# A Fungistatic Chromene from *Ageratum conyzoides*

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*Ageratum conyzoides* L. is an annual herb in the tropics and subtropics whose extracts are known to possess pharmacological and biocidal activity. We report on the bioactivity of a secondary metabolite (a chromene) isolated from the shoots of *A. conyzoides* against some plant pathogenic fungi. Organic solvent extracts from the shoots were tested for antifungal activity against the plant pathogenic fungi *Rhizoctonia solani, Sclerotium rolfsii, Botryodiplodia theobromae, Phomopsis theae* and *Fusarium* species growing *in vitro* on potato dextrose agar medium. The crude *n*-hexane extract completely inhibited the growth of *R. solani* and *S. rolfsii*. The *n*-hexane extract was chromatographed over a column of silica gel followed by activity-guided fractionation to give an antifungal principle. Structure elucidation by detailed analysis of $^{1}H$, $^{13}C$ NMR and mass spectroscopy identified the active compound as precocene II. The growth of *R. solani* and *S. rolfsii* was completely inhibited by precocene II at a concentration of 80–100 ppm. The sclerotia of *R. solani* and *S. rolfsii* were also completely suppressed by 150 ppm of precocene II. Sub-culture of these inhibited fungi onto precocene II-free medium restored growth of the fungus, indicating that precocene II is fungistatic. Crude or refined extracts from *A. conyzoides* offer the possibility of biocontrol of plant pathogenic fungi.
KEY WORDS: *Ageratum conyzoides*; Asteraceae; antifungal activity; fungistasis; *Rhizoctonia solani; Sclerotium rolfsii*; chromene; precocene II.

## INTRODUCTION

*Ageratum conyzoides* L. (Asteraceae) is an annual herbaceous plant considered to be an obnoxious weed. Although a native of the Americas, it is now a common weed in several tropical and sub-tropical countries. It is abundant throughout Sri Lanka on roadsides and cultivated lands.

The plant has a history of use in the traditional medicine of various cultures worldwide and is commonly used to treat wounds, burns, bacterial diseases, etc. (7). Its ethnopharmacology, phytochemistry and bioactivity are reviewed by Okunade (8). Bioactivity of the plant extract has been reported against bacteria and insects. Various extracts of *A. conyzoides* inhibited the *in vitro* development of *Staphylococcus aureus* (3). Methanolic extracts inhibited the growth of *S. aureus, Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa* (1). Insecticidal activity against larvae of *Musca domestica* (5), morphogenetic abnormalities in mosquito larvae (11), and growth inhibition with juvenile hormone mimicry against the larvae of *Culex quinquefasciatus* (10) have been reported. In the absence of an immune system and due to their sedentary nature, plants

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defend themselves from pests and pathogens through synthesizing secondary metabolites. Bioactive products of plant origin are less persistent in the environment than commercial insecticides and safe for non-target organisms. The investigation of indigenous flora, as in this study, is a first step to identify such environmentally friendly compounds for further investigation and eventual use.

This plant was selected for our studies due to its widespread occurrence as a weed and apparently pathogen-free status observed in its natural habitat. The objective of this study was to determine the antifungal activity of organic solvent extracts from shoots of *A. conyzoides*, the nature of their activity and the identification of active constituent(s).

## MATERIALS AND METHODS

The fungi *Rhizoctonia solani, Sclerotium rolfsii, Botryodiplodia theobromae, Phomopsis theae* and *Fusarium* spp. were obtained from the Department of Agriculture, Peradeniya, Sri Lanka. The $^1H$ and $^{13}C$ NMR spectra were recorded on a JEOL Lambda-400 (400/100 MHz for $^1H/^{13}C$) spectrometer in $CDCl_3$ solution with tetramethylsilane as an internal reference.

**Plant material** The shoots of *A. conyzoides* were collected in the Kandy district of Sri Lanka in May 2001 and identified by comparison with herbarium specimens in the National Herbarium, Royal Botanic Gardens, Peradeniya. A voucher specimen is deposited at the Institute of Fundamental Studies.

**Extraction** Fresh mature shoots of *A. conyzoides* were washed in running tap water followed by distilled water and dried in the shade. The dried ground shoots (342 g) were sequentially extracted with cold *n*-hexane, dichloromethane and methanol. Evaporation of the solvents gave *n*-hexane extract (14 g), dichloromethane extract (9 g) and methanol extract (42 g).

**Antifungal activity test** The three extracts were tested for antifungal activity against *R. solani, S. rolfsii, B. theobromae, P. theae* and *Fusarium* spp. Fungal cultures were maintained by regular sub-culture on potato dextrose agar (PDA) medium. For the bioassay, a 10-mm disc taken from the edge of actively growing fungal colonies was placed in the center of a petri dish containing specific amounts of the plant extract dissolved in dichloromethane. Control, extract-free petri dishes contained only the solvent and the medium. The bioassay was repeated with a commercially obtained precocene II (Aldrich 19491-3). All the cultures were incubated in the dark at 27°C and colony diameters were determined. The inhibition of mycelial growth was determined by:

$$\%\ mycelial\ inhibition = \frac{Control\ (area) - Treatment(area)}{Control\ (area)} \times 100$$

Each experiment had five replicates and was repeated.

**Activity on sclerotia** To test the activity of precocene II on resting structures, sclerotia were isolated from 9-day-old cultures of *R. solani,* of diameter 1–1.2 mm. They were surface-sterilized in 95% ethanol and immediately rinsed in sterile distilled water and blotted dry on sterile filter paper. Petri dishes with 100 ppm and 150 ppm of commercial precocene II and control petri dishes with only the solvent were inoculated as described above. Three sclerotia were placed on a petri dish, replicated thrice.

**Minimum inhibitory concentration (MIC)** The active constituent was tested on *R. solani* and *S. rolfsii* from 500 ppm downwards at intervals of 100 ppm; from 100 ppm, the concentration was reduced at 10 ppm intervals. The MIC was determined as that concentration above which the fungi were totally suppressed by the active constituent and below which the fungi resumed growth. This was obtained by plotting the growth inhibition of the fungi against the concentration of the active constituent.

The precocene II isolated from *A. conyzoides* and the commercially obtained precocene II were directly compared on a TLC plate (Merck Art. 1.05554, $F_{254}$) using 10% methanol in chloroform as eluent and HPLC analysis under a reversed phase $C_{18}$ column (4.6 mm i.d. × 150 mm); UV detection at $\gamma$ 254 nm; isocratic solvent 15% $H_2O$/MeOH.

**Isolation and structure elucidation** To isolate the active constituent(s), the *n*-hexane extract (12 g) was chromatographed over a column of silica gel (Merck Art. 7734) with *n*-hexane – dichloromethane as eluent, which gave seven fractions (F1 to F7). Only fraction F5 showed antifungal activity. Further purification of fraction F5 by column chromatography with 40–60% *n*-hexane-dichloromethane gave three sub-fractions. Of these, only the first two fractions inhibited the growth of the fungi. They were determined to be identical by thin layer chromatography (TLC) and were combined to give 430 mg of a pure compound. The structure of the pure compound was determined by $^{1}H$, $^{13}C$ NMR and mass spectral data to be precocene II (ageratochromene; 6,7-dimethoxy-2, 2-dimethyl-2H-chromene).

Precocene II (1): gummy solid; $^{1}H$ NMR ($CDCl_3$): $\delta$ 6.53(1H, *s*, H-5), 6.42(1H, *s*, H-8), 6.24 (1H, *d*, *J*=10 Hz, H-4), 5.48 (1H, *d*, *J*=9.8 Hz, H-3), 3.84 (3H, *s*, -$OCH_3$ at C-6/7), 3.83(3H, *s*, -$OCH_3$ at C-6/7), 1.42(6H, *s*, 2 × Me at C-2); $^{13}C$ NMR ($CDCl_3$): $\delta$ 149.6 (C-6), 147.2(C-7), 143.1(C-8a ), 128.2(C-3), 122.0(C-4), 113.0 (C-4a), 109.7(C-5), 101.0(C-8), 75.4(C-2), 56.5(C-6/7 -O$\underline{C}H_3$), 55.9(C-6/7 -O$\underline{C}H_3$), 27.7(2x Me at C- 2); EIMS *m/z*: 220 ($M^+$), 205 (base peak), 189, 173, 161, 144, 132, 119, 115, 105, 103, 91, 77, 69, 65, 63.

## RESULTS

The antifungal activities of the crude *n*-hexane, dichloromethane and methanol extracts from the shoots of *A. conyzoides* were evaluated against the plant pathogenic fungi *R. solani, S. rolfsii, B. theobromae, P. theae* and *Fusarium* spp. by the PDA bioassay. The methanol and dichloromethane extracts did not show a significant effect in suppressing any of the fungi (Table 1). The *n*-hexane extract completely inhibited the growth in PDA medium of *R. solani* and of *S. rolfsii* (Table 1) at a concentration of 1000 ppm (0.1%). This same concentration also suppressed the growth of *B. theobromae* by 75% (Table 1) and of *P. theae* by 37%. However, with time, the partially suppressed fungi eventually covered the petri dish with their mycelia although at a slower rate than their corresponding controls. The *n*-hexane extract was further assayed against these fungi to determine the minimum inhibitory concentration required to suppress their growth. A concentration of 500 ppm (0.05%) of the crude *n*-hexane extract was found to inhibit completely the growth of these fungi. The other fungi assayed, *P. theae, B. theobromae* and *Fusarium* spp., showed a dose-dependent response (Table 2), with a concentration of 500 ppm producing a significant suppression of fungal growth in the medium. Those fungi that were not totally suppressed grew at a slower rate than their untreated control. The mycelia of *Fusarium* spp. grew in a vertical direction in the petri dishes with the extract, rather than spreading horizontally as

a mycelial mat as in the control cultures. The mycelia of *B. theobromae* were suppressed by 40% after 11 days of culture on 500 ppm *n*-hexane extract.

TABLE 1. Antifungal activity of crude solvent extracts (0.1%) of *Ageratum conyzoides* on five plant pathogenic fungi after 5 days of incubation

| Fungus | % Inhibition over control | | |
|---|---|---|---|
| | *n*-hexane | dichloromethane | methanol |
| *Rhizoctonia solani* | 100 | 0 | 0 |
| *Phomopsis theae* | 37.7 | 32.5 | 9.4 |
| *Fusarium* spp. | 92.2 | 14.1 | 5.5 |
| *Botryodiplodia theobromae* | 75.7 | 22.8 | 6.5 |
| *Sclerotium rolfsii* | 100 | nd | 0 |

nd, not determined.

TABLE 2. Antifungal activity of different concentrations of crude *n*-hexane extract of *Ageratum conyzoides* on five plant pathogenic fungi after 5 days of incubation

| Fungus | % Inhibition over control | | |
|---|---|---|---|
| | 3000 ppm | 500 ppm | 200 ppm |
| *Rhizoctonia solani* | 100 | 100 | 56.6 |
| *Phomopsis theae* | 84.2 | 35.2 | nd |
| *Fusarium* spp. | 97 | 77 | 35 |
| *Sclerotium rolfsii* | 100 | 100 | nd |
| *Botryodiplodia theobromae* | nd | 40.1 | nd |

nd, not determined.

TABLE 3. Mycelia growth (mm) *in vitro* of *Rhizoctonia solani* and *Sclerotium rolfsii* by fractions of the *n*-hexane extract of *Ageratum conyzoides* (S.E. was zero, since the treatments produced either complete or no growth)

| Fraction | *R. solani* | | | *S. rolfsii* | | |
|---|---|---|---|---|---|---|
| | Day after inoculation | Control | 500 ppm | Day after inoculation | Control | 500 ppm |
| F2 | 7 | 85 | 82 | 4 | 85 | 13 |
| F4 | 6 | 85 | 85 | 5 | 85 | 85 |
| F5 | 4 | 85 | 0 | 4 | 85 | 0 |
| F6 | 6 | 85 | 85 | 6 | 85 | 85 |
| F7 | 6 | 85 | 85 | 6 | 85 | 85 |
| F5-1 | 4 | 73 | 0 | 4 | 73 | 0 |
| F5-2 | 5 | 70 | 0 | 7 | 62 | 0 |
| F5-3 | 4 | 75 | 75 | 4 | 75 | 75 |

Investigations to identify the component(s) responsible for antifungal activity by activity-guided fractionation of the crude *n*-hexane extract were restricted to *R. solani* and *S. rolfsii*. The crude *n*-hexane extract was chromatographed over a column of silica gel with *n*-hexane and dichloromethane as eluent to give seven fractions, F1–F7. Of the seven fractions, the yield of fractions F1 and F3 was insufficient for further evaluation. The remaining five fractions were assayed against *R. solani* and *S. rolfsii* at 500 ppm. Fraction F5 completely inhibited the growth of *R. solani* and *S. rolfsii* (Table 3).

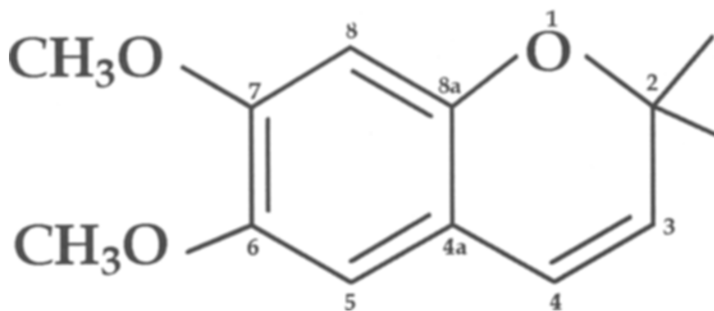


Fig. 1. Structure of precocene II.

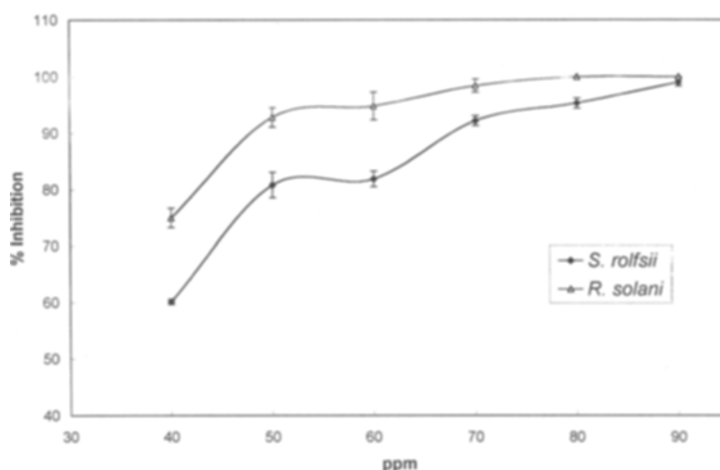


Fig. 2. Minimum inhibitory concentrations of precocene II on *Sclerotium rolfsii* and *Rhizoctonia solanii.* Data are shown as means ±S.E. (n=3) from two independent experiments.

Fraction F5 was further fractionated to isolate the active constituent against the fungi. We obtained two sub-fractions (F5-1 and F5-2) that were chemically similar, as shown by identical Rf values by TLC. They completely inhibited the growth of the fungi (Table 3). The third sub-fraction was inactive against the fungi. The sub-fractions F5-1 and F5-2 were combined and identified as precocene II (Fig. 1) by a detailed analysis of the high-resolution $^{1}$H, $^{13}$C NMR and mass spectral data.

The isolated precocene II from *A. conyzoides* completely inhibited the growth of both fungi at 500 ppm (Table 2) and 400, 300, 200, and 100 ppm (results not shown). The minimum inhibitory concentration that completely suppressed the fungi was determined to be in the range 80–100 ppm (Fig. 2).

We further compared the isolated precocene II from *A. conyzoides* with a commercially available precocene II. Both compounds were found to be identical by TLC and HPLC, with identical retention flow (Rf) values and retention times (Rt) observed, respectively, for the isolated and the commercial compound. The antifungal bioassay was done with the commercial precocene II at a concentration of 100 ppm on *R. solani* and *S. rolfsii.*

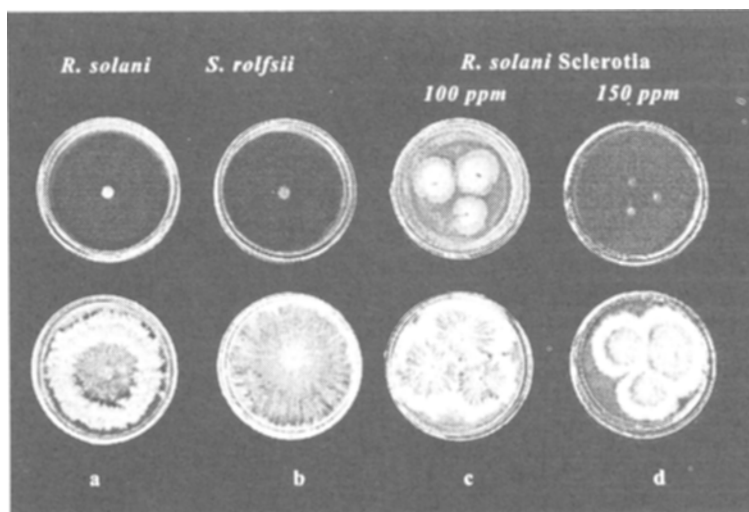


Fig. 3. Growth of the fungi after 3 days of culture with (top row) and without (bottom row) precocene II. a and b, mycelial cultures treated with 100 ppm; c and d; sclerotia treated with 100 ppm and 150 ppm, respectively.

The fungi were completely inhibited after 3 days of culture while the control cultures completely covered the petri dish (Fig. 3). These results confirmed that the antifungal activity of precocene II isolated from *A. conyzoides* was the same as the commercially available product.

To determine the nature of the antifungal activity, the inoculum discs of *R. solani* and *S. rolfsii* that were completely suppressed by the *n*-hexane extract were transferred to extract-free PDA medium. The suppressed inoculum discs of both fungi immediately resumed growth.

To determine the activity on resting structures, sclerotia from *R. solani* were inoculated on medium with and without 100 ppm of commercial precocene II. After 3 days of culture, the control cultures of *R. solani* achieved maximum growth in the petri dishes whereas the precocene II-treated cultures showed a suppressed and lower rate of growth. The mycelia in the control cultures radiated from the point of inoculation of the sclerotia, ending terminally as a cottony aerial growth, whereas the mycelia in the precocene II-treated culture were powdery and not vigorous (Fig. 3). Increasing the precocene II concentration to 150 ppm completely suppressed the growth of mycelia from the sclerotia of *R. solani* and *S. rolfsii.*

## DISCUSSION

The weed *A. conyzoides* synthesizes a variety of secondary metabolites, which include flavonoids, alkaloids, coumarins, essential oils, and tannins, many of which are biologically active (7). Investigations on its bioactivity have been confined to antibacterial and insecticidal activity and medicinal uses. This study investigated the potential use of crude organic solvent extracts of *A. conyzoides* shoots on some plant pathogenic fungi *in vitro*. The *n*-hexane extract effectively inhibited the growth of *R. solani* and *S. rolfsii*. Various extracts of the plant are used as a bactericide (1,4). Fungicidal activity was shown mostly by the *n*-hexane extract. Whereas *R. solani* and *S. rolfsii* were effectively controlled,

the three other fungi assayed were only moderately inhibited. The insignificant effect of dichloromethane and methanol extracts suggests that the active principle responsible for antifungal activity is confined to the *n*-hexane extract. Antifungal compounds reported from the family Compositae are mostly phenolics belonging to different classes (6). This study determined the active constituent of the *n*-hexane extract of *A. conyzoides* as precocene II, which is a chromene. The isolated precocene II from *A. conyzoides* was compared with the commercially available compound. Comparison by TLC and HPLC showed both compounds to be identical. The commercial precocene II also completely suppressed the growth of *R. solani* and *S. rolfsii* at a similar concentration. The growth of mycelia from the sclerotia of *R. solani* and *S. rolfsii* were completely suppressed at 150 ppm.

Eleven chromenes were reported from *A. conyzoides* other than precocene I (7-methoxy-2, 2-dimethyl-2H-chromene) and precocene II (5). Of these, ten were common to other members of the sub-family Asteraceae. The bioactivity of precocene was shown to affect insect development. The hormonal action of precocene induced precocious metamorphosis in insects (4). Antijuvenile hormone-like activity was also shown on the mosquito *Culex quinquefasciatus* by extracts of *A. conyzoides* (10).

Chromenes showing antifungal activity were reported from the roots of *Eupatorium riparium*, which was toxic to the fungus *Colletotrichum gloeosporioides* (2), and from *Piper aduncum* (9), which was active against the fungus *Penicillium oxalicum*. Whereas chromenes from other plant species have evinced antifungal activity, precocene has not been shown to have antifungal activity.

The precocene II from *A. conyzoides* completely inhibited the growth of *R. solani* and *S. rolfsii* at a concentration of 80–100 ppm. Regrowth of the inhibited inoculum discs on medium without the chromene showed that the nature of bioactivity was fungistatic. The antifungal chromene from *E. riparium* was similarly fungistatic to *C. gloeosporioides*; however, none of the fungi assayed was completely inhibited (2).

*Ageratum conyzoides* is a successful annual weed conspicuous by its fungus-free status, although it is an alternate host for the yellow mosaic virus. The inflorescence contains 30–50 flowers which are self-incompatible. Thus, there is an increased opportunity for recombination and segregation in its life cycle. This has apparently enabled the species to evolve faster and successfully against pests and pathogens by accumulating effective secondary metabolites. Other species from the family Compositae with similar attributes merit further investigation.

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REFERENCES

1. Almagboul, A.Z., Farroq, A.A. and Tyagi, B.R. (1985) Antimicrobial activity of certain Sudanese plants used in folkloric medicine: Screening for antibacterial activity Part II. *Fitoterapia* 56:103-109.
2. Bandara, B.M.R., Hewage, C.M., Karunaratne, V., Wannigama, G.P. and Adikaram, N.K.B. (1992) An antifungal chromene from *Eupatorium riparium*. *Phytochemistry* 31:1983-1985.
3. Durodola, J.I. (1977) Antibacterial property of crude extracts from herbal wound healing remedy-*Ageratum conyzoides* L. *Planta Med.* 32:388-390.
4. Ekundayo, O., Sharma, S. and Rao, E.V. (1988) Essential oil of *Ageratum conyzoides*. *Planta Med.* 54:55-57.

5. Gonzalez, A.G., Aguiar, Z.E., Grillo, T.A., Luis, J.G., Rivera, A. and Calle, J. (1991) Chromenes from *Ageratum conyzoides*. *Phytochemistry* 30:1137-1139.
6. Grayer, R.J. and Harborne, J.B. (1994) A survey of antifungal compounds from higher plants. *Phytochemistry* 37:19-42.
7. Ming, L.C. (1999) *Ageratum conyzoides*: A tropical source of medicinal and agricultural products. *in:* Janick, J. [Ed.] Perspectives on New Crops and New Uses. ASHS Press, Alexandria, VA, USA. pp. 469-473.
8. Okunade, A.L. (2002) *Ageratum conyzoides* L. (Asteraceae). *Fitoterapia* 73:1-16.
9. Orjala, J., Erdelmeier, C.A.J., Wright, A.D., Rali, T. and Sticher, O. (1993) Two chromenes and a prenylated benzoic acid derivative from *Piper aduncum*. *Phytochemistry* 34:813-818.
10. Saxena, R.C., Dixit, O.P. and Sukumaran, P. (1992) Laboratory assessment of indigenous plant extracts for anti-juvenile hormone activity in *Culex quinquefasciatus*. *Indian J. Med. Res.* 95:204-206.
11. Sujatha, C.H., Nisar, S. and Jadhi, C. (1988) Evaluation of plant extracts for biological activity against mosquitoes. *Int. Pest Control* 7:122-124.