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Acaconin, a chitinase-like antifungal protein with cytotoxic and anti-HIV-1 reverse transcriptase activities from *Acacia confusa* seeds

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From the seeds of *Acacia confusa*, a chitinase-like antifungal protein designated as acaconin that demonstrated antifungal activity toward *Rhizoctonia solani* with an IC_{50} of 30±4 μM was isolated. Acaconin demonstrated an N-terminal sequence with pronounced similarity to chitinases and a molecular mass of 32 kDa. It was isolated by chromatography on Q-Sepharose, SP-Sepharose and Superdex 75 and was not bound by either ion exchanger. Acaconin was devoid of chitinase activity. The antifungal activity against *Rhizoctonia solani* was completely preserved from pH 4 to 10 and from 0°C to 70°C. Congo Red staining at the tips of *R. solani* hyphae indicated inhibition of fungal growth. However, there was no antifungal activity toward *Mycosphaerella arachidicola, Fusarium oxysporum, Helminthosporium maydis,* and *Valsa mali*. Acaconin inhibited proliferation of breast cancer MCF-7 cells with an IC_{50} of 128±9 μM but did not affect hepatoma HepG2 cells. Its IC_{50} value toward HIV-1 reverse transcriptase was 10±2.3 μM. The unique features of acaconin include relatively high stability when exposed to changes in ambient pH and temperature, specific antifungal and antitumor actions, potent HIV-reverse transcriptase inhibitory activity, and lack of binding by strongly cationic and anionic exchangers.

Keywords: antifungal protein, antitumor, anti-HIV-1 reverse transcriptase, *Acacia confusa*

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INTRODUCTION

The seeds of flowering plants contain an abundance of proteins, some of which are storage proteins but may also play a role of protection from pathogenic microbes and phytophagous insects. Antifungal proteins (Patil *et al.*, 2009), antiviral proteins, lectins/hemagglutinins, ribosome-inactivating proteins, proteinase inhibitors, arcelins, and α-amylase inhibitors are some examples of the myriad of seed proteins. The aforementioned proteins are collectively called defense proteins in view of their antipathogenic activities.

Chitinase is one kind of antifungal proteins. It has been purified from different organisms including algae (Shirota *et al.*, 2008), fungi (Nguyen *et al.*, 2008; 2009), including plant seeds (Patil *et al.*, 2009), bacteria (Liu *et al.*, 2009), insects (Genta *et al.*, 2006), including silkworms (Kabir *et al.*, 2006), amphibians (Oshima *et al.*, 2002), fish (Ikeda *et al.*, 2009), etc.

Research on antifungal proteins has intensified in recent years. Transgenic crops expressing genes encoding antifungal proteins are expected to have augmented resistance against pathogenic fungi. There are various types of antifungal proteins with distinctly different amino-acid sequences. They include thaumatin-like proteins (Chua *et al.*, 2007), chitinases (Patil *et al.*, 2009), glucanases (Buchner *et al.*, 2002), ribosome-inactivating proteins, lipid transfer proteins (Choi *et al.*, 2008) and others. The intent of the present study was to isolate and characterize an antifungal protein from fresh *Acacia confusa* seeds, and to compare its characteristics and activities with previously reported antifungal proteins. Such information would enrich the scarce literature data that is currently available about *A. confusa.*

MATERIALS AND METHODS

Purification of acaconin. Seeds were collected from *Acacia confusa* trees. The seeds have been authenticated by Professor Shiuying Hu, Honorary Professor of Chinese Medicine, The Chinese University of Hong Kong, and deposited with the voucher number NG2009023 in Lab 302, Basic Medical Science Building, The Chinese University of Hong Kong. The seeds (150 g) were first extracted with distilled water using a Waring blender. The extract obtained after centrifugation of the slurry was saved. NH_4HCO_3 buffer (1 M, pH 8.8) was then added to the extract to 10 mM. The extract was then loaded on a 5 cm × 13 cm column of Q-Sepharose (GE Healthcare), unadsorbed proteins were eluted with 10 mM NH_4HCO_3 buffer (pH 8.8) while adsorbed proteins were desorbed by stepwise addition of 1 M NaCl in the starting buffer. The unadsorbed fraction was subsequently fractionated on SP-Sepharose after dialysis against 10 mM NH_4OAc buffer (pH 4.5), adsorbed proteins were desorbed with the NH_4OAc buffer containing 1 M NaCl. The unadsorbed fraction was then lyophylized and subjected to gel filtration in 10 mM NH_4HCO_3 buffer (pH 8.8) on a Superdex 75 HR 10/30 column by fast protein liquid chromatography using AKTA purifier (GE Healthcare). The third peak (S3) represented purified antifungal protein designated as acaconin.

Molecular mass determination using SDS/PAGE gel filtration, and N-terminal amino acid sequencing. Purified acaconin was analyzed by SDS/PAGE

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Abbreviations: MTT, Thiazolyl Blue Tetrazolium; SDS/PAGE, sodium dodecyl sulphate/polyacrylamide gel electrophoresis

(Laemmli & Farve, 1973). Gel filtration on a fast protein liquid chromatography Superdex 75 column HR 10/30 (GE Healthcare) by employing an AKTA Purifier (GE Healthcare) was performed to ascertain the molecular mass of the antifungal protein. The column had previously been calibrated with molecular mass markers, including Blue Dextran 2000 (to determine void volume), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), myoglobulin (17.6 kDa), ribonuclease A (13.7 kDa), aprotinin (6.5 kDa) and vitamin B12 (1.3 kDa) (GE Healthcare). The N-terminal sequence of acaconin was determined as described in reference (Lam *et al.*, 2009), using a Hewlett-Packard sequencer.

Protein determination. The protein concentration was determined by Bradford reagent (dye-binding method) using bovine serum albumin as standard.

Determination of chitinase activity. Acid-swollen chitin was prepared by adding 0.5 g of chitin to 50 ml of dilute aqueous acetic acid (pH 2.5) and stirring the mixture for 72 h at 4 °C. The pH of the suspension was adjusted to 5.0 by adding 2 M NaOH (Pegg, 1998). The assay of chitinase activity was performed by incubating swollen chitin in 50 mM ammonium acetate (pH 5.5) at 37 °C for half hour. One unit of enzyme activity using chitin as substrate is defined as the amount of enzyme required to form 30 nmol of *N*-acetylglucosamine per 30 min. The amount of reducing sugar was estimated by using dinitrosalicyclic acid (Miller, 1959).

Assay of antifungal activity. The various chromatographic fractions were monitored for antifungal activity by agar diffusion assay using a 90 mm × 15 mm petri dish containing 10 ml of potato dextrose agar. A small amount of fungus was inoculated in the center of Petri dish. After the mycelial colony had developed on the agar, at a distance of 1 cm away from the rim of the colony were placed sterile blank paper disks (0.625 cm in diameter). The samples were applied to the disks. The plates were incubated at 25 °C until mycelial growth from the center of disk had enveloped peripheral disks containing the control and formed crescents of inhibition around the paper disks with samples possessing antifungal activity. The pathogenic fungi species used included *Mycosphaerella arachidicola, Fusarium oxysporum, Helminthosporium maydis, Valsa mali* and *Rhizoctonia solani* (Lam & Ng, 2009). Nystatin (Sigma) was used as a positive control.

To determine the IC_{50} value, different concentrations of acaconin were added separately to aliquots, each containing 2 ml of potato dextrose agar at 45 °C, mixed rapidly, and poured into separate 3.3-cm Petri dishes. After the agar had cooled down, a small amount of mycelia was inoculated. Buffer only served as a negative control. The dishes were incubated at 25 °C for 3 days, the area of the mycelial colony was measured and the IC_{50} value for the antifungal activity of acaconin against *R. solani* was determined (Lam & Ng, 2009). IC_{50} is the concentration of the antifungal protein required to produce 50% reduction of the area of mycelial colony.

To test the effect of different pH and temperature on the antifungal activity of acaconin against *R. solani*, the protein was incubated at various pH values and temperatures for 15 min. The pH was restored to 7 and the thermal treatment stopped by instant placing in ice before the assay of antifungal activity was carried out.

Assay of growth inhibition at hyphal tips. This assay was conducted to observe growth inhibition at hyphal tips of *R. solani*. Following incubation of *R. solani* with acaconin for 4 h, Congo Red was added to a final concentration of 1 mM. Fluorescence was examined 2 h later by fluorescence microscopy using an excitation wavelength of 543 nm and an emission wavelength of 560–635 nm. The staining condition of growing hyphae was observed (Moreno *et al.*, 2006).

Assay of HIV-1 reverse transcriptase inhibitory activity. An enzyme-linked immunosorbent assay kit from Boehringer Mannheim was used to assay acaconin for the ability to inhibit HIV-1 reverse transcriptase (Zhang *et al.*, 2009). *Brassica campestris* lipid transfer protein (Lin *et al.*, 2007) was used as a positive control.

Assay of cytotoxic activity. The assay of the cytotoxic activity of the isolated acaconin was carried out on human hepatoma HepG2 cells and human breast cancer MCF-7 cells (Lam & Ng, 2009). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin, in a humidified atmosphere of 5% CO_2 at 37 °C. The cells (5000 cells/100 µl per well) were seeded in a 96-well culture plate and serial dilutions of a solution of acaconin or doxorubicin (as the positive control) in 100 µl medium were added. A solution of medium only was added as the negative control. After incubation for 48 h, the cells were harvested, standard MTT assay was performed to determine the level of its inhibitory activity. All reported values are means of triplicate samples.

RESULTS

Isolation of antifungal protein

Seeds (150 g) collected from *A. confusa* trees were homogenized in 1 liter of 10 mM NH_4HCO_3 buffer (pH 8.8). The homogenate was centrifuged and the supernatant collected. Ion exchange chromatography of the seed extract on Q-Sepharose yielded a large unadsorbed fraction (Q1) and one adsorbed fraction (Q2) desorbed with 1 M NaCl (Fig. 1A). The unadsorbed fraction Q1 was resolved on SP-Sepharose into an unadsorbed fraction (SP1) and an adsorbed fraction (SP2) of similar size eluted with 1 M NaCl (Fig. 1B). SP1 was then chromatographed on Superdex 75. Five fractions, S1 to S5, were obtained (Fig. 1C). The antifungal activity resided in fraction S3. Fraction S3 was re-chromatographed on Superdex 75 and was eluted as a single homogeneous peak with the same elution volume as before corresponding to a molecular mass of 32 kDa (not shown). It demonstrated a single 32-kDa band in SDS/PAGE (Fig. 2). The purified chitinase was designated as acaconin. A summary of the purification of acaconin is included in Table 1. The N-terminal sequence of acaconin was homologous to those of other chitinases (Table 2).

Biological activities of isolated antifungal protein

Acaconin was deficient in chitinase activity when tested up to 100 µM. It inhibited mycelial growth of *R. solani* in a dose-dependent manner with an IC_{50} of 3±4 µM (Fig. 3) but not of *M. arachidicola, H. maydis, F. oxysporum,* and *V. mali* when tested with 50 µg (10 µl of 5 mg/ml acaconin solution was added) in agar diffusion assay (not shown). The antifungal activity of acaconin was stable throughout the pH range 4–10. Only 50% of the antifungal activity remained at pH 3 and 11. It was deactivated at pH 0–2 or 12–14. It was stable in the temperature range 0 °C–70 °C. Only 55% of antifungal activity remained at 80 °C. It was lost above 90 °C.

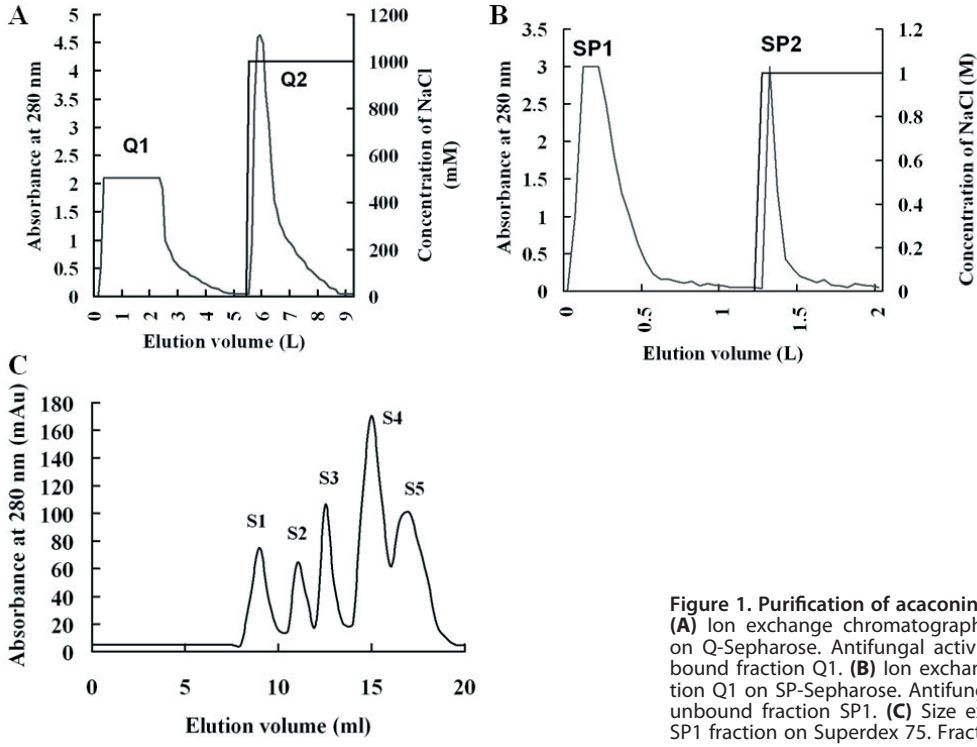

Figure 1. Purification of acaconin
(A) Ion exchange chromatography of *Acacia confusa* extract on Q-Sepharose. Antifungal activity was confined to the unbound fraction Q1. **(B)** Ion exchange chromatography of fraction Q1 on SP-Sepharose. Antifungal activity was found in the unbound fraction SP1. **(C)** Size exclusion chromatography of SP1 fraction on Superdex 75. Fraction S3 represents acaconin.

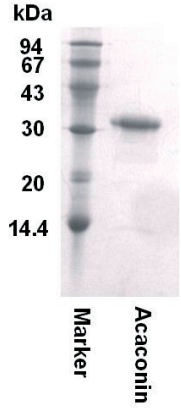

Figure 2. SDS/PAGE
Left: Molecular mass markers from GE Healthcare including phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa). Right: Fraction S3 representing acaconin.

Hyphal growth in *R. solani* was impeded, as evidenced by stainable with Congo Red at the hyphal tip (Fig. 4). It inhibited proliferation of MCF-7 tumor cells with an IC_{50} of 128±9 μM (Fig. 5), but there was no inhibition against HepG2 cells. It inhibited HIV-1 reverse transcriptase with an IC_{50} of 10±2.3 μM (Fig. 6).

Table 1. Yields of various active chromatographic fractions obtained at different stages of purification

Column	Chromatographic fraction	Yield (mg)*	Yield (%)
	Crude extract	1200	100
Q-Sepharose	Q1	253	21.1
SP-Sepharose	SP1	128	10.6
Superdex 75	S3 (acaconin)	22	1.8

*From 150 g seeds

DISCUSSION

Acaconin is an antifungal protein with an N-terminal sequence highly homologous to chitinases. The N-terminal sequence of acaconin is homologous to those of other chitinases, but it does not possess a chitinase activity. Acaconin is similar to chitinase-like antifungal proteins from emperor banana fruits (Ho & Ng, 2007), and black turtle bean (Chu & Ng, 2005) in demonstrating an N-terminal sequence with homology to chitinases. In this context, it is reminiscent of the observation that passiflin possesses a β-lactoglobulin-like N-terminal sequence but does not cross-react with an anti-β-lactoglobulin antiserum. Passiflin shows antifungal activity and cytotoxicity which are absent in β-lactoglobulin. Passiflin is immunologically and biologically unrelated to β-lactoglobulin (Lam & Ng, 2009). Similarly, thaumatin-like proteins have antifungal activity but no sweet taste while the converse is true of thaumatin although they are highly homologous in structure (Ye *et al.*, 1999).

Chitinases have a variety of molecular masses. The molecular mass of small chitinases, e.g. *Panax notoginseng* chitinase (Lam & Ng, 2001), is as low as 15 kDa. The molecular masses of some chitinases are higher, e.g. that of *Oreochromis niloticus* chitinase (Molinari *et al.*, 2007) and *Bombyx mori* chitinase (Kabir *et al.*, 2006) is 75 kDa. The range of molecular masses of most chitinases is from 30 kDa to 60 kDa (Patil *et al.*, 2009; Liu *et al.*, 2009; Ikeda *et al.*, 2009). The molecular mass of acaconin (32 kDa) is within the range reported for chitinases.

The chromatographic behavior of acaconin is distinct from that of antifungal proteins in general. Most of the chitinases are adsorbed on anion exchangers. *Aeromonas schubertii* chitinases (Liu *et al.*, 2009) are adsorbed on DEAE-Sepharose and Mono Q. Some are adsorbed on cation exchangers, e.g. emperor banana chitinase (Ho & Ng, 2007). Some are adsorbed on both kinds of ion exchangers, e.g. *Bacillus cereus* chitinase (Wang *et al.*, 2009).

Table 2. N-terminal amino-acid sequence of acaconin in comparison with counterparts from other species

	Residue No.	N-terminal sequence	Homology (%)
A. confusa chitinase	1	EQHGRQAGGALCMGG	–
Phaseolus vulgaris class I chitinase	1	EQCGRQAGGALCPGG	87
Vitis vinifera chitinase	23	EQCGRQAGGALCSGG	87
Euonymus europaeus chitinase	20	EQCGRQAGGALCPGG	87
Hevea brasiliensis class I chitinase	1	EQCGRQAGGALCPGG	87

Identical amino-acid residues are underscored

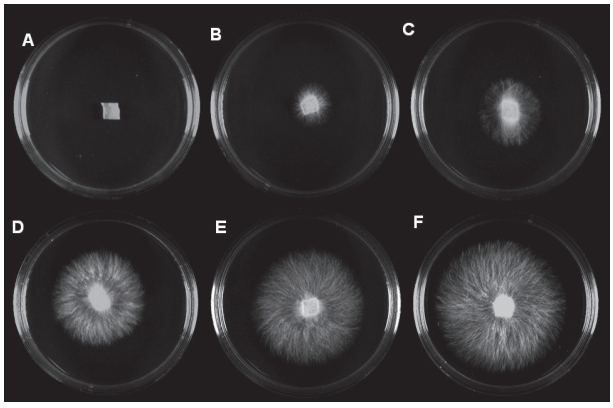

Figure 3. Antifungal activity of acaconin toward *Rhizoctonia solani*
(A) 100 μM acaconin, (B) 75 μM acaconin, (C) 50 μM acaconin, (D) 25 μM acaconin, (E) 12.5 μM acaconin, (F) buffer control. The IC_{50} was 27±3 μM (mean ±S.D., n=3). Results shown here are from one of the three experiments.

In some cases, the chitinase was purified by affinity chromatography, e.g. *Tamarindus indica* chitinase (Rao & Gowda, 2008). Acaconin is unadsorbed on Q-Sepharose (anion exchanger) and SP-Sepharose (cationic exchangers), in contrast to other chitinases.

Not all chitinases have antifungal activity. Some chitinases can inhibit the growth of a number of fungi. *P. notogingseng* chitinase exerts antifungal activity against *Coprinus comatus, F. oxysporum* and *M. arachidicola* (Lam & Ng, 2001). Black turtle bean chitinase specifically inhibits *F. oxysporum* and *M. arachidicola* (Chu & Ng, 2005). Mung bean chitinase inhibits *Fusarium solani*, *F. oxysporum*, *M. arachidicola*, *Pythium aphanidermatum*, and *Sclerotium rolfsii* (Wang *et al.*, 2005). Acaconin exhibits specific antifungal activity towards *R. solani* but not to a number of other fungal species such as *M. arachidicola, H. maydis, F. oxysporum,* and *V. mali*. The specificity of the antifungal action of acaconin is similar to that of some chitinases listed below. *Ananas comosus* chitinase can inhibit *Trichoderma viride* (Taira *et al.*, 2005). Emperor banana chitinases inhibit mycelial growth in *F. oxysporum* (Ho & Ng, 2007). *Trichoderma aureoviride* DY-59 and *Rhizopus microsporus* VS-9 chitinases inhibit the growth of *F. solani* (Nguyen *et al.*, 2008).

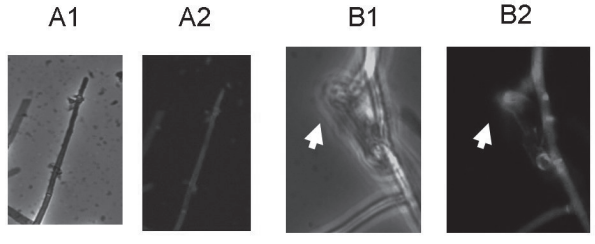

Figure 4. Congo Red staining showing growth inhibition at hyphal tips of *R. solani*
(A) Buffer control. Pictures A1 and A2 were taken under light microscope and fluorescence microscope, respectively. (B) Treatment with 50 μM acaconin. Pictures B1 and B2 taken under light microscope and fluorescence microscope, respectively. Hyphal tips were stained with Congo Red (white arrow) showing that the growth was inhibited.

Acaconin demonstrates a relatively high pH stability from pH 4 to pH 10, which is higher than that reported for some chitinases (Kabir *et al.*, 2006). *Bacillus licheniformis* SK-1 chitinase is more stable (from pH 4 to pH 9) than other chitinases (Kudan & Pichyangkura, 2009), in which it is similar to acaconin. Some antifungal proteins have not been tested for pH stability (Morre *et al.*, 2004). However, acaconin is similar to other chitinases in thermostability (Wang *et al.*, 2008).

To the best of our knowledge, very few chitinases have been reported to have cytotoxicity toward mammalian cells. *Serratia marcescens* and *Streptomyces griseus* chitinases show potent cytotoxicity toward cancer cells (breast cancer MCF-7 cells) (Pan *et al.*, 2005). Acaconin manifests a specific antitumor action against MCF-7 cells (IC_{50} = 128 μM) but not against hepatoma HepG2 cells. The cytotoxic potency of acaconin is similar to that of *Allium tuberosum* chitinase (Lam *et al.*, 2000).

Only a few chitinases show HIV-1 reverse transcriptase inhibitory activity. Acaconin has a highly potent inhibitory action (IC_{50} = 10 μM) against HIV-1 reverse transcriptase, with a similar potency to chestnut chitinase (Wang & Ng, 2003). Pinto bean chitinase (Ye & Ng, 2003), *Delandia umbellata* chitinase (Ye & Ng, 2002) and *Allium tuberosum* chitinase (Lam *et al.*, 2000) show a much weaker inhibitory potency toward HIV-1 reverse transcriptase, while mung bean chitinase lacks such activity (Lin *et al.*, 2007).

Chitinases act on chitin in the fungal cell wall to exert their antifungal activity. The antifungal activity of chitinases is directly correlated with the proportion of chitin in the fungal cell wall (Yan *et al.*, 2008). Congo Red, a dye that binds strongly to β-glucans, is used to stain the fungal cell wall. A strong Congo Red signal was detected at the site where acaconin attacks the fungal cell wall. The hyphae are probably damaged by acaconin causing accumulation of chitin and so inhibition of hyphal growth. Similarly, the hyphal tips of rice blast fungus *Magnaporthe grisea* are stained by Congo Red after treatment with *Aspergillus giganteus* antifungal protein (Moreno *et al.*, 2006).

Phytochemicals with a small molecular mass have been identified in the flowers (Wu *et al.*, 2008b), leaves (Lee *et al.*, 2000), heartwood (Wu *et al.*, 2008a) and bark (Tung *et al.*, 2009) of *A. confusa*. However, there is a dearth of information pertaining to their protein constituents. Only a trypsin inhibitor (Lin *et al.*, 1991) has been purified from seeds. Hence, the present report of an antifungal protein from *A. confusa* seeds adds to the scanty literature.

The novel aspects of the isolated chitinase include unique chromatographic behavior on cation and anion exchangers, relatively high thermostability and pH stabil-

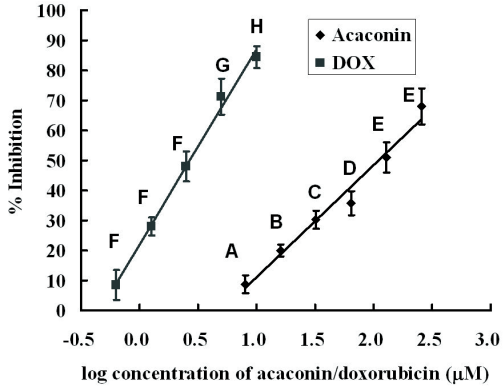

Figure 5. Effects of acaconin on viability of breast cancer MCF-7 cells
The viability of MCF-7 cells after incubation for 48 h in the presence of various concentrations of acaconin was determined as described in Materials and Methods. Viability of cells in RPMI medium only was taken as 100%. Values are expressed as mean ±S.D. (n=3). Data points marked with different letters (A, B, C, D and E) are significantly different ($P<0.05$) from one another when analyzed by ANOVA followed by Duncan's multiple range test. The IC_{50} was 128 ± 9 μM (mean ±S.D., n=3). Results shown here are from one of the three experiments.

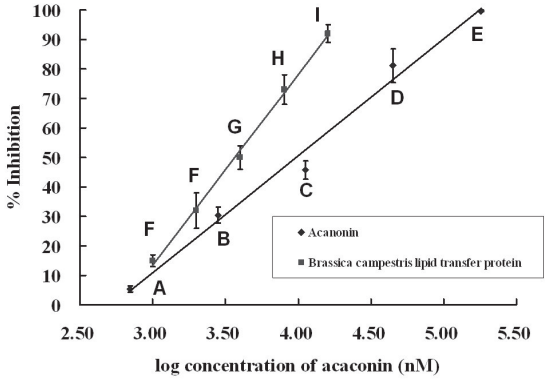

Figure 6. Dose-dependent inhibitory effect of acaconin on HIV-1 reverse transcriptase
Data are represented as the mean ±S.D. (n=3). Data points marked with different letters (A, B, C, and D) are significantly different ($P<0.05$) from one another when analyzed by ANOVA followed by Duncan's multiple range test. The IC_{50} was 10±2.3 μM (mean ±S.D., n=3). Results shown here are from one of the three experiments.

ity and specificity of antifungal action toward *R. solani* and absence of activity toward a number of other fungi, specificity of cytotoxicity toward breast cancer cells and lack of activity toward hepatoma cells, and highly potent inhibitory activity against HIV-1 reverse transcriptase.

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