarium nygami* and *Rhizoctonia solani*. All the extracts at three concentrations (10, 20 and 30%) inhibited fungal mycelium growth with varying degrees of effectiveness as compared to the control. Garlic extract exhibited the most growth inhibition against *P. exigua*, *F. nygami* and *R. solani* especially at 20 and 30% concentrations [5].

Both the volatile oil and curcumin of *Curcuma longa* exhibited a significant inhibitory effect of aflatoxin AFB_1 and AFB_2 production by *A. flavus* [6]. Wilson et al. studied the antifungal activity against *Botrytis cinerea* for extracts from 345 plants and 49 essential oils, they found that allium and capsicum extracts as

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well as the essential oils of *Cymbopogon martini*, *Thymus zygis*, *Cinnamomum zeylanicum* and *Eugenia caryophyllata* exhibited the most antifungal activity [7]. An investigation on the antifungal effects of rosemary, cumin, sater (savory), basil and pickling herb hydrosols against *R. solani*, *Fusarium oxysporum*, *B. cinerea* and *Alternaria citri* was carried out and the result showed that the hydrosols of sater and pickling herb showed the most relevant fungicidal activity [8].

The antifungal effects of essential oils derived from twenty spices were investigated against *A. niger*, *Candida albicans*, *Candida blanki*, *Candida cylindracea*, *Candida glabrata*, *Candida krusei*, *Candida tropicalis*, and *Saccharomyces cerevisiae* using the disc diffusion method [9]. Essential oil of cassia, allspice, clove, cumin, coriander, thyme, basil, anise, curry leaf, and asafetida inhibited all tested fungi, while oils from ginger, turmeric, and pomegranate were ineffective [9].

Strawberry anthracnose can be devastating since several plant parts may be infected in addition to the fruit. This fungal disease causes millions of dollars in crop loss each year [10]. *Phomopsis obscurans* causes a disease known as leaf blight of the cultivated strawberry and can infect foliage, runners, petioles, and fruits with a dark brown center surrounded by light-brown rings with purplish halos [11,12].

Phomopsis viticola causes phomopsis cane and leaf spot, which is an important disease of grapes worldwide, it affects most parts of the grapevine, such as leaves, rachis, flowers, and berries and up to 30% losses of the crop has been reported [13].

In this study, the antifungal activity of 22 common spices using direct bio-autography, coupled to a *Colletotrichum* species was performed to evaluate their potential utility in managing these plant pathogens. Turmeric, nutmeg, ginger, clove, oregano, cinnamon, anise, fennel, black pepper, basil and black cumin showed antifungal activity against three *Colletotrichum* species. Turmeric and nutmeg extracts were chosen for bioassay-guided fractionation, because they were the two most active spices among the tested spices. Three curcuminoids (**1**–**3**) were isolated from turmeric and two 8-*O*-4′-neolignans (**4**, **5**) along with 5-hydroxyeugenol (**6**) were isolated from nutmeg. The isolated compounds were subsequently evaluated using a 96-well micro-dilution broth assay against plant pathogens.

2. Materials and methods

2.1. General experimental procedures

UV spectra were obtained in MeOH using a Varian Cary 50 Bio UV–visible spectrophotometer and IR spectra were recorded using a Brucker Tensor 27 spectrophotometer. 1D and 2D NMR spectra were obtained on a Varian AS 400 spectrometer. High resolution electrospray ionization mass spectroscopy (HRESIMS) was recorded on a Brucker Bioapex FTMS in ESI mode. Classical TLC analysis was performed on silica gel 60 F_{254} 20 × 20 cm on an aluminium sheet (EMD). Detection was carried out under UV light (254 nm, 366 nm) and visualization made with vanillin–H_2SO_4 (1 g vanillin in 100 mL of 20% H_2SO_4 in EtOH) reagent followed by heating. Column chromatographic separations were performed on Si gel (Merck, 70–230 Mesh, and 63–200 μm), Sephadex (LH-20, Aldrich) and Strata Si-SPE (Phenomenex). Purity of the isolated compounds was confirmed by HPLC (Waters, PDA detector, C18 analytical Luna Phenomenex columns). The absorbance of the well plates was recorded on a Packard Spectra Count Microplate photometer (Downers Grove, IL).

2.2. Plant materials and chemicals

Spices were purchased as powders from McCormick & CO., Hunt Valley, MD, USA. ACS-grade solvents, methanol, dichloromethane (DCM), acetone, petroleum ether, ethyl acetate (EtOAc), and *n*-hexane were purchased from Fisher Scientific. Fungicide standards benomyl, cyprodinil, azoxystrobin, and captan were purchased from (Chem Service Inc., West Chester, PA).

2.3. Extraction

Each powdered material (30 g each) was extracted with MeOH (200 mL × 3) at room temperature for three days. Extracts were filtered and evaporated until dryness under reduced pressure.

2.4. Biossay guided isolation of compounds **1**–**3** from turmeric

Dried turmeric methanol extract (8.0 g) was fractionated on Si gel VLC (200 g, 10 × 30 cm) using EtOAc/*n*-hexane [25:70, 50:50, 75:25, 100:0 (500 mL each fraction)], followed by MeOH/EtOAc [50:50, 100:0 (1 L each)]. All the fractions were concentrated in vacuo and submitted for antifungal evaluation, fraction C (75% EtOAc/*n*-hexane) was the most active fraction against *Colletotrichum* species. Fraction C (2.8 g) was subsequently subjected to Si gel flash column chromatography (80 g, 2 × 60 cm) eluted with MeOH/DCM (0:100 to 15:85) to afford 8 subfractions (C-1 to C-8). The active fraction, C-4 (233 mg) was further purified by sephadex LH-20 using MeOH as an eluent followed by Si SPE column (10 g), eluted with isocratic 30% EtOAc in petroleum ether to yield 3 pure curcumenoids; curcumin (**1**, 40.0 mg), demethoxyated curcumin (**2**, 17.6 mg) and bisdemethoxy curcumin (**3**, 86.5 mg).

2.5. Bioassay guided isolation of compounds **4**–**6** from Nutmeg

Nutmeg methanol extract (5.0 g) was fractionated on Si VLC using the same method applied for turmeric, to yield 9 fractions (A-I). Fraction D [(75% EtOAc/*n*-hexane)] displayed antifungal activity against *Colletotrichum* species. Fraction D (644 g) was chromatographed on Si SPE (20 g) eluted by 10% EtOAc/*n*-hexane to afford 10 subfractions (D1–D10). Fraction D2 (430 mg) was subjected to amino SPE column (10 g) with a mobile phase of isopropanol/DCM (0:100 to 100:20), giving 9 fractions (D2a–D2i). Compound **4** (*erythro*-(7*R*,8*R*)-$\Delta^{8'}$-4,7-dihydroxy-3,3′,5′-trimethoxy-8-*O*-4′-neolignan, 155 mg) was isolated from fraction D2d by washing with cold methanol. Fraction D2a (45 mg) was further purified on C8 SPE column eluted with 65% MeOH/H_2O to provide **5** (*erythro*-(7R,8R)-$\Delta^{8'}$-7-acetoxy-3,4,3′,5′-tetra-methoxy-8-*O*-4′-neolignan, 11.5 mg). Fraction D2h (37.9) was subjected to C18 SPE column using 75% MeOH/H_2O as an eluent to yield compound **6** (5-hydroxy-eugenol 16.6 mg).

2.6. Antifungal assays

2.6.1. Fungal isolates and media

Isolates of *Colletotrichum acutatum* Simmonds, *Colletotrichum fragariae* Brooks, *Colletotrichum gloeosporioides* Penz. and Sacc and *P. obscurans* were isolated from strawberry (*Fragaria* x *ananassa* Duchesne), while *Phomopsis viticola* and *B. cinerea* were isolated from commercial grape (*Vitis vinifera* L.) and *F. oxysporum* was isolated from the orchid (*Cynoches* sp.) at the USDA-ARS, Natural Products Utilization Research Unit, Oxford, MS. Direct Bioautography Assay for Activity Against Plant Pathogenic Fungi. Matrix bioautography was used to screen crude extracts at 80 and 160 μg/spot on glass silica gel thin layer chromatography (TLC) plate. Three *Colletotrichum* species were used as test organisms to identify the antifungal fractions. Conidia of *C. fragariae*, *C. acutatum and C. gloeosporioides* suspensions were adjusted to 3.0×10^5 conidia/mL with liquid potato-dextrose broth (PDB, Difco, Detroit, MI) and 0.1% Tween-80. Using a 50 mL chromatographic sprayer, each TLC plate with fluorescent indicator (250 mm, Silica Gel GF Uniplate, Analtech, Inc., Newark, DE) was sprayed lightly (until damp) three times with the conidial suspension. Inoculated plates were placed in a $30 \times 13 \times 7.5$ cm moisture chamber (398-C, Pioneer Plastics, Inc. Dixon, KY) and incubated in a growth chamber at 24 ± 1 °C with 12 h photoperiod under 60 ± 5 $\mu mols \cdot m^{-2} \cdot s^{-1}$ light. Inhibition of fungal growth was measured 4 d after treatment. Sensitivity of each fungal species to each test compound was determined by comparing the size of the inhibitory zones. Fungicide standards benomyl, cyprodinil, azoxystrobin, and captan were used as controls at 2 mM in 2 μL of EtOH.

2.6.2. Micro-dilution broth assay

A standardized 96-well micro-dilution broth assay developed by Wedge and Kuhajek was used to evaluate the antifungal activity of the extracts and pure isolates against *B. cinerea*, *C. acutatum*, *C. fragariae*, *C. gloeosporioides*, *P. viticola*, *P. obscurans* and *F. oxysporum* [14]. The fungicide captan was used as an internal fungicide standard in all assays. Each fungus was challenged in a dose–response format using test compounds where the final treatment concentrations were 0.3, 3.0 and 30.0 μM. Sixteen wells containing broth and inoculum served as positive controls, eight wells containing solvent at the appropriate concentration and broth without inoculum were used as negative controls. The experiments were repeated three times. Fungal growth was then evaluated by measuring the absorbance of each well at 620 nm using a microplate photometer (Packard Spectra Count, Packard Instrument Co., Downers Grove, IL). Mean absorbance values and standard errors were used to evaluate fungal growth at 48 h and 72 h except for *P. obscurans* and *P. viticola* in which the data were recorded at 144 h. Means for percent inhibition of each fungus at each dose of test compounds relative to the untreated positive growth controls

Table 1
Fungal growth inhibition for tested spices against three *Colletotrichum* species.

Plant name/Part used	Mean fungal growth inhibition[a] (mm) ± SD					
	C. acutatum 80 μg/spot	*C. fragariae* 80 μg/spot	*C. gloeosporioides* 80 μg/spot	*C. acutatum* 160 μg/spot	*C. fragariae* 160 μg/spot	*C. gloeosporioides* 160 μg/spot
Turmeric/Rhizome (*Curcuma longa*)	5.5 ± 0.71	6.0 ± 0.00	5.5 ± 0.71	10.5 ± 0.71	11 ± 0.71	10.5 ± 0.71
Ginger/Rhizome (*Zingiber officinale*)	4.5 ± 0.71	5.0 ± 0.00	4.5 ± 0.71	6.0 ± 0.71	6.5 ± 0.71	6.0 ± 0.71
Cardamom/Fruit	NA	NA	NA	NA	NA	NA
Nutmeg/Fruit (*Myristica fragrans*)	4.5 ± 0.71	5.0 ± 0.00	4.5 ± 0.71	7.0 ± 0.71	7.5 ± 0.00	7.0 ± 0.71
Cinnamon/Bark (*Cinnamomum cassia*)	5.0 ± 0.71	5.5 ± 0.00	4.5 ± 0.71	6.0 ± 0.71	6.5 ± 0.71	6.0 ± 0.71
Black pepper/Fruit (*Piper nigrum*)	5.5 ± 0.71	6.0 ± 0.00	5.5 ± 0.71	6.0 ± 0.00	6.5 ± 0.71	6.0 ± 0.71
Clove/Fruit (*Syzygium aromaticum*)	5.5 ± 0.71	6.0 ± 0.00	6.0 ± 0.00	6.5 ± 0.71	7.0 ± 0.00	5.5 ± 0.71
Garlic/Seed	NA	NA	NA	Diffuse	Diffuse	Diffuse
Poppy/Seed	NA	NA	NA	NA	NA	NA
Paprika/Fruit	NA	NA	NA	Diffuse	Diffuse	Diffuse
Cayenne/Fruit	NA	NA	NA	NA	NA	NA
Black cumin/Seed (*Nigella sativa*)	4.5 ± 0.71	5.5 ± 0.71	4.5 ± 0.00	5.5 ± 0.71	6.0 ± 0.00	5.5 ± 0.71
Fennel/Fruit (*Foeniculum vulgare*)	5.5 ± 0.71	6.0 ± 0.00	5.5 ± 0.71	6.0 ± 0.00	6.5 ± 0.71	6.0 ± 0.00
Anise/Fruits (*Pimpinella anisum*)	5.6 ± 0.14	6.2 ± 0.21	5.2 ± 0.71	6.1 ± 0.14	6.7 ± 0.21	6.1 ± 0.14
Coriander/Fruits	NA	NA	NA	NA	NA	NA
Cumin/Fruits	NA	NA	NA	NA	NA	NA
Dill/Fruits	NA	NA	NA	Diffuse	Diffuse	Diffuse
Cilantro/Herb	NA	NA	NA	Diffuse	Diffuse	Diffuse
Oregano/Herb (*Origanum vulgare*)	4.5 ± 0.71	5.5 ± 0.71	5.0 ± 0.00	6.0 ± 0.71	6.5 ± 0.71	6.0 ± 0.71
Basil/Herb (*Ocimum basilicum*)	Diffuse	Diffuse	Diffuse	4.5 ± 0.71	5.0 ± 0.00	4.5 ± 0.71
Rosemary/Herb	NA	NA	NA	Diffuse	Diffuse	Diffuse
Mint/Herb	NA	NA	NA	Herb	NA	NA

Agrochemical standards

Agrochemical fungicides[b]	Benomyl at 1.16 μg	Captan at 1.2 μg	Cyprodinil at 0.9 μg	Azoxystrobin at 1.61 μg
C. acutatum	Diffuse	11.7 ± 1.77	Diffuse	Diffuse
C. fragariae	22 ± 2.16	14.75 ± 0.96	19 ± 1.83	21.25 ± 2.63
C. gloeosporioides	Diffuse	12.25 ± 1.26	Diffuse	Diffuse

Diffuse zones represents for not clear antifungal zones. NA: Not active at tested concentration.

[a] Mean inhibitory zones and standard deviations (SD) were used to determine the level of antifungal activity against each fungal species.

[b] Technical grade agrochemical fungicides (without formulation) with different modes of action were used as internal standards.

were used to evaluate fungal growth. The SAS system analysis of variance procedure (Statistical Analysis System, Cary, North Carolina) was used to identify significant factors, and Fisher's protected LSD was used to separate means [15].

3. Results and discussion

In our search for new naturally occurring plant protectants from aromatic and medicinal plants, four spices from the Lamiaceae family (mint, basil, oregano and rosemary), six from the Apiaceae family (fennel, anise, coriander, cilantro, cumin and dill), three from the Zingiberaceae family (cardamom, ginger and turmeric) and nine spices (garlic, cinnamon, nutmeg, clove, poppy seed, paprika, cayenne, black pepper and black cumin) from miscellaneous families were evaluated for their antifungal activity against plant pathogens. All methanolic extracts were tested against *C. acutatum*, *C. fragariae* and *C. gloeosporioides* and fungal growth inhibition was determined using direct bioautography techniques. Of the tested spice extracts, turmeric (*Curcuma longa*), nutmeg (*Myristica fragrans*), ginger (*Zingiber officinale*), clove (*Syzygium aromaticum*), oregano (*O. vulgare*), cinnamon (*Cinnamomum cassia*), anise (*Pimpinella anisum*), fennel (*Foeniculum vulgare*), basil (*Ocimum basilicum*), black cumin (Nigella sativa) and black pepper (*Piper nigrum*) showed antifungal activity against *Colletotrichum* species (Table 1).

Turmeric and nutmeg extracts showed the largest fungal growth inhibition zones and these two extracts were selected for further evaluation to investigate the bioactive secondary metabolites using the bioassay-guided fractionation. Turmeric and nutmeg methanol extracts demonstrated non-specific growth inhibition against *Colletotrichum* fungal species at 80 μg/spot and 160 μg/spot (Table 1). Biological guided fractionation of turmeric extract resulted in the isolation of three curcumenoids (**1**–**3**) from the active column fraction and their chemical structures (Fig. 1) were determined by using a combination of 1D and 2D NMR techniques as well as HR-MS and confirmed by comparing their spectroscopic data with those reported for curcumin (**1**), demethylated curcumin (**2**) and bisdimethyl curcumin (**3**) [16–18].

From nutmeg, compounds **4** and **5** (Fig. 1) were isolated as optically active ($[\alpha]_D^{25}$ + 20.6 (c 0.5, $CHCl_3$, **4**; $[\alpha]_D^{25}$ + 23.8 (c 0.5, $CHCl_3$, **5**)); colorless viscous substances. Their molecular formulae were determined as $C_{21}H_{26}O_6$ and $C_{24}H_{30}O_7$ respectively by HRESIMS. Their ^{13}H and ^{13}C NMR spectroscopic data (supporting information) were similar to those reported for *erythro*-(7*R*,8*R*)-$\Delta^{8'}$-4,7-dihydroxy-3,3′,5′-trimethoxy-8-*O*-4′-neolignan (**4**), and *erythro*-(7*R*,8R)-$\Delta^{8'}$-7-acetoxy-3,4,3′,5′-tetra-methoxy-8-*O*-4′-neolignan (**5**) [19–21]. The small coupling constants between the H-7-and H-8 methine protons in compounds **4** (J = 2.4 Hz) and **5** (J = 3.2 Hz) confirmed their relative configuration, which was supported by measuring their optical rotation [19–21]. Compound **6** was also isolated form nutmeg and chemically identified as 5-hydroxyeugenol (**6**) by comparing its spectroscopic data with those published in literature [22,23].

The isolated compounds (**1**–**6**) were evaluated individually using a 96 well micro-dilution broth bioassays against *B. cinerea*, *C. acutatum*, *C. fragariae*, *C. gloeosporioides*, *P. viticola*, *P. obscurans* and *F. oxysporum*. Compound **3** showed weak antifungal activity

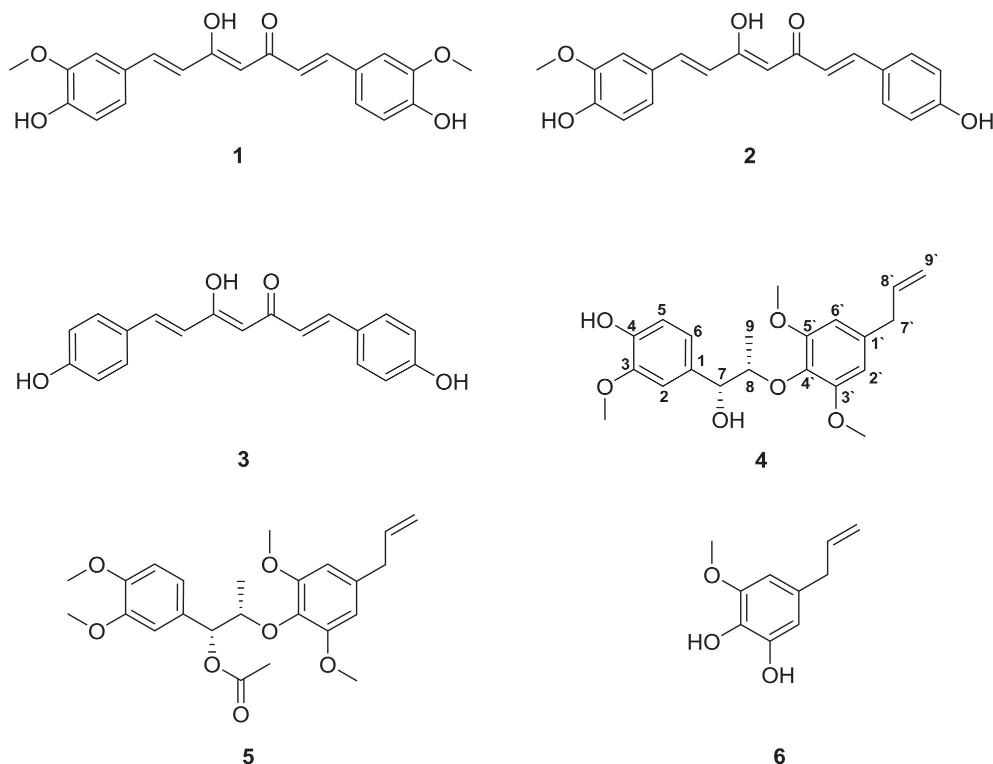

Fig. 1. Chemical structures of compounds **1**–**6**.

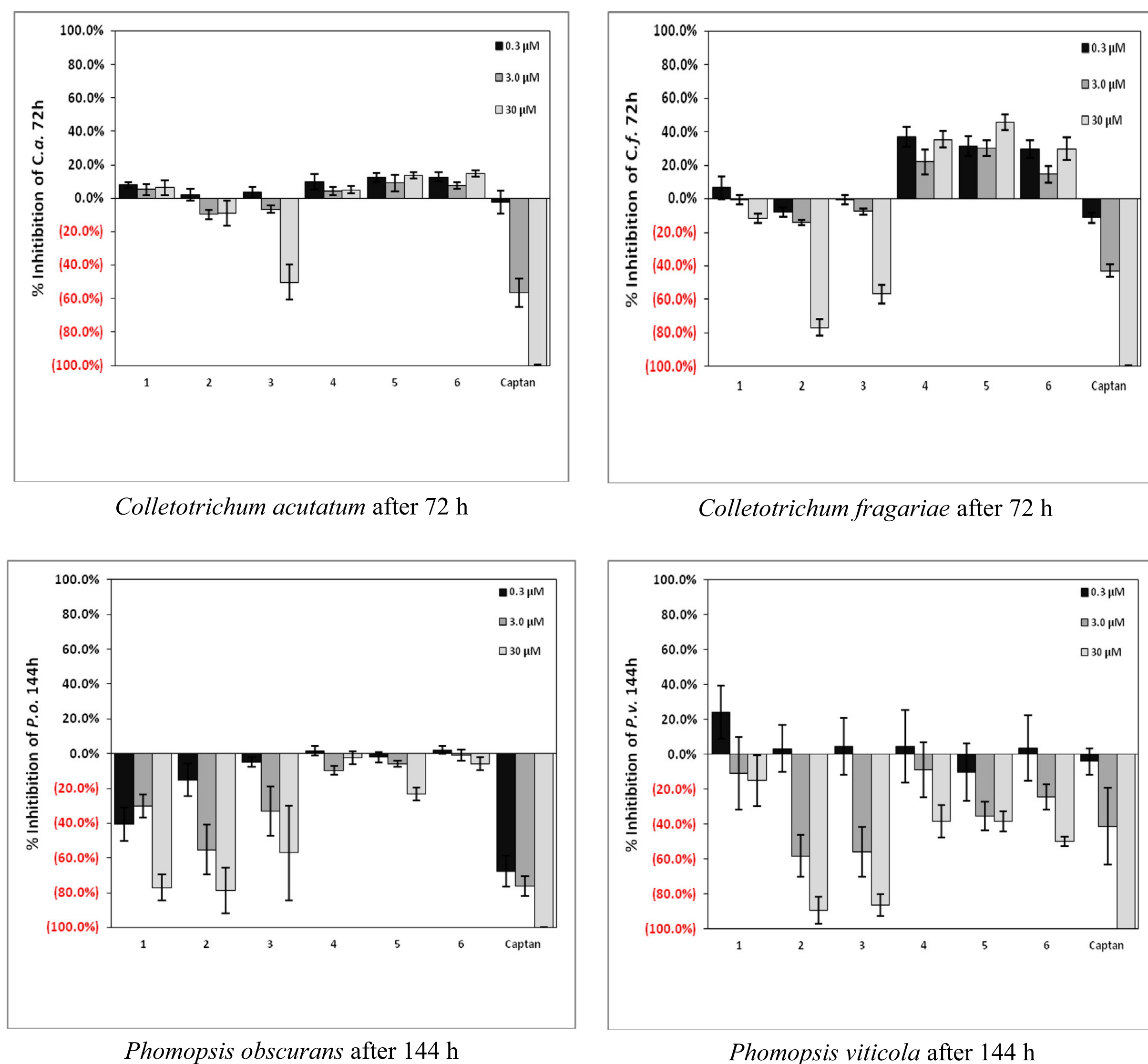

Fig. 2. Growth inhibition of *Colletotrichum*, and *Phomopsis* species using 96 well microdilution broth assays in a dose response to isolated compounds (**1**–**6**) with the commercial fungicide standard captan.

at 30 μM with 50.45% and 56.9% inhibition against *C. acutatum*, *C. fragariae* after 72 h, respectively. Compound **2** possessed good antifungal activity with 76.8% growth inhibition of *C. fragariae* at 30 μM (Fig. 2). However, compounds **2** and **3** showed very weak activity against *C. gloeosporioides* at the highest concentration (30 μM). Compounds **1**, **4**, **5** and **6** were inactive against three *Colletotrichum* species (Fig. 2). Curcumenoids (**1**–**3**) exhibited the most antifungal activity against *Phomopsis* species with 77.0%, 78.6% and 56.9% growth inhibition of *P. obscurans* at 30 μM, respectively. Compounds **2** and **3** showed more potent and selective growth inhibition of *P. viticola* at 30 μM with 82.8% and 90.7% at 144 h (Fig. 2). Compounds **4**, **5**, and **6** had very weak antifungal activity with 28.7%, 28.1%, and 46.6% growth inhibition of *P. viticola*, respectively (Fig. 2). No compounds showed any antifungal activity against *F. oxysporum* (data not shown). Jun-Young et al. also reported that curcumenoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin) showed good to moderate in vitro antifungal activity against *Colletotrichum coccodes*, *C. acutatum* and *C. gloeosporioides*. However, only demethoxycurcumin demonstrated in vivo antifungal activity against *C. coccodes* causing red pepper anthracnose at the concentrations of 500 and 1000 μg/ml [24].

In conclusion, we used bioautography technique to identify antifungal compounds in plant extracts against three *Colletotrichum* species and the isolated pure compounds were subsequently followed in dose response 96-well microtiter assays against wide range of plant pathogens. In this current study, out of the six isolated compounds, two curcumenoids demethylated curcumin (**2**) and bisdimethyl curcumin (**3**) possessed the highest antifungal activity against *P. viticola*. Although curcumenoids were previously reported their anti-fungal activity against *Colletotrichum* species, this is the first report on the evaluation of the antifungal activity of turmeric and nutmeg extracts as well as compounds (**1**–**6**) against

P. obscurans, *P. viticola*, *F. oxysporum and B. cinerea*. These two curcumenoids (**2**, **3**) could be potential lead molecules to develop new natural based chemical controls for phomopsis diseases with low mammalian and environmental toxicity. This research also indicated that the biological activity of curcumenoids extends beyond post-harvest pathogens of foods such as phomopsis, which cause serious diseases of strawberry and grape plants. Curcumenoids might be a good source of lead compounds for pesticides. To find a natural product that has wide range of fungicide application, there are continuing researches on natural compounds for this goal.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2014.08.021.

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