Antifungal Activity of Camptothecin, Trifolin, and Hyperoside Isolated from *Camptotheca acuminata*

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Leaf spots and root rots are major fungal diseases in *Camptotheca acuminata* that limit cultivation of the plant for camptothecin (CPT), a promising anticancer and antiviral alkaloid. Bioassays showed that pure CPT and flavonoids (trifolin and hyperoside) isolated from *Camptotheca* effectively control fungal pathogens in vitro, including *Alternaria alternata*, *Epicoccum nigrum*, *Pestalotia guepinii*, *Drechslera* sp., and *Fusarium avenaceum*, although antifungal activity of these compounds in the plant is limited. CPT inhibited mycelial growth by approximately 50% (EC_{50}) at 10–30 μg/mL and fully inhibited growth at 75–125 μg/mL. The flavonoids were less effective than CPT at 50 μg/mL, particularly within 20 days after treatment, but more effective at 100 or 150 μg/mL. CPT, trifolin, and hyperoside may serve as leads for the development of fungicides.

KEYWORDS: *Camptotheca*; camptothecin; trifolin; hyperoside; antifungal activity; chemical defense

INTRODUCTION

Camptotheca Decaisne (happytrees) of the family Nyssaceae is the major source of camptothecin (CPT) and its analogues, promising anticancer and antiviral indole alkaloids (*1*). The structure of CPT isolated from *Camptotheca acuminata* Decaisne was reported in 1966 (*2, 3*). Two CPT drugs, Hycamtin and Camptosar, have received FDA approval for the treatment of ovarian and lung cancers and for colorectal cancer, respectively (*3*). Together they account for nearly 8% of the total $15 billion of the global cancer drug market. Orathecin is being considered by the FDA for accelerated approval for the treatment of pancreatic cancer, with 14 other CPT drugs currently undergoing clinical trials (*4*). CPTs also show promising potential as antiviral (HIV and herpes) (*1*), antifungal (*Candida*) (*5*), and antipsoriasis (*1*) drugs, and as pesticides (*6*). To date, commercial CPT synthesis is not feasible, and supplies of CPT required to manufacture the drugs are now extracted from the fruits of *C. acuminata*, listed as an endangered species in China since 1997 (*4*). Cultivation of the tree is limited because it grows in subtropical climates. Moreover, the tree takes approximately 10 years to produce a stable fruit yield. Recently, two new species of *Camptotheca* were added to the previously monotypic genus, and several high-yielding cultivars such as Katie have been developed to harvest intact young vegetative tissues (*4*). Furthermore, "trichome management" was developed to induce the biosynthesis of CPTs to levels 16–20 times that normally observed in *Camptotheca* (*7*). Eleven years of greenhouse and field trials in Texas have shown great potential for future supply of CPTs (Camptotech, Inc., unpublished data).

Fungal diseases, particularly leaf spots and root rots, are the main limiting factors in the cultivation of *Camptotheca* in plantations for vegetative biomass and CPT production. In a preliminary study, seven fungal pathogens have been isolated from various tissues of *C. acuminata*: *Alternaria alternata*, *Epicoccum nigrum*, and *Pestalotia guepinii* caused leaf spots; *Discula umbrinella*, *Drechslera* sp., and *Nectria* sp.; *Fusarium avenaceum* caused root rots (*8*). Interestingly, however, we observed that fungal diseases were primarily found on the *Camptotheca* varieties and tissues with lower CPT contents, while fungal inoculation on young leaf tissues with high CPT content failed. Considering CPT's potent bioactivity against tumors, viruses, pests, and *Candida* (*1, 5, 6*), we conducted this in vitro antifungal assay to determine if CPT inhibits the fungi that often infect the *Camptotheca* trees. In addition to the alkaloid CPT, trifolin and hyperoside (**Figure 1**), two flavonoids abundantly occurring in *C. acuminata* leaves (*9*), are included in this study. These flavonoids commonly occur in a wide range of plants and have shown bactericidal activity (*10, 11*). Abou Zeid found that crude extracts and flavonoids isolated from banana (*Musa*), including trifolin and hyperoside, have some antifungal activities (*12*). However, several anomalous reports lead to uncertainty as to the antifungal activities of trifolin and hyperoside. Funayama et al. (1995) found that trifolin was inactive against yeasts at 1000 μg/mL (*13*). Lu et al. (2002) found that hyperoside was inactive in in vitro bioassays against yeasts, *Fusarium*, and other fungi at >100 μg/mL (*14*). Dall'Agnol et al. reported that crude extracts of *Hypericum*,

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Table 1. Experimental Concentrations of Three Compounds (CPT, Trifolin, and Hyperoside) Isolated from *Camptotheca* Leaves for in Vitro Tests against Five Fungi Isolated from *C. acuminata*

fungus	concn (µg/mL)					
	control	CPT	trifolin	hyperoside	Maneb[a]	Bravo[b]
A. alternata	0	10, 25, 50, 75, 100, 125, 150			3000	10000
E. nigrum	0	10, 25, 50, 75, 100, 125, 150			3000	10000
P. guepinii	0	10, 30, 50, 70, 100, 125, 150	50, 100, 150	50, 100, 150	3000	10000
Drechslera sp.	0	10, 30, 50, 70, 100, 125, 150	50, 100, 150	50, 100, 150	3000	10000
F. avenaceum	0	10, 30, 50, 70, 100, 125, 150	50, 100, 150	50, 100, 150	3000	10000

[a] The concentration of active ingredient chlorothalonil. [b] The concentration of active ingredient manganous ethylenebis[dithiocarbamate].
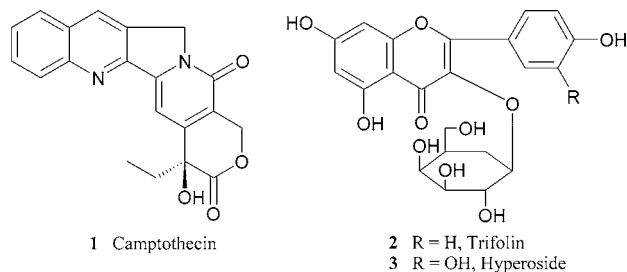
Figure 1. CPT and flavonoids, namely, trifolin and hyperoside, isolated from *C. acuminata* leaves.

including hyperoside, showed no activity against yeast (*15*). The present study investigates the in vitro antifungal activity of CPT, trifolin, and hyperoside, naturally occurring in *C. acuminata*, against five fungi isolated from the leaves. The results will provide a basis for developing strategies to control fungal pathogens in *Camptotheca* plantations and to enhance CPT production.

MATERIALS AND METHODS

Plant Materials. Leaves of *C. acuminata* were collected in Nacogdoches, TX, in June 2002. A voucher specimen (S. Y. Li and Z. Z. Zhang US-TX-AH-02-0601012) has been deposited at the Center for Medicinal Plant Research of Stephen F. Austin State University.

Chemical Analyses. Camptothecin (**1**), trifolin (**2**), and hyperoside (**3**) (**Figure 1**) were previously isolated from the leaves of *C. acuminata* (*9*). Their structures were determined by spectroscopic methods. The ^{1}H and ^{13}C NMR data of these compounds are in agreement with those from refs *16−18*. General experimental procedures and extraction and isolation methods are described in detail in ref *9*.

Antifungal Assays. *A. alternata*, *E. nigrum*, *P. guepinii*, *Drechslera* sp., and *F. avenaceum* were isolated from infected leaves and roots of *C. acuminata* grown in Nacogdoches, TX, in 2001 and 2002. The strains were cultured and maintained on potato dextrose agar (PDA) medium at 24 °C.

CPT, trifolin, and hyperoside isolated from *C. acuminata* were tested for their ability to inhibit these fungi with two standard classical fungicides, Bravo (active ingredient chlorothalonil) and Maneb (active ingredient manganous ethylenebis[dithiocarbamate]). The concentrations of the fungicides were those recommended by the manufacturers (Bravo, 10000 µg/mL; Maneb, 3000 µg/mL). In addition to a negative control (0) and two fungicides as positive controls for each of the five isolated pathogens, seven concentrations of CPT were tested with each of the fungi and three levels of trifolin and hyperoside were tested with *P. guepinii*, *Drechslera* sp., and *F. avenaceum*, respectively (**Table 1**). Trifolin and hyperoside treatments were applied at concentrations of 50, 100, and 150 µg/mL, respectively. For all cultures, final concentrations were made in molten (50 °C) potato dextrose agar (Difco), and 10 mL aliquots were poured into Petri dishes (85 mm in diameter). Within 24 h after pouring, each of the plates was inoculated with one of the five fungi. One 5 × 5 mm mycelial plug was cut from the actively growing front of a 2 week old colony, then placed with the inoculum side down in the center of each treatment plate, and incubated at 24
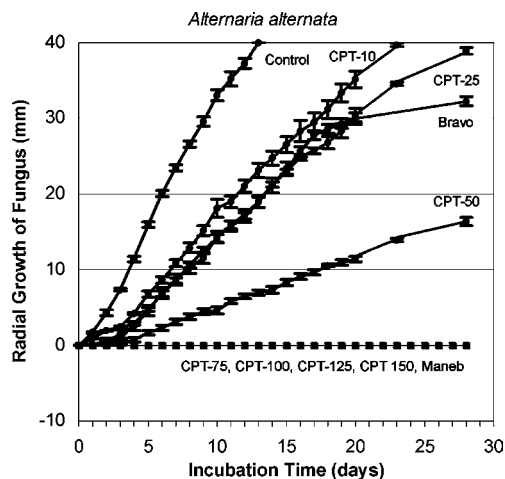
Figure 2. Effect of different concentrations of CPT, Bravo, and Maneb on mycelial growth of *A. alternata*: CPT, 10 µg/mL (CPT-10), 25 µg/mL (CPT-25), 50 µg/mL (CPT-50), 75 µg/mL (CPT-75), 100 µg/mL (CPT-100), 125 µg/mL (CPT-125), and 150 µg/mL (CPT-150); Bravo, 10000 µg/mL; Maneb, 3000 µg/mL. Points and bars represent the means and standard errors of five replicates.

°C. For all experiments, five replicate plates were inoculated for each treatment.

Mycelial growth on each plate was observed daily, recorded on a transparent film for the first two weeks, and then recorded on the 16th, 20th, 23rd, and 28th days. Colony radii were measured along four vertical radial directions. The mean of the four measurements was calculated as the growth rate on each plate. The mean and standard error were calculated from the five replicates of each treatment. For each of the fungi, values of EC_{50} and MIC of each compound were estimated.

RESULTS

Antifungal Activity against *A. alternata*. CPT significantly inhibited *Alternaria* growth at 10 µg/mL (CPT-10) ($p < 0.001$). Colonies exposed to CPT-10 were inhibited 41−66%; it took 23 days for mycelium under the CPT-10 treatment to completely cover the agar surface, compared to 13 days under the control treatment. CPT-25 (CPT at 25 µg/mL) was similar to Bravo in its ability to inhibit *A. alternata* (**Figure 2**). Colonies in both treatments started to grow on the second day of the experiment, and their radii were always within about 1 mm of one another. CPT-25 colony expansion was reduced by 215% (28 days to cover the agar surface vs 13 days for the control). On day 13, colonies in the CPT-25 treatment were 52.8% smaller than those of the controls. Thus, it is estimated that the EC_{50} of CPT for *A. alternata* is <25 µg/mL. The CPT-50 treatment was able to inhibit fungal growth by more than 180%. The fungus was totally controlled by CPT at 75 µg/mL (CPT-75) and above, and by Maneb.

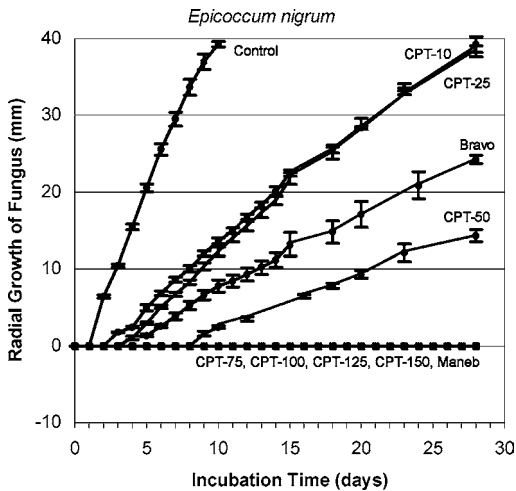**Figure 3.** Effect of different concentrations of CPT, Bravo, and Maneb on mycelial growth of *E. nigrum*: CPT, 10 μg/mL (CPT-10), 25 μg/ mL (CPT-25), 50 μg/mL (CPT-50), 75 μg/mL (CPT-75), 100 μg/mL (CPT-100), 125 μg/mL (CPT-125), and 150 μg/mL (CPT-150); Bravo, 10000 μg/mL; Maneb, 3000 μg/mL. Points and bars represent the means and standard errors of five replicates.

Antifungal Activity against *E. nigrum.* *E. nigrum* grew faster than *A. alternata* under control conditions in the experiment, with colonies covering the agar surface in 10 days (**Figure 3**). However, this fungus was more strongly inhibited by CPT and fungicide than *A. alternata*. CPT at 10 and 25 μg/mL delayed mycelial growth of *E. nigrum* by 280% (28 days to cover the agar surface vs 10 days for the control). CPT-10 and CPT-25 treatments were not significantly different in inhibiting *E. nigrum*. On day 10, colonies in the CPT-10 treatment were 65% smaller than those of the controls. Thus, the EC_{50} of CPT for *E. nigrum* is estimated to be <10 μg/mL. Similar to results with *A. alternata*, CPT at 50 μg/mL inhibited growth of *E. nigrum* by >90% while CPT at 75 μg/mL and higher concentrations, as well as Maneb, totally inhibited growth. Bravo treatment was slightly superior to CPT-10 and CPT-25 treatment. Colonies exposed to Bravo did not start to grow until the fifth day; by day 15 they had radii of 13 mm compared to 20 mm in the CPT-25 treatment. The CPT-50 treatment was superior to Bravo treatment. Under this treatment, growth did not begin until the eighth day of the experiment. On day 20, colony radii measured 19 mm under Bravo treatment but only 8 mm when exposed to CPT-50.

Antifungal Activity against *P. guepinii.* *P. guepinii* showed a growth pattern similar to that of *E. nigrum* under control conditions (**Figure 4**). CPT-10 reduced mycelial growth by 43.5% on the 11th day when the fungus under the control treatment had covered the agar surface. Colonies exposed to CPT-10 treatment did not reach the Petri dish margins until day 20. Thus, it is estimated that the EC_{50} of CPT for *P. guepinii* is approximately 10 μg/mL. However, CPT totally inhibited growth only at $\geq$125 μg/mL. Both fungicides also successfully controlled *P. guepinii*. Trifolin and hyperoside were similar in their abilities to inhibit *P. guepinii*. On day 11, colonies exposed to either trifolin-50 or hyperoside-50 were inhibited by 53.4% and 53.8%, respectively. The EC_{50} of both flavonoids for *P. guepinii* is estimated to be approximately 50 μg/mL. However, as concentrations increased to 100 μg/mL, the flavonoids were more effective in inhibition of *P. guepinii* than CPT; MICs of both trifolin and hyperoside for *P. guepinii* are most likely below 125 μg/mL, the level at which CPT successfully controlled the fungus.
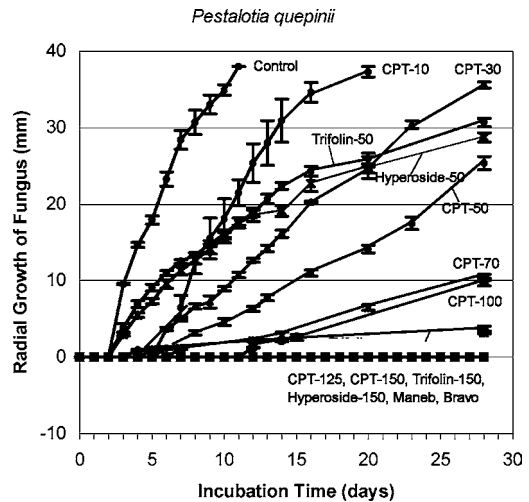

Figure 4. Effect of different concentrations of CPT, trifolin, hyperoside, Bravo, and Maneb on mycelial growth of *P. quepinii*: CPT, 10 μg/mL (CPT-10), 30 μg/mL (CPT-30), 50 μg/mL (CPT-50), 70 μg/mL (CPT-70), 100 μg/mL (CPT-100), 125 μg/mL (CPT-125), and 150 μg/mL (CPT-150); trifolin, 50 μg/mL (Trifolin-50), 100 μg/mL (Trifolin-100), and 150 μg/mL (Trifolin-150); hyperoside, 50 μg/mL (Hyperoside-50), 100 μg/mL (Hyperoside-100), and 150 μg/ mL (Hyperoside-150); Bravo, 10000 μg/ mL; Maneb, 3000 μg/mL. Points and bars represent the means and standard errors of five replicates.
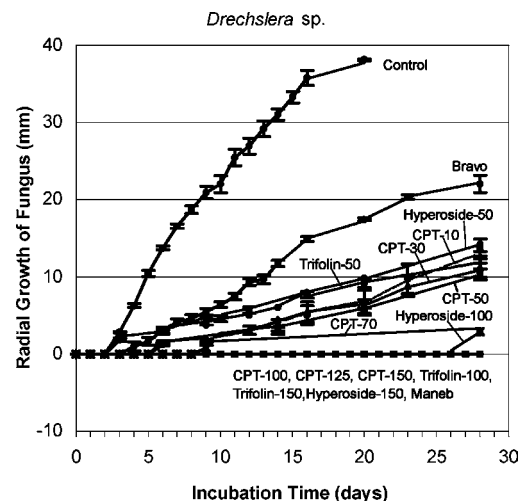

Figure 5. Effect of different concentrations of CPT, trifolin, hyperoside, Bravo, and Maneb on mycelial growth of *Drechslera* sp.: CPT, 10 μg/ mL (CPT-10), 30 μg/mL (CPT-30), 50 μg/mL (CPT-50), 70 μg/mL (CPT-70), 100 μg/mL (CPT-100), 125 μg/mL (CPT-125), and 150 μg/mL (CPT-150); trifolin, 50 μg/mL (Trifolin-50), 100 μg/mL (Trifolin-100), and 150 μg/mL (Trifolin-150); hyperoside, 50 μg/mL (Hyperoside-50), 100 μg/mL (Hyperoside-100), and 150 μg/mL (Hyperoside-150); Bravo, 10000 μg/ mL; Maneb, 3000 μg/mL. Points and bars represent the means and standard errors of five replicates.

Antifungal Activity against *Drechslera* sp. *Drechslera* sp. grew more slowly than *A. alternata*, *E. nigrum*, and *P. guepinii* under control conditions but was also more sensitive to CPT, flavonoids, and fungicides than the other fungi tested (**Figure 5**). CPT at all experimental levels showed greater inhibition rates than Bravo. This fungicide reduced mycelial growth by 54.4% on day 20, when control mycelium completely covered the agar surface. Thus, the EC_{50} of CPT for *Drechslera* is <10 μg/mL. However, CPT only completely inhibited fungal growth at $\geq$100 μg/mL. Trifolin-50 and hyperoside-50 (trifolin and hyperoside

Antifungal Activity of Camptothecin, Trifolin, and Hyperoside
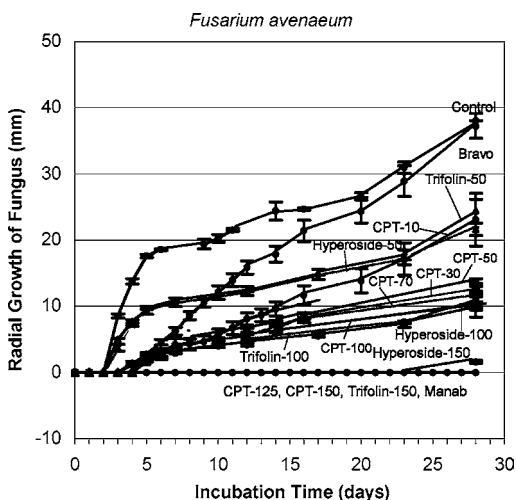
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Figure 6. Effect of different concentrations of CPT, trifolin, hyperoside, Bravo, and Maneb on mycelial growth of *F. avenaceum*: CPT, 10 μg/mL (CPT-10), 30 μg/mL (CPT-30), 50 μg/mL (CPT-50), 70 μg/mL (CPT-70), 100 μg/mL (CPT-100), 125 μg/mL (CPT-125), and 150 μg/mL (CPT-150); trifolin, 50 μg/mL (Trifolin-50), 100 μg/mL (Trifolin-100), and 150 μg/mL (Trifolin-150); hyperoside, 50 μg/mL (Hyperoside-50), 100 μg/mL (Hyperoside-100), and 150 μg/mL (Hyperoside-150); Bravo, 10000 μg/mL; Maneb, 3000 μg/mL. Points and bars represent the means and standard errors of five replicates.

at 50 μg/mL) were similar in their ability to inhibit *Drechslera*. On day 20, colonies exposed to either trifolin-50 or hyperoside-50 were inhibited by 76.1% and 74.3%, respectively. Thus, the EC$_{50}$ values of both flavonoids against *Drechslera* are <50 μg/mL. Hyperoside-100 (hyperoside at 100 μg/mL) inhibited fungal growth successfully, with little growth by day 28, while trifolin-100 (trifolin at 100 μg/mL) totally controlled the fungus over the course of the entire experiment. CPT at ≥100 μg/mL, trifolin at ≥100 μg/mL, hyperoside at 150 μg/mL, and Maneb completely inhibited *Drechslera*.

Antifungal Activity against *F. avenaceum*. *F. avenaceum* exhibited the slowest growth rate of all experimental fungi under control conditions, but was somewhat less sensitive to CPT, flavonoids, and fungicides (**Figure 6**). Bravo showed effective inhibition of mycelial growth during the first several days of the experiment but was less effective in the later stages. On day 28, mycelium with Bravo treatment completely covered the agar surface similar to the control colonies. CPT-10 was much more effective than Bravo, with 70−80% inhibition of mycelial growth during the first several days of the experiment and approximately 40% inhibition in the later stages. CPT-30 inhibited the rate of mycelial growth by >60%. Thus, the EC$_{50}$ of CPT for *F. avenaceum* is estimated to be between 10 and 30 μg/mL. Higher levels of CPT more effectively inhibited mycelial growth, but complete inhibition was not achieved until 125 μg/mL. Trifolin and hyperoside exhibited similar inhibition patterns at 100 μg/mL, but trifolin was more effective than hyperoside at 50 μg/mL. Trifolin-50 and hyperoside-50 were less effective against *F. avenaceum* than Bravo and CPT-10 at the beginning of the experiment but more effective than Bravo and similar to CPT-10 during the later stages of the experiment. On day 28, trifolin and hyperoside inhibited fungal growth by 35.8% and 31.6%, respectively, at 50 μg/mL, and by 74.8% and 72.6%, respectively, at 100 μg/mL. Thus, it is estimated that EC$_{50}$ values of both flavonoids against *F. avenaceum* are between 50 and 100 μg/mL. Hyperoside at 150 μg/mL completely inhibited fungal growth during the first four weeks, while trifolin at the

Table 2. Inhibition of Alkaloid and Flavonoids Isolated from *C. acuminata* against Mycelial Growth of Fungi[a]

fungus	EC$_{50}$ (μg/mL)			MIC (μg/mL)		
	CPT	trifolin	hyperoside	CPT	trifolin	hyperoside
A. alternata	<25			<75		
E. nigrum	<10			<75		
P. guepinii	10	<50	<50	<125	100–150	100–150
Drechslera sp.	<10	<50	<50	<100	<100	100–150
F. avenaceum	10–30	75	75	<125	<125	>150

[a] Both EC$_{50}$ (effective concentration that caused 50% inhibition of mycelial growth) and MIC (minimum inhibitory concentration) are the average of five replicates measured when the fungus completely covered the agar surface under control conditions.

same level totally controlled the fungus during the entire experiment. CPT at ≥125 μg/mL and Maneb also completely inhibited growth of *F. avenaceum*.

DISCUSSION

Fungal Pathogens of *C. acuminata*. Leaf spots caused by *Alternaria*, *Epicoccum*, and *Pestalotia* and root rots caused by *Fusarium* are major fungal diseases affecting biomass and CPT production in *C. acuminata* cultivation. *Alternaria* is commonly found on leaf spots and dead tissues of *C. acuminata*. Leaf spots often increase in severity as the season progresses and leaves become mature (*19*). Leaves die and drop off prematurely, and yields decreased as a result of the plant's decreased ability to carry out photosynthesis. *E. nigrum* is a common cause of leaf spots in *C. acuminata*. It is a cosmopolitan saprophyte found on many plants, textiles, paper products, and foodstuffs, in soils, and in air samples. *P. guepinii* is primarily a secondary pathogen. It is saprophytic on dead and dying tissues and is weakly parasitic, infecting through wounds under moist conditions. *Drechslera* sp. are either plant pathogens or saprobes. *Fusarium* is a soil-borne fungus with worldwide distribution, particularly throughout tropical and subtropical areas.

Antifungal Activity of Alkaloid and Flavonoids Isolated from *C. acuminata*. Bioassays showed that the pure alkaloid CPT and the flavonoids trifolin and hyperoside isolated from *C. acuminata* can effectively inhibit the above fungal pathogens in vitro. CPT can effectively inhibit mycelial growth by 50% (EC$_{50}$) at relatively low concentrations: approximately 10 μg/mL for *E. nigrum*, *P. guepinii*, and *Drechslera* sp., <25 μg/mL for *A. alternata*, and <30 μg/mL for *F. avenaceum* (**Table 2**). Higher levels of CPT more effectively inhibited mycelial growth, but the minimal inhibitory concentration varied among the fungi: approximately 75 μg/mL for *A. alternata* and *E. nigrum*, 100 μg/mL for *Drechslera* sp., 125 μg/mL for *P. guepinii* and *F. avenaceum*. The flavonoids were less effective than the alkaloid CPT at 50 μg/mL, particularly during the first three weeks, but more effective than the alkaloid at ≥100 μg/mL during the whole experimental period. Maneb successfully controlled all fungi, while Bravo failed to completely suppress all tested fungi except *P. guepinii*. Both the alkaloid and flavonoids at all experimental concentrations were more potent in inhibiting *Drechslera* sp. and *F. avenaceum* than Bravo, while the alkaloid at ≥50 μg/mL more effectively suppressed growth of *A. alternata* and *E. nigrum* than Bravo.

The fungi varied in their sensitivity to the alkaloid, flavonoids, and fungicides. The leaf spot fungi *A. alternata* and *E. nigrum* grew fast in control plates, but both were inhibited by CPT at relatively low concentrations. *P. guepinii*, another fast-growing fungus associated with leaf spots, required higher concentrations

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of CPT for complete inhibition. *Drechslera* sp. grew slowly and were more sensitive to all tested chemicals than *P. guepinii* and *F. avenaceum. F. avenaceum* was the slowest growing isolate but least sensitive to all chemicals at lower concentrations.

Dithiocarbamates are organic fungicides commonly used for the treatment of soil, seeds, and foliar and postharvest diseases of some crops. Rafin et al. found that dithiocarbamates, including Maneb, *N,N*-diethyldithiocarbamic acid sodium salt, and some new synthetic compounds, inhibited growth of *Fusarium oxysporum* f. sp. *lini* by 7−30% at 100 ppm (100 μg/mL) (*20*). At the same concentrations CPT, trifolin, and hyperoside inhibited growth of the related fungal pathogen *F. avenaceum* by 70% in vitro.

CPT, trifolin, and hyperoside also showed more potent antifungal activity than some recently discovered natural antifungal products. As the most potent antifungal compound of the essential oils of 13 herbs selected by Sridhar et al., geraniol isolated from cymbopogan (*Cymbopogan martini*) inhibited growth of *Botrytis cinerea* in both in vitro tests and TLC bioautography with an MIC of 160 μg/mL (*21*). Essential oils of *Salvia sclarea* L. (Lamiaceae) inhibited growth of *F. oxysporum* f. sp. *dianthi* by 72% at 2000 μL/L (*22*). β-Asarone from rhizomes of *Acorus gramineus* Solander (Araceae) was effective against mycelial growth of *Alternaria mali* (with MIC > 100 μg/mL) and *F. oxysporum* f. sp. *lycopersici* (with MIC > 100 μg/mL) (*23*). Vanillin, 4-hydroxy-3-methoxycinnamaldehyde, and ($\pm$)-pinoresinol isolated from *Melia azedarach* L. (Meliaceae) controlled *Fusarium verticillioides* at higher concentrations (with MICs of 600, 400, and 1000 μg/mL, respectively) (*24*).

Due to a dramatic increase in pathogen resistance to both agrochemical and pharmaceutical fungicides, discovery of new antimicrobial compounds with new modes of action is becoming increasingly important. CPT, trifolin, and hyperoside have more potent antifungal activity than many newly discovered antifungal agents, including some fungicides on the market. CPTs are potent inhibitors of the enzyme DNA topoisomerase I (*1*), which has not been previously targeted in fungicide development. Thus, CPTs with this unique mechanism of action, particularly some water-soluble CPT analogues, should be further investigated as antifungal agents. The flavonoids trifolin and hyperoside are abundant in *Camptotheca* and some other plants. Although their mechanisms of action against fungi are unknown, their effectiveness, resource availability at low cost, and probable low toxicity to humans give the flavonoids potential as prototypes for fungicides.

Chemical Defense of *C. acuminata*. Two interesting patterns were observed upon correlation of concentrations of defensive compounds and fungal pathogen distribution. First, *C. acuminata* plants can be infected by *A. alternata*, *E. nigrum*, *P. guepinii*, *Drechslera* sp., and *F. avenaceum*, although the affected tissues may have concentrations of CPT and flavonoids much higher than the MICs of these compounds against these fungi in vitro. For example, mature and old leaves have CPT concentrations of at least 0.01−0.025% (on a fresh mass basis, equivalent to 100−250 μg/mL) (*7*) but are frequently affected by leaf spots (*A. alternata*, *E. nigrum*, and *P. guepinii*). Trifolin and hyperoside may have contents 20 times higher than that of CPT; thus, the total content of all three antifungal compounds in these mature and old leaves may reach 0.4−1.0% (on a fresh mass basis), approximately 40−100 times higher than their in vitro MICs. Clearly, the defense of CPT and flavonoids in plants cannot be measured by in vitro testing of isolates alone. On the other hand, however, fungal diseases are less serious in some higher CPT yielding varieties and during seasons with higher CPT concentrations. Cultivars with higher CPT contents have fewer fungal problems. Fungal pathogens are less serious in fast-growing seasons (May and June) with higher CPT contents than early spring or fall when tissues have much lower CPT concentrations. From this point of view, resistance against fungal pathogens of plants can be improved by cultivar development and culture technology.

CPT, trifolin, and hyperoside exhibit potent activity against all five fungal pathogens isolated from *C. acuminata* in in vitro assays, but their antifungal activity in the plant is very limited. Further investigations on the balance of fungal infection and chemical defense are needed to understand this puzzle and to enhance alkaloidal biosynthesis in *Camptotheca*. In fact, a question about autotoxicity of CPTs in *Camptotheca* has never been addressed. CPTs are potent inhibitors of DNA topoisomerase and affect cell growth of almost all organisms, but why do CPTs not poison cells in living *Camptotheca* plants? The bioavailability of CPTs in plants may provide a starting point for further analysis to understand this question.

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