

IN VITRO ACTIVITY OF EXTRACTS OF FIVE MEDICINAL PLANT SPECIES ON PLANT PATHOGENIC FUNGI

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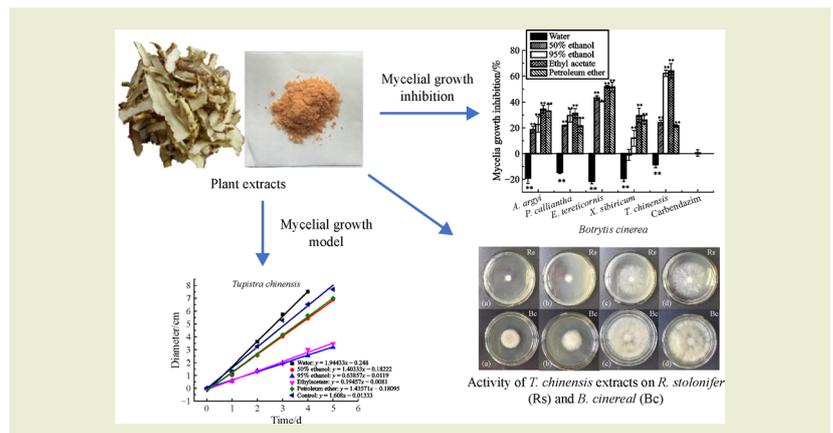
KEYWORDS

Botrytis cinerea, food preservation, plant diseases, plant pathogenic fungi, *Tupistra chinensis*

HIGHLIGHTS

- Ethyl acetate and 95% ethanol extracts from *T. chinensis* rhizomes gave great inhibition on six plant pathogenic fungi.
- *T. chinensis* has potential to be a new natural fungicide resource.
- *T. chinensis* has potential to be used for the preservation of agricultural and forestry products such as fruits and vegetables.

GRAPHICAL ABSTRACT



Received July 31, 2019;
 Accepted October 16, 2019.

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ABSTRACT

The antifungal effectiveness of extracts of five medicinal plant species was determined. The inhibitory activity of extracts of *Eucalyptus tereticornis*, *Xanthium sibiricum*, *Artemisia argyi*, *Tupistra chinensis* and *Pyrola calliantha* were evaluated against the mycelial growth of the plant pathogenic fungi *Aspergillus niger*, *Botrytis cinerea*, *Penicillium digitatum*, *P. expansum*, *P. italicum* and *Rhizopus stolonifer*. All plant extracts were prepared at 60°C using solvents (either water, 50% ethanol (v/v), 95% ethanol (v/v), ethyl acetate or petroleum ether). Fungicidal effects of all plants tested were confirmed. Different extracts from the same plant species gave different degrees of inhibition. All aqueous extracts had weak or no activity on all fungi tested. Ethyl acetate and 95% ethanol extracts from *T. chinensis* rhizomes gave greater inhibition and a broader spectrum inhibition than the other extracts. *T. chinensis* may have potential as a new natural fungicide and may be used for the preservation of agricultural and forestry products such as fruits and vegetables.

1 INTRODUCTION

Many plant pathogens are fungi that cause considerable damage by postharvest decay of fruits and vegetables^[1]. Common pathogenic fungi causing postharvest rots are in the genera *Aspergillus*, *Botrytis*, *Colletotrichum*, *Penicillium* and *Rhizopus*, with major species being *Aspergillus niger*, *Botrytis cinerea*, *Colletotrichum musae*, *Penicillium digitatum*, *P. expansum*, and *Rhizopus stolonifer*^[2].

Postharvest fungal rots affect the quality and shorten the shelf life of fruit and vegetables, leading to considerable economic loss and the waste of important resources. In addition to causing disease in fruit and vegetables, many species of *Alternaria*, *Aspergillus* and *Penicillium* are also sources of mycotoxins that are of concern in animal and human health^[3,4]. Aflatoxins produced by *Aspergillus* may cause DNA damage and liver cancer^[5,6] and gliotoxin, citrinin and patulin affect interferon- γ production^[7].

Synthetic fungicides have long been the most important method of protecting agricultural and forestry products against fungal damage. However, most synthetic fungicides on the market are toxic and have undesirable effects on non-target organisms in the environment^[8]. Furthermore, some synthetic fungicides such as benzimidazole accumulate in plants, waters and soils and can affect humans by transfer via the food chain^[9].

In recent years, studies have focused on natural antifungicidal agents such as essential oils^[10,11], phenolics, flavonoids^[12,13] and saponins^[14]. Many extracts from Chinese medicinal plants have antifungal effects against pathogenic fungi, for example methanol extracts from *Artemisia argyi* and *Xanthium sibiricum* which inhibit *Peronophythora litchii*^[15]. Extracts of *Tupistra chinensis* and *Menispermum dauricum* also inhibit *C. musae*^[16] and *B. cinerea*^[17].

Five plant species investigated to find more effective broad-spectrum natural fungicides were *A. argyi* (leaves), *Pyrola calliantha* (stalks and leaves), *Eucalyptus tereticornis* (leaves), *X. sibiricum* (leaves), and *T. chinensis* (rhizomes). Crude extracts were made with five polar solvents to screen comprehensively for antifungal constituents.

2 MATERIALS AND METHODS

2.1 Materials

Plant materials were purchased from different Chinese herbal

medicine stores. Leaves of *A. argyi* were obtained from Qichun Jingchu Qiai Products Co., Ltd., Huanggang, China and *P. calliantha* (whole stalks and leaves) from Bozhou Zhongyitang Traditional Chinese Medicine Sales Co., Ltd., Bozhou, China. Leaves of *E. tereticornis* were purchased from commercial suppliers: (Aegean Traditional Chinese Medicine Health Club, Bozhou, China). Leaves of *X. sibiricum* were bought from Caomuyu Wild Chinese Medicine Store, Jining, China and rhizomes of *T. chinensis* from a Chinese herbal medicine store in Shennongjia Forestry District, Hubei Province, China. Plant materials were cleaned, dried, and ground using a laboratory grinding mill (Taisite, Tianjin, China), sieved (20 mesh) and stored hermetically until used.

Fungal strains *P. expansum* ACCC 37275, *P. italicum* ACCC 30399, *A. niger* ACCC 30005, *P. digitatum* ACCC 30389, *B. cinerea* ACCC 37273, and *R. stolonifer* ACCC 36973 were purchased from the Agricultural Culture Collection, Beijing, China. The fungal strains were grown on potato dextrose agar (PDA; Beijing SanYao Science & Technology Development Co., Beijing, China). Ethanol, ethyl acetate and petroleum ether were used to prepare extracts.

The instruments used were an HH-ZK4 electro thermal water bath (Nanjing Jia Meilun Scientific Instrument Co., Ltd., Nanjing, China), a rotary evaporator (Shanghai Ailang Instrument Co., Ltd., Shanghai, China), a biochemical incubator (Shanghai Jinhong Laboratory Instrument Co., Ltd., Shanghai, China), an autoclave (LDZX-50KBS, Shanghai Shenan Medical Instrument Factory), and a UV-visible spectrophotometer (UV-2450, Shimadzu, Kyoto, Japan).

2.2 Methods

2.2.1 Preparation of plant extracts ether

Ethanol, ethyl acetate and petroleum ether were used to prepare extracts. Dried ground plant material (100 g) was soaked separately in 1 L of water, 50% ethanol (v/v), 95% ethanol (v/v), ethyl acetate, and petroleum ether in 1 L conical flasks sealed with plastic film. All conical flasks were placed in an ultrasonic cleaner for 30 min^[18] and allowed to stand for 3 h at 60°C for the extraction process^[19]. The supernatant from each flask was filtered through Whatman filter paper and vacuum evaporated at 45–55°C. The plant materials underwent three further extractions to obtain the maximum metabolite yield. Extracts were freeze-dried or vacuum dried, weighed separately, transferred into small vials, and the percentage yield from the extraction calculated. These samples were stored at 4°C before use.

2.2.2 Activation of strains and fungal cultures

Lyophilized fungal strains were revived in accordance with the supplier's instructions. PDA slopes were inoculated with the fungal strains and incubated at 25–28°C in the dark for 7 d then sub-cultured in the same way.

Spores were harvested by adding sterile water and glass spheres (6 mm) to the flask and shaking for 3–5 min. Spore concentration was determined using a hemacytometer^[20] and was adjusted to a final concentration of 1×10^6 spores mL⁻¹. Spore suspensions (10 mL) containing 1×10^6 spores mL⁻¹ were added to PDA plates and incubated at 25±2°C in the dark for 3–5 d.

2.2.3 Mycelial growth inhibition

Three main steps were followed to assess the mycelial growth inhibition of the plant extracts^[21]. First, extracts were dissolved separately into the extraction solvents water, 50% ethanol, 95% ethanol, ethyl acetate and petroleum ether. Carbendazim was dissolved in ethanol. All samples were then diluted and emulsified with 0.1% Tween 80 (cell culture level) to final concentrations of extracts of 10 g·L⁻¹. Secondly, PDA medium containing 1 g·L⁻¹ solvent extract and a separate control medium with carbendazim (1 g·L⁻¹) were prepared. Two types of negative control were also prepared, PDA medium and PDA medium containing the corresponding solvents used for extraction (including the 0.1% Tween 80) from the first step. The final concentrations of the organic solvents were < 2%. The broad-spectrum fungicide carbendazim was used to make comparisons with extracts. Thirdly, plates with extracts (1 g·L⁻¹) were inoculated with the six fungal strains using a 6-mm-diameter agar disk (placed centrally) from an actively growing region of the fungus on PDA agar plates. The inoculated plates were incubated in the dark at 25±2°C. Colony diameters were measured using a caliper every 24 h until control plates were filled with fungal growth. The diameter of mycelial growth was calculated using the equation $D = D_0 - 0.6$ (cm), where D_0 represents the diameter of mycelial growth measured. Mean growth values were converted into the inhibition percentage of mycelial growth relative to the control treatment using the following equation:

$$MGI(\%) = \left[\frac{(D_c - D_t)}{D_c} \right] \times 100\%$$

where D_c and D_t represent mycelial growth diameter in control and treated Petri dishes, respectively. Three replicate plates of each treatment were assessed.

2.2.4 Total phenol and flavonoid contents in the extracts

The total phenol and flavonoid contents in the extracts were assayed as described by Hossain et al.^[22]. The absorbance of all samples was measured at fixed wavelengths of 760 nm (total phenol) and 510 nm (total flavonoids) using a UV-visible spectrophotometer. Gallic acid (total phenol) and rutin (total flavonoids) standards were used for the calibration curve.

2.2.5 Statistical analysis

All analysis was carried out on at least three replicates and results are expressed as the mean values±SE. Analysis of variance (ANOVA) of data and a multiple comparison of mean values using Duncan's multiple range test at the 5% protection level were conducted using the IBM SPSS 20.0 statistical software package.

3 RESULTS

3.1 Extraction yields

Figure 1 shows that the yield of extraction was significantly affected by the solvent used. It is likely that the solvent polarity was the main factor determining the ability to extract soluble substances. The polar solvents water, 50% ethanol and 95% ethanol extracted greater proportions of plant constituents than the non-polar solvents ethyl acetate and petroleum ether.

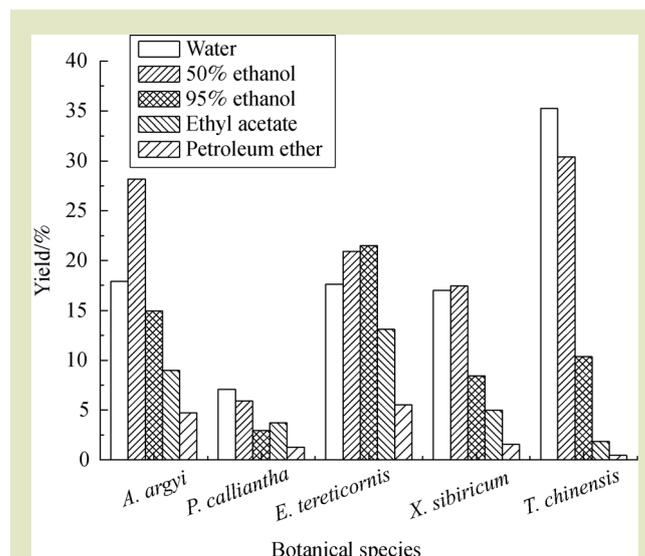


Fig. 1 Percentage yields of extracts of *Artemisia argyi*, *Eucalyptus tereticornis*, *Pyrola calliantha*, *Tupistra chinensis* and *Xanthium sibiricum* using five solvents.

3.2 Mycelial growth inhibition

The inhibitory effects of 1 g·L⁻¹ crude extracts on mycelial growth were tested on *P. expansum*, *P. italicum*, *A. niger*, *P. digitatum*, *B. cinerea*, and *R. stolonifer* (Fig. 2).

A. argyi extract inhibited all the microorganisms tested (Fig. 2(a)). Except for the aqueous extracts of *A. argyi*, *P. calliantha* and *X. sibiricum*, ethyl acetate extracts of *E. tereticornis*, and 95% ethanol extracts of *X. sibiricum*, all extracts had *in vitro* inhibitory effects on *A. niger*. *T. chinensis* extracts using 95% ethanol and ethyl acetate had the greatest inhibitory effect on *A. niger* (>60%).

Aqueous extracts of all species and all *P. calliantha* extracts had effectively no inhibitory effect on *P. expansum* (Fig. 2(b)). *T. chinensis* extracts using 95% ethanol and ethyl acetate had stronger inhibitory effects on *P. expansum* than the other extracts.

The inhibitory effects of the plant extracts on *P. digitatum* are shown in Fig. 2(c). *P. calliantha* and *E. tereticornis* extracts had effectively no inhibitory effect on *P. digitatum*. Ethyl acetate and 95% ethanol extracts of *T. chinensis* had greater inhibitory effects on *P. digitatum* than the other extracts.

All ethyl acetate extracts, and 95% ethanol and petroleum ether extracts from *T. chinensis* significantly inhibited *P. italicum* (Fig. 2(d)). Water and 50% ethanol extracts gave less inhibition than the ethyl acetate extracts, 95% ethanol and petroleum ether extracts.

Most crude extracts (except aqueous extracts) significantly inhibited all the fungal pathogens (Fig. 2(e) and Fig. 3). Overall, the mycelial growth inhibition associated with 95% ethanol, ethyl acetate, and petroleum ether extracts of *E. tereticornis*, *X. sibiricum* and *T. chinensis* were greater than for the others tested after 24 h. However, most mycelial diameters of *R. stolonifer* were >8.4 cm after 3 d, except with 95% ethanol and ethyl acetate extracts of *T. chinensis*. Mycelial growth diameters with 95% ethanol and ethyl acetate extracts of *T. chinensis* were only 2.07 cm and 5.88 cm, respectively, after 5 d (Table 1).

The inhibitory effects of the extracts on *B. cinerea* are shown in Fig. 2(f) and Fig. 3. All extracts except aqueous extracts inhibited *B. cinerea*; 95% ethanol and ethyl acetate extracts gave the greatest inhibition of *B. cinerea*, followed by *E. tereticornis* extracts. The aqueous extracts gave the greatest mycelial growth.

3.3 Model of mycelial radial growth of *Botrytis cinerea*

The fitted models and equations for mycelial radial growth of *B. cinerea* with the addition of water, 50% ethanol, 95% ethanol, ethyl acetate and petroleum ether extracts are shown in Fig. 4. The mycelial growth diameter of *B. cinerea* became linear with time (during the first 6 d). The fitted equation indicates the growth rate and linear correlation coefficients (*r*).

Figure 4(a) shows the effect of *A. argyi* extracts on *B. cinerea* growth. Water extracts promoted the growth of *B. cinerea* compared to the control. All other extracts inhibited growth to varying degrees, and the inhibitory effects of ethyl acetate and petroleum ether extracts were stronger than the 95% ethanol and 50% ethanol extracts.

Figure 4(b) shows the effect of *P. calliantha* extracts on *B. cinerea* growth. With the exception of aqueous and 50% ethanol extracts, growth was inhibited by the extracts in the sequence petroleum ether extracts < 95% ethanol < ethyl acetate.

Figure 4(c) shows the effect of *E. tereticornis* extracts on *B. cinerea* growth. With the exception of aqueous extract, *E. tereticornis* extracts had a stronger effect than *A. argyi* or *P. calliantha* extracts on growth; the slopes of the mycelial radial growth equation for *B. cinerea* with the addition of *E. tereticornis* extracts were <1.12. The growth inhibition with ethyl acetate and petroleum ether *E. tereticornis* extracts was greater than with 95% ethanol or 50% ethanol extracts.

The effect *X. sibiricum* extracts on *B. cinerea* growth is shown in Fig. 4(d). The inhibition with *X. sibiricum* extracts was slightly weaker than with extracts from *A. argyi*, *E. tereticornis* and *P. calliantha*; the slopes of the mycelial radial growth equation of *B. cinerea* with *X. sibiricum* extracts were >1.34. The growth inhibition with ethyl acetate and petroleum ether extracts was greater than with 95% ethanol and 50% ethanol extracts.

The effect of *T. chinensis* extracts on *B. cinerea* growth is shown in Fig. 4(e). The inhibition with 95% ethanol and ethyl acetate extracts was greater than with petroleum ether and 50% ethanol extracts, and the slopes of the growth equation were <0.72. Aqueous extract promoted the growth of *B. cinerea* compared to the control.

In summary, ethyl acetate extracts gave the greatest inhibition of *B. cinerea* mycelial radial growth of the extracts tested. Conversely, all water extracts gave the least negative effect on growth, or promoted growth. Ethyl acetate and 95% ethanol

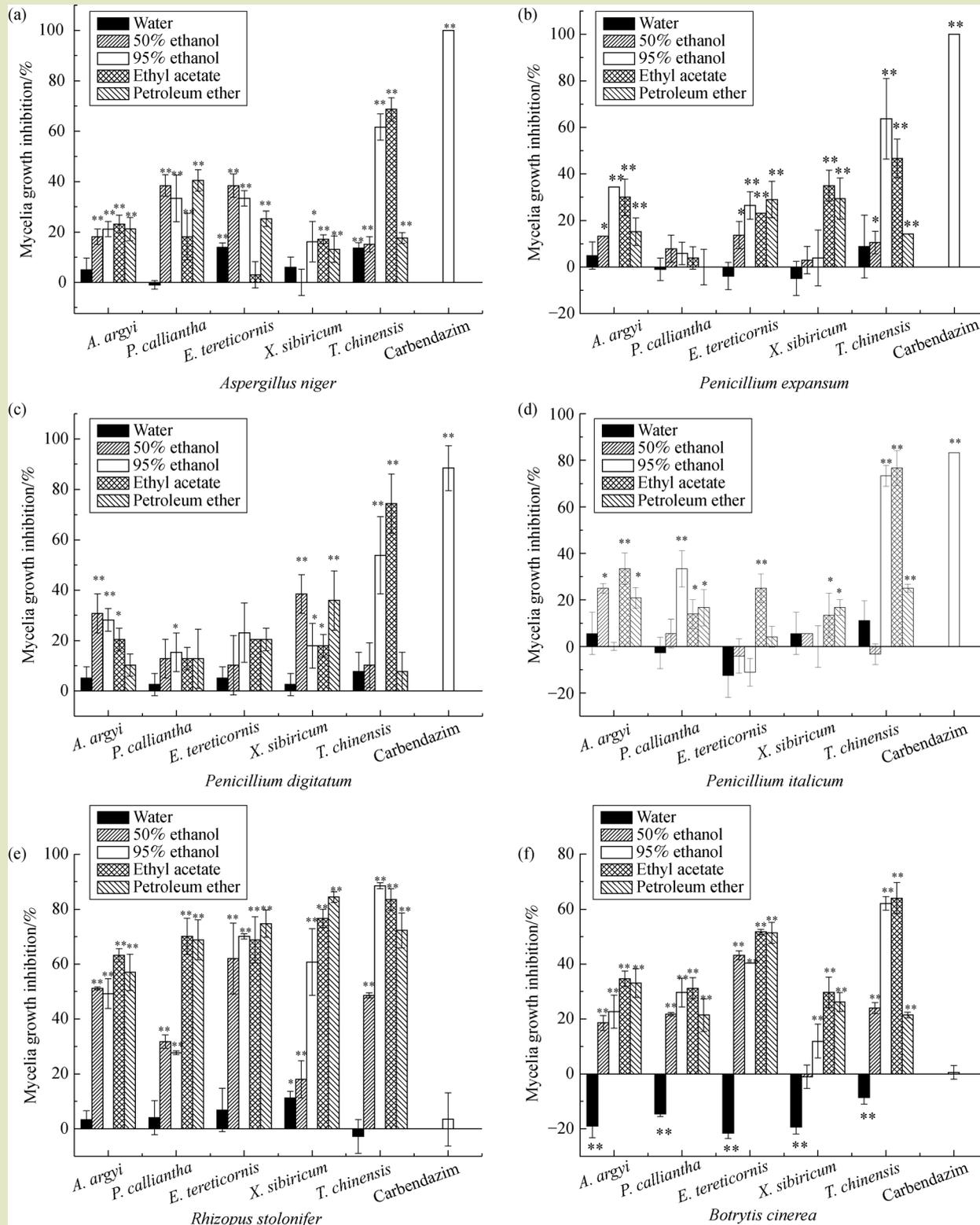


Fig. 2 In vitro inhibition by five solvent extracts of *Artemisia argyi*, *Eucalyptus tereticornis*, *Pyrola calliantha*, *Tupistra chinensis* and *Xanthium sibiricum*, and a carbendazim control against the growth of (a) *Aspergillus niger* (after 2 d); (b) *Penicillium expansum* (after 3 d); (c) *Penicillium digitatum* (after 2 d); (d) *Penicillium italicum* (after 3 d); (e) *Rhizopus stolonifer* (after 1 d); and (f) *Botrytis cinerea* (after 3 d).

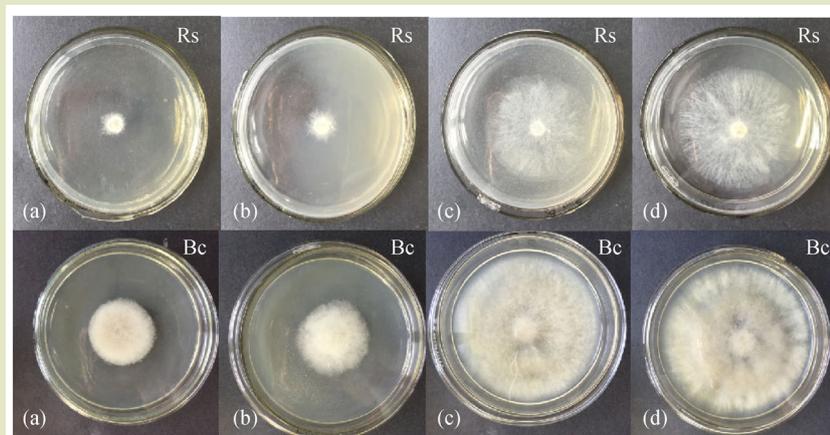


Fig. 3 Effects of *Tupistra chinensis* extracts on the mycelial growth of *Rhizopus stolonifer* (Rs) (after 1 d) and *Botrytis cinerea* (Bc) (after 5 d). (a) 95% ethanol extracts; (b) ethyl acetate extracts; (c) carbendazim; and (d) water control.

Table 1 Effect of *Tupistra chinensis* (rhizome) extracts with different solvents on the mycelial growth of *Rhizopus stolonifer* ($d \cdot cm^{-1}$)

Crude extract	Day 1	Day 2	Day 3	Day 4	Day 5
Water	5.50±0.28	> 8.4	–	–	–
50% ethanol	2.75±0.05	5.45±0.30	> 8.4	–	–
95% ethanol	0.62±0.07	1.08±0.19	1.50±0.25	1.63±0.25	2.07±0.31
Ethyl acetate	0.88±0.18	2.03±0.21	3.70±0.50	4.32±0.41	5.88±0.54
Petroleum ether	1.48±0.28	3.25±0.24	> 8.4	–	–
Water control	5.35±0.17	> 8.4	–	–	–
Carbendazim	5.17±0.45	> 8.4	–	–	–

Note: –, not measurable.

extracts of *T. chinensis* gave the greatest inhibition of *B. cinerea* growth.

3.4 Phenol and flavonoid contents of the plant extracts

The total phenol contents of the extracts are given in Fig. 5(a). Total phenol contents in water extracts, 50% ethanol extracts and 95% ethanol extracts from leaves of *A. argyi* and *E. terebinthifolius* were greater than in the other extracts. However, total phenol contents in extracts from *T. chinensis* were less than the other extracts, which did not parallel their antifungal activity, thus other compounds must be responsible for the antifungal activity of *T. chinensis* extracts.

Total flavonoid contents in different extracts are shown in

Fig. 5(b). Total flavonoid contents in water extracts, 50% ethanol extracts and 95% ethanol extracts from *A. argyi* were also higher than other extracts. In general, the total flavonoid contents of extracts from *T. chinensis* were less than the other extracts, in the sequence ethyl acetate extracts > 95% ethanol extracts > 50% ethanol and petroleum ether extracts > water, which effectively parallels their antifungal activity. Flavonoids might be one of the antifungal constituents of extracts from *T. chinensis*.

4 DISCUSSION

The antifungal activity of botanical extracts indicates that *T. chinensis* might be a natural source of fungicidal compounds. The experiments confirm the strong antifungal efficacy shown *in vitro* by ethyl acetate and 95% ethanol extracts of *T. chinensis*. The results indicate that 95% ethanol and ethyl acetate extracts from *T. chinensis* in $1 \text{ mg} \cdot \text{mL}^{-1}$ caused considerable growth

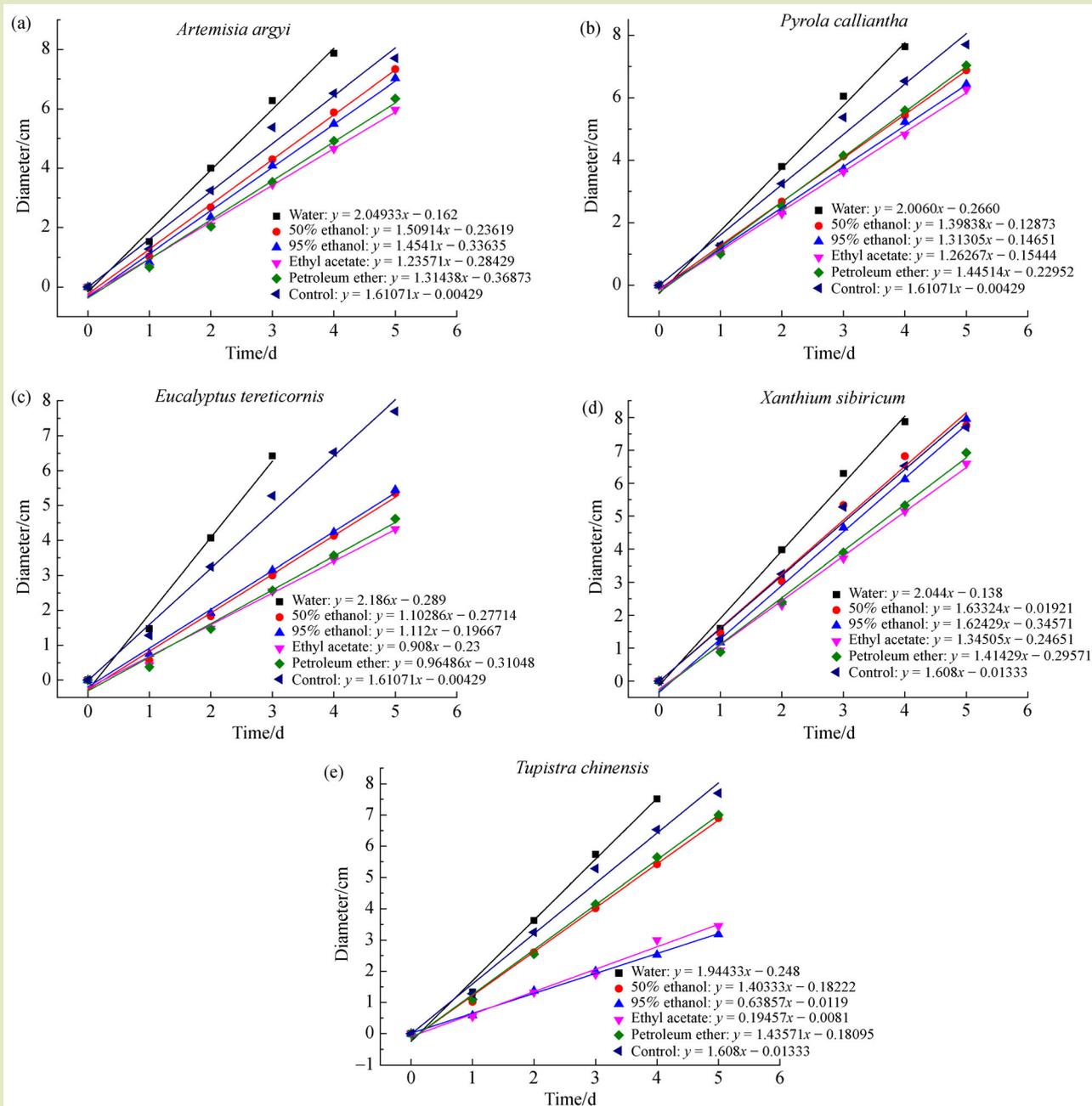


Fig. 4 Fitted models for mycelial radial growth of *Botrytis cinerea* in the presence of water, 50% ethanol (v/v), 95% ethanol (v/v), ethyl acetate, and petroleum ether extracts ($1 \text{ g} \cdot \text{L}^{-1}$) from (a) *Artemisia argyi* (leaves); (b) *Pyrola calliantha* (stalks and leaves); (c) *Eucalyptus tereticornis* (leaves); (d) *Xanthium sibiricum* (leaves); and (e) *Tupistra chinensis* (rhizomes). Growth diameters were diameters determined by the subtraction of the 6-mm-diameter agar plug used as inoculum.

inhibition in most of the fungi, with 60% to 80% inhibition of *B. cinerea*, *R. stolonifer*, *P. italicum*, *A. niger*, *P. digitatum* and *P. expansum*. Previous studies show that antifungal activity of some medicinal plants extracts was lower with 5 to 30%

inhibition of *A. niger* at $1 \text{ mg} \cdot \text{mL}^{-1}$ *in vitro* [23].

In addition, different solvent extracts from the same plant showed different degrees of inhibitory effect on fungal growth.

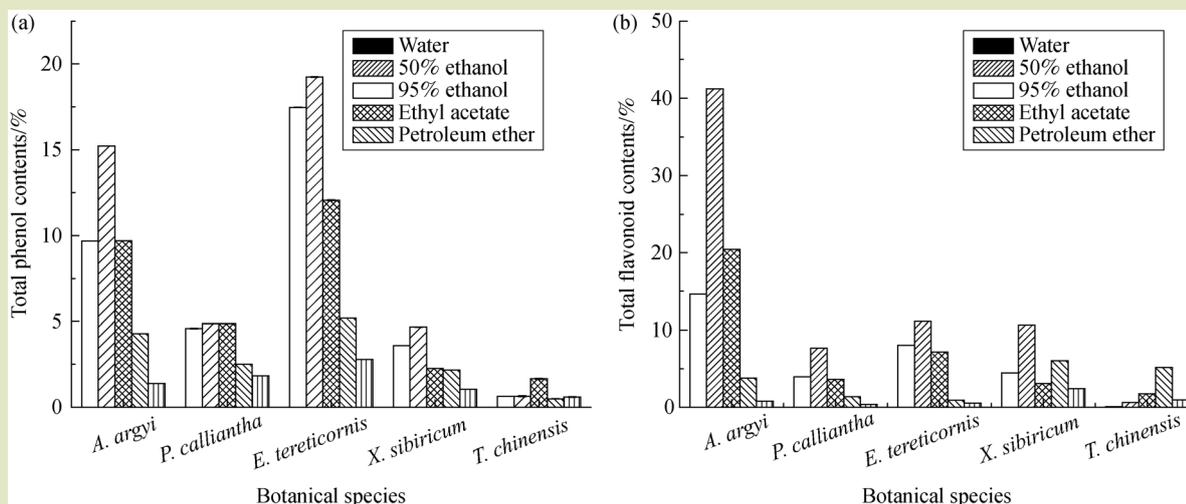


Fig. 5 Total phenol contents (a) and total flavonoid contents (b) of water, 50% ethanol (v/v), 95% ethanol (v/v), ethyl acetate, petroleum ether extracts of *Artemisia argyi* (leaves), *Pyrola calliantha* (stalks and leaves), *Eucalyptus tereticornis* (leaves), *Xanthium sibiricum* (leaves), and *Tupistra chinensis* (rhizomes).

The ethyl acetate and 95% ethanol extracts of *T. chinensis* had the greatest antifungal effects, with lower effects of water, 50% ethanol and petroleum ether extracts. This observation agrees with Thembo et al.^[24] who found that all extracts except aqueous extracts inhibited *Fusarium* spp. with the most active being methanol and hexane extracts of *Vigna unguiculata* and *Amaranthus spinosus*. Similarly^[25], alcoholic extracts of *Melia azedarach* had greater antimicrobial activity than methanol, petroleum ether or water extracts. Bakht et al.^[26] found that the butanol extracts of *Allium sativum* gave the greatest inhibition of *Bacillus cereus*. However, petroleum ether, methanol and water did not inhibit the test microbes.

B. cinerea is a plant pathogen that attacks over 200 crop species worldwide, especially grapes, vegetables and berries^[27,28]. *B. cinerea* is difficult to control because it has a wide range of hosts as inoculum sources and various modes of infection. It can also survive as conidia, mycelia and/or sclerotia for extended periods as in crop debris^[29]. The present study indicates that all extracts other than water extracts can inhibit *B. cinerea*. However, carbendazim, a common fungicide, had almost no *in*

vitro activity on *B. cinerea*. The 95% ethanol and ethyl acetate extracts of *T. chinensis* rhizomes and ethyl acetate and petroleum ether extracts of *E. tereticornis* leaves inhibited *B. cinerea* by > 50% at 1 mg·mL⁻¹, indicating that these plant species are good prospects as potential sources of antifungal agents for control of *B. cinerea*.

5 CONCLUSIONS

In conclusion, *in vitro* tests were conducted with plant extracts against six plant pathogenic fungi. The 95% ethanol and ethyl acetate extracts of *T. chinensis* gave the greatest antifungal activity against these fungi. Based on *in vitro* tests, 95% ethanol and ethyl acetate extracts of *T. chinensis* rhizomes might provide good antifungal agents for controlling *R. stolonifer* and *B. cinerea* in postharvest fruits. *T. chinensis* has potential to be a new natural fungicide resource and may be used for the preservation of agricultural and forestry products such as fruits and vegetables. Clearly, further studies are justified on antifungal compounds from plant sources for extending fruit storage.

Compliance with ethics guidelines

Shufeng Yao, Jiali Xu, Haibo Zhang, Hong Gao, Shibin Shang, and Dan Wang declare that they have no conflicts of interest or financial conflicts to disclose. This article does not contain any studies with human or animal subjects performed by any of the authors.

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