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Research Note

Inhibition of plant pathogens in vitro and in vivo with essential oil and organic extracts of *Torreya grandis* 'Merrilli' aril

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Abstract

The seed aril of *Torreya grandis* 'Merrilli', a native conifer from China, has recently become popular for its food and pharmaceutical properties. The aims of this study were to comprehensively evaluate the efficacy of essential oil and other aril extracts as antifungal agents. The chemical components of the oil were analyzed by GC–MS. The results revealed the existence of more than 57 compounds in the volatile fraction, of which the main compounds were limonene (16.789%) and β-pinene (8.553%). The aril oil and organic extracts showed remarkable antifungal property against five commercially important plant pathogenic fungi (*Fusarium oxysporum* f. sp. *cucumerinum*, *Pyricularia oryzae*, *Bipolaris maydis*, *Pseudoperonospora cubensis*, *Alternaria solani*). The EC50 ranged from 0.608-1.249 mg mL^{-1} and 0.241-1.350 mg mL^{-1}, respectively. The oil had a strong detrimental effect on spore germination of all tested plant pathogens *in vitro*. Moreover, aril essential oil significantly reduced the damage areas produced by *Pseudoperonospora cubensis* and *Alternaria solani* on cucumber leaves and tomato fruits, respectively. The relative control efficiency (92.25%) was higher for essential oil treated cucumber leaves than that of Carbendazim treated leaves (84.27%). This is the first study that demonstrates *T. grandis* 'Merrilli' aril oil and its organic extracts possess a wide range of antifungal activity. They therefore have the potential of becoming an alternative to synthetic fungicides for controlling economically important plant fungal diseases.

Keywords: Antifungal activity; essential oil; phytopathogens; plant extracts; seed aril; *Torreya grandis* 'Merrilli'.
Abbreviations: GC-MS–gas chromatography-mass spectrometry; PDA_potato dextrose agar; DMSO_dimethyl sulfoxide; EC50_50% effective concentration.

Introduction

Plant diseases enormously reduce crop productivity and food/grain quality. About 80% of plant diseases are caused by plant pathogenic fungi. The global yield lost to fungal diseases can extend to 12% or even higher in some developing countries (Oerke, 2006). For example, the deadly rice blast disease caused by *Pyricularia oryzae* costs rice farmers millions of dollars every year in lost harvests. Other pathogenic fungi such as *Bipolaris maydis*, *Pseudoperonospora cubensis* and *Alternaria solani* also severely damage agricultural crops worldwide. Chemical control remains the main strategy for reducing the incidence of plant diseases in various fruits and vegetables. A serious problem against the suatainable use of these chemicals is the development of resistance by the fungi (Isman, 2000). Therefore, the development of natural crop protective products as alternative to synthetic fungicides has gained attention in the last decade (Tripathi and Dubey, 2004). Natural antifungal agents such as plant-based form of essential oils and other plant extracts are also considered to be environmentally safe and suatainable (Wilson et al., 1997). Considering the importance of natural antifungal agents, research in the area has intensified in the past few years. Screening of natural agents is oriented more towards molecules that possess a selective action against plant fungi, without being toxic to man or the ecosystem. Since most plants have their own defense systems against fungal pathogens, it is logical to investigate if antifungal compounds or phytochemicals from one species can protect another (Gurgel et al., 2005; Sridhar et al., 2003; Chauhan et al., 2007).

Torreya grandis 'Merrilli' is a conifer species of the Taxaceae family. It is native to China and grows semi-wild in the Kuaiji Mountain of Shaoxing, Zhejiang province Eastern China. In the recent years, the cultivation of *Torreya grandis* has increased due to high economic benefits gained through novel product development. It is well-known for its edible seeds that taste like nuts with a unique flavor and aroma. The succulent seed aril which completely encloses the seeds represents about 50% of the fresh seed weight. Traditionally, people peel off the aril from the woody seed

coat and discard it before drying the seeds. This is because the aril emits an unpleasant odor and pollutes the surrounding environment. However, Chinese scientists have recently generated great interest in the aril because of its potential utility in both, food and pharmaceutical industries. Preliminary studies have identified distinct monoterpenes, sesquiterpenes, diterpenes and their oxygenated compounds from the aril (Saeed et al., 2007). However, the biological activity of the essential oil from *T. grandis* 'Merrilli' seed aril including its antimycotic activity has received less attention.

In this study, we examined the chemical composition of the essential oil isolated from *T. grandis* 'Merrilli' seed aril and tested the efficacy of the essential oil and other seed aril extracts (prepared using petroleum ether, chloroform, ethyl acetate and n-butyl alcohol) against economically important plant pathogenic fungi *in vitro* and *in vivo*. Since *T. grandis* 'Merrilli' is now commonly cultivated in China, our findings will increase the economic benefits to farmers, reduce use of toxic chemicals and prevent stinking from a large amount of discarded seed aril. This is the first comprehensive investigation of effect of *T. grandis* 'Merrilli' seed aril oil and other extracts on plant pathogenic fungi.

Results

Analysis of volatile compounds from seed aril

The hydrodistillation of the essential oil from *T. grandis* 'Merrilli' aril produced light yellowish oil. The chemical composition of the essential oil was determined by GC-MS analysis (Table 1). More than 57 compounds were identified, which constituted over 96.65% of the entire volatile fraction. Monoterpenes and sesquiterpenes were the main components in the oil. The most abundant constituents were limonene (16.789%), β-pinene (8.553%), epi-bicyclosesquiphelandrene (7.016%) and cadina-4,9-diene (6.536%). α-Terpinene (0.184%), terpinolene (0.069%), ε-elemene (0.212%), copaene (0.097%), α-gurjunene (0.087%), linalool (0.072 %) and diisobutyl phthalate (0.075 %) were also found to be present in trace amounts.

In vitro antifungal assay

The *T. grandis* 'Merrilli' aril oil exhibited a moderate to high antifungal activity against all the plant pathogens tested (Table 2). The EC50 ranged from 0.608-1.249 mg mL^{-1}[*Pseudoperonospora cubensis* (1.157 mg mL^{-1}), *Fusarium oxysporum* f. sp. *cucumerinum* (1.249 mg mL^{-1}), *Bipolaris maydis* (0.608 mg mL^{-1}), *Pyricularia oryzae* (0.677 mg mL^{-1}), *A. solani* (0.905 mg mL^{-1})], indicating notably different susceptibility among the fungal species. Results showed that the strongest inhibitory effect of the oil was on *B. maydis* (Fig 1). The EC50 value was 0.608 mg mL^{-1}. Dimethyl sulfoxide (DMSO), at levels equivalent to those in the test compound solutions, did not affect the growth of the fungi investigated. The antifungal activity of different solvent extracts was also tested. The solvent extracts also exhibited strong antifungal activity against all the plant pathogens tested, the EC50 ranging from 0.241 to 1.350 mg mL^{-1} (Table 3). Petroleum ether extract exhibited strong antifungal activity against all the plant pathogens tested (according to respective fungal growth inhibition percentage). One of the fungal pathogens, *Pyricularia oryzae*, displayed more susceptibility to the petroleum ether (Fig 2) and ethyl acetate extracts with EC50 of 0.327 and 0.241 mg mL^{-1}, respectively. The results from the spore germination assay for each fungus in response to essential oil and different solvent extracts are presented in Table 4 and Table 5. Essential oil from *T. grandis* 'Merrilli' aril and different solvent extracts displayed a range of inhibitory action on fungal spore germination. Toxicity test results indicate that the ethyl acetate extract, chloroform extract, n-butyl alcohol extract and essential oil greatly inhibited the spore germination of *Pyricularia oryzae*; EC50 were 0.0007, 0.0056, 0.0165 and 0.084 mg mL^{-1}, respectively. The EC50 was lowest for ethyl acetate treatment, reaching 0.0007 mg mL^{-1} after 24 h incubation with *Pyricularia oryzae*.

In vivo antifungal activity of the aril oil

To evaluate the field antifungal potential of the essential oil from *T. grandis* 'Merrilli' aril, it's *in vivo* antifungal activity was determined and compared to that obtained with Carbendazim, the principal commercially available plant fungicide used in China. According to the results presented in Table 6 and Fig 3, the oil exhibited a wide range of *in vivo* antifungal activity. Both, Carbendazim and the essential oil inhibited the infection of cucumber leaves by *Pseudoperonospora cubensis*. The essential oil inhibited disease development in tomatoes and cucumbers leaves to different extent. The relative control efficiency for essential oil treated leaves (92.25%) was higher than that of Carbendazim treated leaves (84.27%), but no statistically significant differences were found. In addition, the level of protection of tomato fruits against *A. solani* was evaluated (Table 6 and Fig 4). The results indicate that both the essential oil and Carbendazim, did not obviously inhibit growth of the fungus. In the essential oil treated fruits, the relative control efficiency reached only 52.25%. However the essential oil activity was significantly higher compared with the control on the infection of tomato fruits by *A. solani.*

Discussion

Consumers are increasingly demanding safer food products with reduced amounts of pesticides. In addition, threat of resistance development to currently used antifungal agents has therefore significant social and economic implications when investigating novel natural plant-based antifungal agents. In general, essential oils are considered as non-phytotoxic compounds and potentially effective in food and agriculture industries against pathogenic fungi (Bajpai et al., 2007). In the present study, the hydrodistillated oil of *T. grandis* 'Merrilli' aril contained oxygenated mono- and sesquiterpenes, and their respective hydrocarbons. More than 57 volatile compounds were identified. The number of compounds detected in this study was different to previous report (Feng et al., 2011). The most abundant constituents were limonene (16.789%) and β-pinene (8.553%). However, Niu et al. (2011) reported that *T. grandis* 'Merrilli' aril essential oils were characterized by their richness of limonene (34.5–43.9%), a-pinene (23.4–34.3%), and d-3-carene (1.9–6.4%). It is often quite difficult to compare the results obtained from different studies because the compositions of the essential oils can vary greatly depending upon the geographical region, the variety, the age of the plant, the method of drying and the method of oil extraction (Zomorodian et al., 2011). This implies that quantitative data on plant oils and extracts are required. In this study, many trace or minor components were found to be present in the *T. grandis* aril oil. Most of them were terpenes and sesquiterpenes. In recent years, several researchers have reported that terpenes and sesquiterpenes are the major components of essential oils from plant origin. These compounds

Table 1. Volatile compound composition of *Torreya grandis* 'Merrilli' aril oil isolated by hydrodistillation.

No.	Compound	Molecular formula	Percentage[a] （%）	RSD (%)[b]
1	2-Methyl-1-heptane	C_8H_{16}	0.268	0.321
2	2,6,6-Trimethyl bicyclol [3,1,1]-2- hepten	$C_{10}H_{16}$	4.038	0.212
3	Camphene	$C_{10}H_{16}$	1.36	0.121
4	α-Phellandrene	$C_{10}H_{16}$	2.393	0.173
5	7,7-Trimethyl bicyclol [4,1,10]-3 -hepten	$C_{10}H_{16}$	5.162	0.343
6	β-Pinene	$C_{10}H_{16}$	8.553	0.366
7	α- Terpinene	$C_{10}H_{16}$	0.184	0.034
8	Limonene	$C_{10}H_{16}$	16.789	0.516
9	α-Phellandrene	$C_{10}H_{16}$	1.252	0.781
10	α-Pinene	$C_{10}H_{16}$	0.643	0.132
11	1-Methyl-4-（1-methylethylidene）-cycl ohexene	$C_{10}H_{16}$	0.237	0.150
12	3,7-Dimethyl-1,3,6- triene	$C_{10}H_{16}$	0.965	0.113
13	Terpinolene	$C_{10}H_{16}$	0.069	0.042
14	3-Carene	$C_{10}H_{16}$	2.659	0.161
15	α- Cubebene	$C_{15}H_{24}$	0.986	0.314
16	ε- Elemene	$C_{15}H_{24}$	0.212	0.172
17	Copaene	$C_{15}H_{24}$	0.097	0.043
18	α- Gurjunene	$C_{15}H_{24}$	0.087	0.052
19	β- cubebene	$C_{15}H_{24}$	1.248	0.671
20	Linalool	$C_{10}H_{18}O$	0.072	0.075
21	3,7-Dimethyl-6- octenoic aci methyl ester	$C_{11}H_{20}O_2$	0.136	0.234
22	Levorotatory acetic acid borneol	$C_{12}H_{20}O_2$	0.154	0.352
23	Syringaldehyde	$C_{10}H_{16}O_2$	0.154	0.472
24	Caryophyllene	$C_{15}H_{24}$	1.166	0.682
25	1-(1- Dimethyl)-4-methyl-3- cyclohexen-1-ol	$C_{10}H_{18}O$	0.583	0.695
26	α- Muurolene	$C_{15}H_{24}$	1.488	0.494
27	Farnesene	$C_{15}H_{24}$	3.075	0.224
28	γ-Cadinene	$C_{15}H_{24}$	0.932	0.283
29	α- Terpineol	$C_{10}H_{18}O$	0.775	0.182
30	2-Borneol	$C_{10}H_{18}O$	0.213	0.231
31	Epi-bicyclosesquiphellandrene	$C_{15}H_{24}$	7.016	1.212
32	Bicyclosesquiphollandrene	$C_{15}H_{24}$	1.960	1.031
33	α- Cadinene	$C_{15}H_{24}$	2.083	0.354
34	Cadina-4, 9-diene	$C_{15}H_{24}$	6.536	1.712
35	3,7-Dimethyl-6-octen-1-ol	$C_{10}H_{20}O$	0.924	0.154
36	Cadina-1,4-diene	$C_{15}H_{24}$	0.142	0.442
37	Eraniol	$C_{10}H_{18}O$	0.106	0.854
38	γ- Elemene	$C_{15}H_{24}$	3.531	0.211
39	2,7-Dimethyl-2,6- dienol	$C_{10}H_{18}O$	0.135	0.194
40	Ledol	$C_{15}H_{26}O$	0.252	0.312
41	4-Methyl-2-(1,5-dimethyl-4-hexenylidene)-3-cyclohexene-1-ol	$C_{15}H_{26}O$	0.489	0.075
42	Lauryl alcohol	$C_{15}H_{26}O$	0.251	0..372
43	9-Tetradecen-1-ol	$C_{14}H_{28}O$	0.134	0.151
44	8-Dodecen-1-ol	$C_{12}H_{14}O$	0.717	0.042
45	Elemene-D-4-alcohol	$C_{15}H_{26}O$	0.617	0.254
46	Cubenol	$C_{15}H_{26}O$	3.476	0.213
47	Eucalypto	$C_{15}H_{26}O$	0.332	0.375
48	Spathuleno	$C_{15}H_{26}O$	0.465	0.341
49	Cadinol	$C_{15}H_{26}O$	6.156	1.061
50	Methyl palmitate	$C_{17}H_{34}O$	0.365	0.142
51	Sclareol	$C_{20}H_{36}O_2$	1.964	0.654
52	Cembrene	$C_{20}H_{32}$	0.314	0.322
53	Nerolidol	$C_{15}H_{26}O$	0.164	0.083
54	Abietatriene	$C_{20}H_{30}$	0.283	0.075
55	Diisobutyl phthalate	$C_{16}H_{22}O_3$	0.075	0.011
56	Methy linoleate	$C_{19}H_{34}O_2$	0.142	0.091
57	Dehydroabietane	$C_{20}H_{30}$	2.071	0.312

[a] Percentage of peak area relative to total peak area;
[b] relative standard deviation.

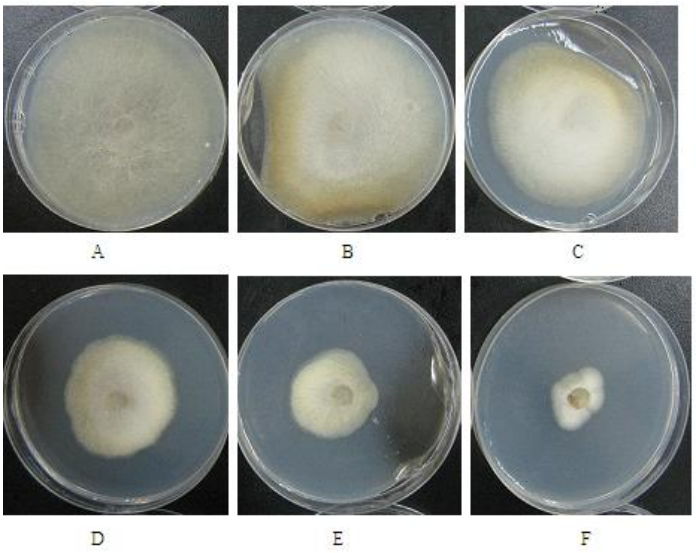

Fig 1. The inhibitory effect of *Torreya grandis* 'Merrilli' aril oil on *B. maydis.* A. Control; B. 0.4 mg mL^{-1}; C. 0.8 mg mL^{-1}; D. 1.2 mg mL^{-1}; E. 1.6 mg mL^{-1}; F. 2 mg mL^{-1}.

compounds have been suggested to have potentially strong anti-microbial properties (Cakir et al., 2004). In the present study, the essential oil from *T. grandis* aril showed potential *in vitro* and *in vivo* antifungal effect against all the plant pathogens tested. Both, the oil and solvent extracts of *T. grandis* aril exhibited strong antifungal property as seen from the EC50 values and the inhibitory effects on hyphal growth and spore germination of the tested plant pathogens. Petroleum ether extract exhibited highest antifungal effect against all the plant pathogens according to respective fungal growth inhibition percentage. Further, various extracts also significantly inhibited growth of fungal mycelia from economically important plant pathogens, although inhibition rate differed according to the extract. This may be attributed to the mode of resistance behavior of the fungi against various substances present in the different solvent extracts. In future research, it may be appropriate to separate and identify individual compounds that are most inhibitory. This research work also describes the complex effect of essential oil and various extracts on fungal spore germination. When comparing data from Table 2, 3, 4 and 5, spore germination stage was more sensitive to the oil and organic extracts than hyphal growth. Historically, many plant oils and extracts have been reported to have antimicrobial properties (Salamci et al., 2007). Earlier papers on the analysis and antifungal properties of the essential oils from various species have shown that they have a varying degree of growth inhibitory effect against some fungal species due to their different chemical compositions (Cakir et al., 2004; Bajpai et al., 2007). It is reasonable to hypothesize that antifungal activity results from a synergistic activity of all the compounds present in the oil. The biological activities could depend on a synergy of the different chemical characteristics of the constituent compounds. Each of the essential oil components has its own contribution to the total biological activity of the oil. For example, the oils of *Pistacia terebinthus*, *Pistacia vera* and *Pistacia lentiscus* had moderate activities against *Rhizoctonia solani*. In these essential oils, α-terpineol, α-pinene, β-pinene and β-caryophyllene were found as major components and they were also characterized in terms of the high contents of monoterpenes and terpenes (Duru et al., 2003). The preliminary analysis by GC-MS shows that *T. grandis* 'Merrilli' aril oil contains a series of secondary metabolites (Table 1), such as α-pinene, β-pinene and β-caryophyllene, which have been reported as antifungal compounds for phytopathogenic fungi (Cakir et al., 2004). We propose that the antifungal activity of *T. grandis* 'Merrilli' aril oil could be explained, at least partly, by the presence of terpenes compounds, such as α-pinene, β-pinene, *etc.* (Filipowicz et al., 2003). Moreover, Marei *et al*. (2012) reported that limonenes are potent antifungal compounds. And we found that the main component in the *T. grandis* 'Merrilli' aril essential oil was limonene (16.789%), followed by β-pinene (8.553%). Further, Sharma and Tripathi (2006) also reported that essential oils from the epicarp of *Citrus sinensis* exhibited absolute fungitoxicity against the 10 post-harvest pathogens and the main components of oil were limonene and β-pinene. Amri *et al.* (2013) reported that β-pinene as predominant constituents in cypress oil and this compound may be responsible for its antifungal property. Our observations are in agreement with the above reports. Nonetheless, more quantitative data on *T. grandis* 'Merrilli' aril oil and organic extracts are required. This is particularly important in the view of increased demand for control of plant pathogens using natural products that are effective against a broad range of pathogens. Finally, the essential oil moderately inhibited *in vivo* development in tomatoes and cucumbers leaves. However, the essential oil activity was significantly higher compared with the control on controlling the infection of tomato fruits by *A. solani.* This may be attributed to the different mode of resistance behavior of the fungi when infected *in vitro* and *in vivo*. It also highlights that the plant, pathogens and the control agents have a complex relationship.

Materials and Methods

Plant materials

The arils of *Torreya grandis* 'Merrilli' were collected from the local area of Kuaiji Mountain of Shaoxing, Zhejiang province, China, in September 2012 and identified by the morphological features by Expert Kaijiang Chu. The aril was deposited at the Laboratory of Yuanpei College (Shaoxing University Yuanpei College, China).

Table 2. Antifungal activity of Torreya grandis 'Merrilli' aril essential oil.

Fungal species	Growth inhibition		
	Virulence regression equation[a]	r	EC50 (mg mL^{-1})
Pseudoperonospora cubensis	Y=4.6362X+4.707	0.972	1.157
Fusarium oxysporum f.sp. cucumerinum	Y=3.567X+4.656	0.944	1.249
Bipolaris maydis	Y=3.6215X+5.7832	0.961	0.608
Pyricularia oryzae	Y=3.4831X+5.5897	0.983	0.677
Alternaria solani	Y=3.3168X+5.1446	0.937	0.905

[a] Y is the probability of the growth inhibition; X is the $\log_{10}$ concentration; r is the sample correlation coefficient.

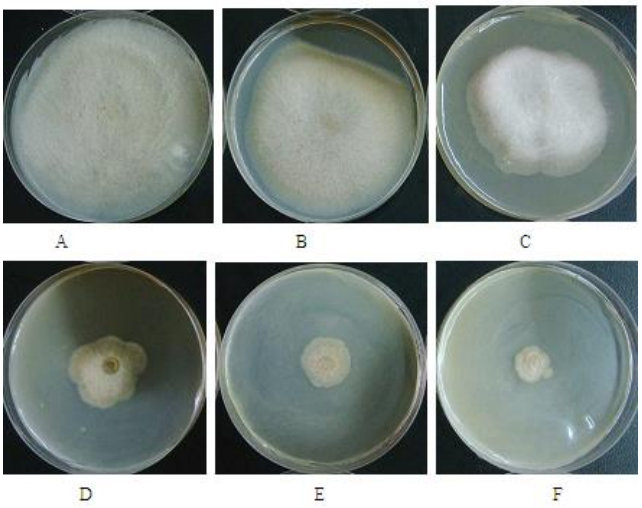

Fig 2. The inhibitory effect of Torreya grandis 'Merrilli' aril Petroleum ether extract on Pyricularia oryzae; A. Control; B. 0.4 mg mL^{-1}; C. 0.8 mg mL^{-1}; D. 1.2 mg mL^{-1}; E. 1.6 mg mL^{-1}; F. 2 mg mL^{-1}.

Isolation of the essential oil

The aril was crushed using a blender (DJ-10A, Shanghai Dianjiu Chinese Machinery Manufacturing Co. Ltd., Shanghai, China). Essential oil was subsequently obtained by hydrodistillation in a modified volatile oil determination apparatus for 4 h. The essential oil was collected and dried on anhydrous sodium sulfate. Clear yellow oil was obtained in a yield of 1.26 % (w/w).

Preparation of aril extracts

The air-dried aril was pulverized into powdered form. The dried powder (1500g) was extracted with ethanol extract and separated by different solvents including petroleum ether, chloroform, ethyl acetate, and n-butyl alcohol at room temperature, and the solvents from the combined extracts were evaporated by vacuum rotary evaporator (RE-52AH, Yarong Machinery Manufacturing Co. Ltd., Shanghai, China). The yields from different solvents in descending order are petroleum ether (300.1 g), chloroform (205.6 g), ethyl acetate (98.2 g) and n-butyl alcohol (15.5 g).

Gas chromatography-mass spectrometry

Essential oil constituents were analyzed by an Agilent 6890N/5975 MSD. A DB-WAX capillary column (30 m*0.25 mm, film thickness 0.25 mm) was used for the analyses. The temperature program used was: 80 ^{o}C for 3 min and then raised to 250 ^{o}C at 3 ^{o}C/min, to 250 ^{o}C at 5 ^{o}C/min final isotherm and held for 10 min. The injector was kept at 250 ^{o}C. The carrier gas was He (99.99 %) at a flow rate of 1 ml min^{-1}. The MS detector operated in the scan mode with 70 eV electron impact, scanning the 20–400 m/z range. The multiplier was at 2 kV. The ion source temperature was 200 ^{o}C. The diluted sample (in methanol) of 0.5µL was injected manually. The relative percentage of the oil constituents were quantified by calculating the relative contents based on the peak area normalization method. Identification of components of the essential oil was assigned by comparison of their retention indices, relative to GC–MS spectra from the MS data (NIST) and literature (Niu et al., 2011).

Fungal pathogens

Five pathogenic fungi (*Fusarium oxysporum* f. sp. *cucumerinum*, *Pyricularia oryzae*, *Bipolaris maydis*, *Pseudoperonospora cubensis*, *Alternaria solani*) were supplied by Shandong Academy of Agricultural Sciences, China. The cultures of each fungal species were maintained on potato dextrose agar (PDA) slants and stored at 4 ^{o}C. The samples of mycelium necessary for the *in vitro* and *in vivo* experiments were taken from cultures grown in test tubes and kept at 28 ±1 ^{o}C on potato dextrose agar (PDA).

In vitro antifungal assays

Effect of aril extracts on mycelial growth

The culture media for the treatments were prepared by adding different volumes of a solution of *T. grandis* 'Merrilli' aril extract in dimethyl sulfoxide (DMSO) to each Petri plate (each still containing potato dextrose agar) to obtain final concentrations of 0.4, 0.8, 1.2, 1.6 and 2 mg mL^{-1}. The DMSO concentration in the final solution was 0.1 %. Equivalent quantities (0.1%) of the solvent (DMSO) were

Table 3. Growth inhibition of different pathogenic fungi by various organic extracts prepared from *Torreya grandis* 'Merrilli' aril.

Fungal species	Growth inhibition											
	Petroleum ether extract			Chloroform extract			Ethyl acetate extract			N-butyl alcohol extract		
	Virulence regression equation[a]	r	EC50 (mg mL^{-1})	Virulence regression equation[a]	r	EC50 (mg mL^{-1})	Virulence regression equation[a]	r	EC50 (mg mL^{-1})	Virulence regression equation[a]	r	EC50 (mg mL^{-1})
Pseudoperonospora cubensis	Y=2.5735X+5.2299	0.913	0.814	Y=2.5119X+5.273	0.942	0.779	Y=3.5328X+5.7008	0.910	0.633	Y=2.6416X+5.7589	0.968	0.516
Fusarium oxysporum f.sp. *cucumerinum*	Y=2.1522X+4.9315	0.951	1.171	Y=1.7702X+4.8823	0.91	1.165	Y=1.5486X+4.7983	0.915	1.350	Y=2.2795X+4.9812	0.924	1.019
Bipolaris maydis	Y=2.1944X+5.7527	0.827	0.454	Y=2.3244X+5.0864	0.981	0.918	Y=2.1104X+5.4602	0.915	0.605	Y=2.1090X+5.4782	0.918	0.593
Pyricularia oryzae	Y=2.8458X+6.3811	0.886	0.327	Y=4.2989X+4.565	0.986	1.262	Y=1.3511X+5.8358	0.826	0.241	Y=2.4869X+5.4658	0.939	0.650
Alternaria solani	Y=1.6907X+5.3837	0.917	0.593	Y=2.4120X+5.1708	0.969	0.850	Y=2.2571X+5.344	0.992	0.704	Y=2.5958X+5.336	0.997	0.742

[a] Y is the probability of the growth inhibition; X is the $\log_{10}$ concentration; r is the sample correlation coefficient.

Table 4. Efficacy of *Torreya grandis* 'Merrilli' aril oil on inhibiting spore germination of 5 plant pathogenic fungi.

Fungal species	Growth inhibition		
	Virulence regression equation[a]	r	EC50 (mg mL^{-1})
Bipolaris maydis	Y=0.9605X+5.7128	0.899	0.181
Fusarium oxysporum f.sp. *cucumerinum*	Y=0.9567X+3.256	0.934	0.149
Pseudoperonospora cubensis	Y=0.9388X+5.9972	0.976	0.087
Alternaria solani	Y=0.9885X+5.8523	0.939	0.137
Pyricularia oryzae	Y=1.0587X+6.1371	0.995	0.084

[a] Y is the probability of the growth inhibition; X is the $\log_{10}$ concentration; r is the sample correlation coefficient.

Table 5. Efficacy of different solvent extracts from *Torreya grandis* 'Merrilli' aril against spores germination of 5 plant pathogenic fungi.

Fungal strain	Growth inhibition											
	Petroleum ether extract			Chloroform extract			Ethyl acetate extract			N-butyl alcohol extract		
	Virulence regression equation[a]	r	EC50 (mg mL^{-1})	Virulence regression equation[a]	r	EC50 (mg mL^{-1})	Virulence regression equation[a]	r	EC50 (mg mL^{-1})	Virulence regression equation[a]	r	EC50 (mg mL^{-1})
Bipolaris maydis	Y=1.2908X+5.8289	0.9816	0.228	Y=1.0397X+6.0354	0.9841	0.076	Y=1.5437X+5.9704	0.986	0.235	Y=0.6384X+5.9086	0.952	0.038
Fusarium oxysporum f.sp.*cucumerinum*	Y=1.3807X+6.8345	0.982	0.108	Y=1.2579X+5.0453	0.9840	0.173	Y=1.2314X+5.4325	0.975	0.278	Y=1.3284X+5.3214	0.964	0.289
Pseudoperonospora cubensis	Y=1.8777X+6.0644	0.985	0.271	Y=0.9778X+6.0865	0.9962	0.077	Y=1.4626X+6.0018	0.966	0.207	Y=1.8082X+6.0476	0.987	0.263
Alternaria solani	Y=0.9005X+6.0369	0.983	0.071	Y=1.1178X+5.943	0.9808	0.143	Y=1.2906X+5.7479	0.972	0.263	Y=1.3666X+5.9695	0.954	0.195
Pyricularia oryzae	Y=0.8483X+5.9668	0.98	0.073	Y=0.3319X+5.7481	0.9483	0.0056	Y=0.3326X+6.042	0.817	0.0007	Y=0.7108X+6.2673	0.998	0.0165

[a] Y is the probability of the growth inhibition; X is the $\log_{10}$ concentration;r is the sample correlation coefficient.

Table 6. The efficacy of the *Torreya grandis* 'Merrilli' aril essential oil against fungal growth *in vivo.* Different letters (a-b) denote significant differences ($p<0.05$) between essential oils/compounds.

Colonize	Essential oil relative control efficiency % (2 mg mL^{-1})	Carbendazim relative control efficiency % (2 mg mL^{-1})
Cucumber leaves (*Pseudoperonospora cubensis)*	92.25±2.9a	84.27±3.5a
Tomato fruits (*Alternaria solani*)	52.25±2.7a	37±4.2b

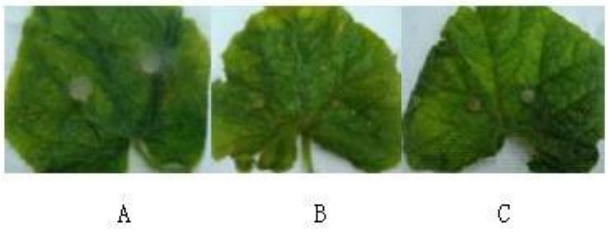

Fig 3. The efficacy of *Torreya grandis* 'Merrilli' aril oil against *Pseudoperonospora cubensis* in cucumber leaves; A. Control; B. *T. grandis* 'Merrilli' aril oil (2 mg mL^{-1}); C. Carbendazim (2 mg mL^{-1})

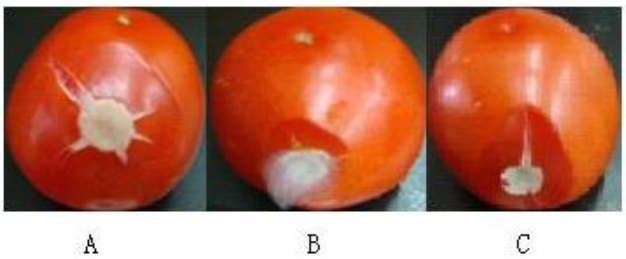

Fig 4. The efficacy of *Torreya grandis* 'Merrilli' aril oil against *Alternaria solani* in tomato fruits; A. Control; B. *T. grandis* 'Merrilli' aril oil (2 mg mL^{-1}); C. Carbendazim (2 mg mL^{-1})

added to the controls. Cultures were obtained by transplanting mycelium disks (diameter, 7 mm) from a single culture in stationary phase. They were then incubated at 28±1 ^{o}C on PDA until the logarithmic phase of growth was reached. Subsequently, the cultures (mycelium disks, diameter, 7 mm) were transferred to Petri plates with medium containing the compounds diluted to the above-mentioned final concentrations. From this moment, the diameter of growth was measured after 3 days. All data are averages from three determinations in three independent experiments. The growth inhibition rate was calculated by the following equation:

inhibition（%）=(1-*D*a/*D*b) ×100

where, *D*a is the mean of three replicates of hyphal extension (mm) of plates treated and *D*b is the mean of three replicates of hyphal extension (mm) of controls.

Effect of aril extracts on spore germination

We set up a novel, more appropriate technique to count the number of colonies formed within 24 h. This assay was undertaken using a modified method described earlier (Cotoras et al., 2004; Mitani et al., 2001). 20 µL aliquots of conidial suspensions were plated on a 1% agar flake (10 mm in diameter) mounted with different concentrations (0, 0.4, 0.8, 1.2, 1.6 and 2 mg mL^{-1}) of oil, and then incubated in petri dishes at 28±1 ^{o}C. After 24 h of incubation, germination rates were assessed by observing approximately 200 spores per treatment replicate under a light microscope. Spore was considered germinated when the length of the germ tube was equal to or greater than the length of the spore. The experiment was conducted in a completely randomized design, and the test was repeated three times. The spore germination inhibition rate was calculated by the following equation: percentage of spore germination（%） = (the number of spore germination / the total number of spores) ×100

spore germination inhibition rate（%）= (C-T)/C×100

where C is the mean of three replicates of spore germination percentage of controls and T is the mean of three replicates of spore germination percentage of treated with essential oil and *T. grandis* 'Merrilli' aril extract.

In vivo antifungal assays

Effect of the T. grandis* 'Merrilli' *aril oil on the ability of Pseudoperonospora cubensis to colonize cucumber leaves

This assay was performed using a modified method described earlier (Ribera et al., 2008; Ghaouth et al., 1992). Detached cucumber (*Cucumis sativus* L.) leaves were disinfected with 10 % sodium hypochlorite for 10 min, washed three times with sterile distilled water, and placed in Petri dishes containing water–agar (1% agar). The commercial fungicide Carbendazim (dissolved in distilled water) was used to compare activity of essential oil and distilled water as a control. The test solution (oil at a final concentration of 2 mg mL^{-1}) was sprayed on the leaf surface with a small spray bottle. Subsequently, treated leaves were inoculated with 0.7 cm agar disks with thin mycelium of *Pseudoperonospora cubensis*. Petri dishes were incubated at 28±1 ^{o}C in the dark. After 3 days of incubation, the diameter of damaged area on cucumber leaves was measured. Each treatment was replicated thrice.

Effect of the T. grandis* 'Merrilli' *aril oil on the ability of Alternaria solani to infect tomato fruits

This assay was performed using a previously described method with some modifications (Wu and Zheng, 2007; Vitoratos et al., 2013). The commercial fungicide of Carbendazim (dissolved in distilled water) was used as positive control. Fruits were sprayed with the test solution (oil at a final concentration of 2 mg mL^{-1}). Subsequently, 0.7 cm agar disks with thin mycelium of *A. solani* were inoculated on the wound of each treated fruit. The inoculated fruits were incubated in a humid chamber at 28±1 ^{o}C in darkness. After 3 days of incubation, the damaged area was measured. Each treatment was replicated thrice.

Statistical analysis

Each treatment was performed in three replicates. Statistical analysis was performed using SPSS 11.5. EC50 was defined

as dilution of the extract that results in at least 50% of the growth inhibition rate. Probability unit regression methods were used to analyze linear regression and EC50. For variance and linear regression analyses, a logarithmic transformation was applied for extract concentration (X). And the probability transformation was applied for the growth inhibition percentage (Y).

The rate of antifungal activity *in vivo* were determined by Student's t-test. Differences at $P<0.05$ were considered significant.

Conclusions

T. grandis 'Merrilli' aril oil and organic extracts have not been previously studied for their antifungal activity. Our results indicate that compared to standard fungicides, the oil and organic extracts extracts have a potentially better *in vitro* and *in vivo* antifungal activity. After more in depth analysis, *T. grandis* 'Merrilli' aril oil and organic extracts could therefore be applied as alternative natural fungicides for use in agriculture. Although further study is needed to fully understand their mechanism of action, *T. grandis* 'Merrilli' aril can serve as an important constituent in products against phytopathogens. In this regard, we have started a program aimed at the evaluation of antifungal activity of essential oil and various organic extracts (petroleum ether, chloroform, ethyl acetate, and n-butyl alcohol) of *T. grandis* 'Merrilli' aril in hope to find new natural products to be used in the control of economically important plant pathogenic fungi. Further studies will involve a detailed assessment to test their safety and efficacy against a broad range of fungal pathogens.

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