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FOUR SOLVENT EXTRACTION OF *CINNAMOMUM CAMPHORA* XYLEM AND ANALYSIS OF THE ANTI-FUNGAL ACTIVITY OF THE EXTRACTIVES

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ABSTRACT

Four solvents including distilled water, acetone, ethyl acetate and petroleum ether were used to extract xylem of *C. camphora*. The differences in chemical compounds of xylem of *C. camphora* were analyzed by gas chromatography/mass spectrometry (GC/MS) and the anti-fungal activity of *C. camphora* extractives on *Coriolus versicolor* (CV), *Trametes versicolor* (TV), *Poria vaporari*a (PP) and *Gloeophyllum trabeum* (GT) were tested. The result showed that the chemical composition and relative content of the four different solvent extracts

were different. A large number of chemical compounds in the *C. camphora* extractives had a variety of biological activity and certain application value. The growth inhibitory rates of ethyl acetate extracts of *C. camphora* on PP, CV, TV and GT were 52.24%, 52.51%, 43.26%, and 54.63%, respectively. According to the concentration for 50% of maximal effect, the inhibitory order on test fungus were GT > PP > CV > TV.

KEYWORDS: *Cinnamomum camphora* extracts, Chemical compound, Soxhlet extraction, Growth inhibitory rates.

INTRODUCTION

It is generally believed that the chemical composition of the decay resistance wood determines the degree of toxicity to wood rot fungi. Extracting anti-fungi compounds with low toxicity to humans, high anti-fungal efficiency and good environmental compatibility from plant as preservative is a new approach to the research and development in the field of wood preservation. *Cinnamomum camphora* (L.) Presl is a highly decay resistance tree species, so the research and development of extracts from *C. camphora* xylem is of a great significance to wood protection. The bioactivity compounds such as camphor oil, which can be extracted from the bark, xylem, root and leaves of *C. camphora*, have the function of resisting to fungus, bacteria and insects (Chen et al. 2018). Current research showed that all kinds of wood species have nature stress response to outside stimulus in long term natural evolution process, which is due to the difference in biological organization, structure, chemical compounds and content caused by the long-term accumulation, hence the grade of natural decay resistance is different. Frizzo et al. (2000) reviewed the resource distribution, chemical compounds of leaves, bark and stem of camphor tree, but did not mention the extraction difference of different solvents from *C. camphora*. The research and application of plant extracts mainly focus on the filed of food preservation (Hao et al. 1998, Ahmadi et al. 2010, El-Nagerabi et al. 2012, Prakash et al. 2012) and antibacterial agents (Lima et al. 1993, Jamalian et al. 2012, Mishra et al. 2012). Ghaedi et al. (2015) also extend the research about natural plant-derived extracts to interdisciplinary (Scalbert et al. 1998), including life sciences, biology and chemistry (Sansone et al. 2014). With the popularity of the environmental concept, more and more scholars began to pay attention to the use of plant extracts on wood preservatives. At present, plant-derived preservatives have an obstacles to large-scale promotion and application due to the complex of separating and extracting from wood. Therefore, it is necessary to deeply analyze the effective anti-fungal compounds and anti-fungal mechanism of wood decay (Chen et al. 2013, Kumari et al. 2015), to lay the foundation for further industrial production and application of *C. camphora* extract as wood preservative. Furthermore, it is equally important to study on extracts from the xylem of *C. camphora*, which will help to get a comprehensive and in-depth understanding of the content of the chemical compositions and discussing its utilization value. This research is of great significance for the research, development, application and promotion of *C. camphora* extractives as a preservative and similar plant-derived extracts as raw material products.

MATERIAL AND METHODS

Material, reagent and equipment

C. camphora wood was 40-50 years old and was collected from the streets of Fuzhou, China. It was crushed with a grinder and filtered through a 40-mesh sieve to obtain test materials. Brown rot fungi (*Poria vaporari*a, *Gloeophyllum trabeum*) and white rot fungi (*Coriolus versicolor*, *Trametes versicolor*) were obtained from Fujian agriculture and forest university (Fuzhou, China) and were used for the anti-fungal test. Acetone, ethyl acetate and petroleum ether were analytically pure purchased from Shanghai pilot chemical corporation. Agilent 5975C/7890N gas chromatography mass spectrometer was manufactured by American Agilent company.

Extraction of *C. camphora*

The xylem of *C. camphora* was pulverized (40-60 mesh) after air drying. 50 g of *C.camphora* powder was placed in a heat reflux extraction. Distilled water, acetone, ethyl acetate and petroleum ether were used as solvents, respectively. The solvents were heated to their respective boiling point temperature and heated for 2 h with a ratio of solid/solvent ratio of 1:10 ($g \cdot mL^{-1}$), then the extractives filtered to obtain the extracted liquid. The solvent was recovered by distillation under reduced pressure, and the *C. camphora* extracts were obtained at the same time. The extracts were collected and stored in labeled brown sample bottles. The extracts obtained were analyzed by gas chromatography/mass spectrometry (GC/MS) and used for anti-fungal test.

GC/MS conditions

Chromatographic conditions: DB-17MS (30 m × 0.25 mm × 0.25 μm) column, 99.999% high purity helium carrier gas, flow rate 1.0 $mL \cdot min^{-1}$, injection volume 0.8 μL, split ratio 10:1, the inlet temperature was 280°C. Program temperature rise: 50°C (maintain 3 min), 20°C·min^{-1} rise to 80°C (maintain 3 min); 2°C·min^{-1} rise to 120°C (maintain 5 min); 1.5°C·min^{-1} rise to 130°C (maintain 5 min); 2°C·min^{-1} rose to 180°C (hold for 5 min); 5°C·min^{-1} rose to 250°C (hold for 5 min).

Mass spectrometry conditions: EI ionization source, electron energy 70 eV; ion source temperature 230°C; scan mass range: 30-600 amu; solvent delay 3 min. The mass spectra were searched using the NIST library, and compared with the manual spectrum analysis and the related literature.

Anti-fungal test

Coriolus versicolor (CV), *Trametes versicolor* (TV), *Poria vaporari*a (PP) and *Gloeophyllum trabeum* (GT) were tested by growth rate of poison medium culture method. The medium containing molten maltose in a 9 cm petri dish were added with a certain amount of extracts and mixed to obtain the final concentration of 0.5 $g \cdot L^{-1}$, 1.0 $g \cdot L^{-1}$, 1.5 $g \cdot L^{-1}$, 2 $g \cdot L^{-1}$, 2.5 $g \cdot L^{-1}$. All kinds of fungal were incubated medium at 28 ± 2°C, 75-85% relative humidity for

several days to mycelia get to petri dishes edge. All tests were performed in triplicate. The effective dose for 50% inhibition (EC_{50}) was calculated by profit analysis. The growth inhibition rates was calculated as follows:

$$\text{Growth inhibition rates (\%)} = \frac{D_0 - Dt}{D_0} \times 100\% \quad (1)$$

where: D_0 was blank control colony growth diameter with an average of three replications (mm), and D_t was preservative treatment colony growth diameter with an average of three replications (mm).

RESULTS AND DISCUSSION

The compound identification and analysis of extracts from *C. camphora* xylem

Total ion chromatogram of the water and acetone extracts of *C. camphora* xylem separated and analyzed by GC/MS was shown in Fig. 1. 41 compounds were separated from the water extracts, and 6 compounds were identified, which accounted for 11.65% of the total peak area. Among these compounds, three of them had mass fraction than 1%, and included 2-pinen-4-one (2.26%), 2,6-dimethoxyphenol (2.07%), di-2-ethylhexyl phthalate (5.34%). 208 compounds were separated from the xylem acetone extracts, and 23 compounds were identified, which accounted for 30.56% of the total peak area (Tab. 1). The compounds with a mass fraction than 1% included camphor (5.18%), tetradecanal (4.16%), myristic acid (1.48%), pentadecanoic acid (1.25%), di-2-ethylhexyl phthalate (3.55%), γ-sitosterol (3.88%), and cyclotetracosane (1.47%) (Silan et al. 2018).

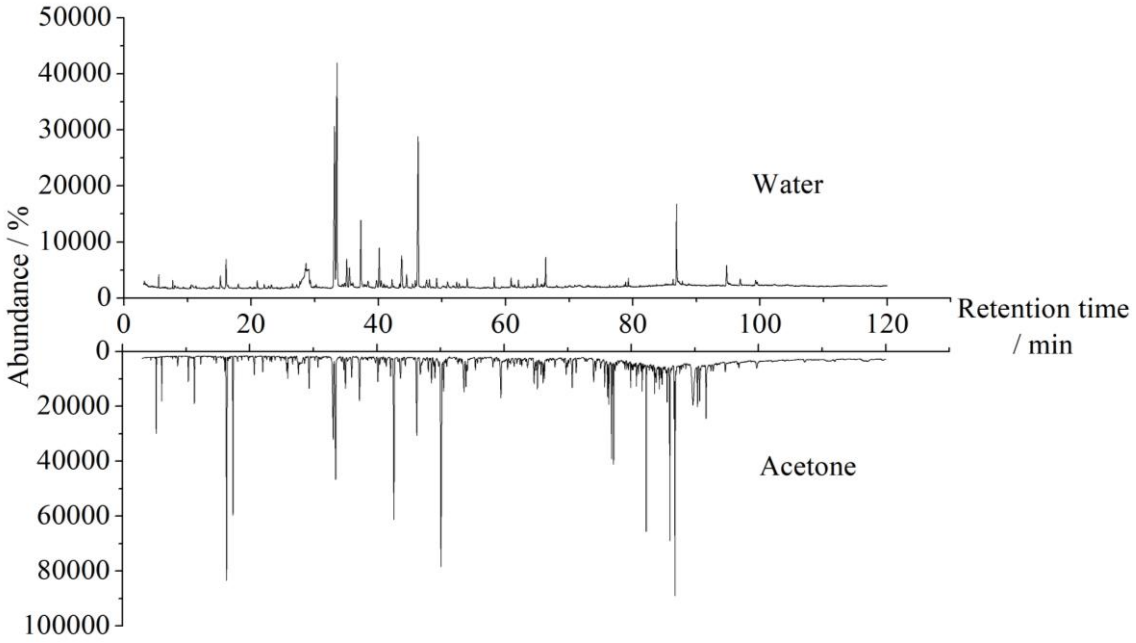

Fig. 1: Total ion chromatogram of the water and acetone extracts of C. camphora xylem.

Tab. 1: Volatile compounds and their relative area of peak extracted by water and acetone solvent extraction.

No.	Compounds	Water			Acetone		
		Retention time (min)	Relative content (%)	Matching rate (%)	Retention time (min)	Relative content (%)	Matching rate (%)
1	(1-Methylethyl)benzene	-	-	-	6.173	0.55	94
2	Linalool	-	-	-	11.315	0.91	92
3	Guaiacol	15.17	0.85	94	-	-	-
4	2-Pinen-4-one	16.076	2.26	78	-	-	-
5	Terpinen-4-ol	-	-	-	16.089	0.27	96
6	(1r,4r)-(+)-Camphor	-	-	-	16.374	5.18	98
7	1,8-Epoxy-p-menthan-2-ol	-	-	-	20.733	0.49	95
8	1,7-Dimethyl-7-(4-methyl-3-pentenyl)-tricyclo[2.2.1.0(2,6)]heptane	-	-	-	25.831	0.36	98
9	Safrole	-	-	-	25.979	0.41	98
10	L-Threitol	28.515	0.33	93	-	-	-
11	Heptadecanal	-	-	-	34.816	0.39	94
12	2,6-Dimethoxyphenol	35.475	2.07	94	-	-	-
13	d-Cadinene	-	-	-	36.045	0.61	95
14	Tetradecanal	-	-	-	42.688	4.16	99
15	2-Isopropyl-5-methyl-9-methylene–bicyclo[4.4.0]dec-1-ene	-	-	-	48.594	0.72	95
16	2-Pentadecanone	-	-	-	49.137	0.61	94
17	Myristic acid	-	-	-	53.704	1.48	99
18	4-Allyl-2,6-dimethoxyphenol	-	-	-	54.021	0.78	83
19	3,4,5-Trimethoxyphenol	58.238	0.8	86	-	-	-
20	Pentadecanoic acid	-	-	-	59.513	1.25	99
21	Palmitic acid	-	-	-	64.727	0.82	96
22	Palmitic Acid Vinyl Ester	-	-	-	65.257	0.7	87
23	1-Heptadecene	-	-	-	70.717	0.87	93
24	1-Pentadecanol	-	-	-	71.344	0.39	95
25	Linoleic acid	-	-	-	75.827	0.71	99
26	Di-2-ethylhexyl phthalate	86.882	5.34	98	86.882	3.55	98
27	γ-Sitosterol	-	-	-	89.708	3.88	95
28	Cyclotetracosane	-	-	-	91.766	1.47	96

Note: Identification was achieved using computer to match the mass spectra with the NIST library.

Total ion chromatogram of the ethyl acetate and petroleum ether extracts of *C. camphora* xylem separated and analyzed by GC/M was shown in Fig. 2. 46 compounds were separated from the ethyl acetate extracts, and 20 compounds were identified, which accounted for 48.72% of the total peak area (Tab. 2). Compounds with high content included di-2-ethylhexyl phthalate (6.04%), tetradecanal (5.32%), eucalyptol (1.18%), linalool (3.45%), terpinen-4-ol (1.02%), camphor (13.85%), safrole (1.48%), (-)-b-santalene (1.41), d-cadinene (1.03%), myristic acid (1.53%), pentadecanoic acid (1.49%), cyclopentadecane (1.19%), γ-sitosterol (3.18%), 1-triacontanol,and 1-acetate (1.82%), etc. 30 compounds were separated from the ethyl acetate extracts, and 14 compounds were identified, which accounted for 49.29% of the total peak area

(Tab. 2). Compounds with high content included linalool (2.8%), camphor (6.60%), 1,7-dimethyl-7-(4-methy-3-pentenyl) tricyclo (1.69%), safrole (1.8%), (-)-b-santalene (1.64%), d-cadinene (1.88%), myristic acid (1.49%), 2-isopropyl-5-methyl-9-methylene-bicyclo[4.4.0] dec-1-ene (1.36%), pentadecanoic acid (1.82%), cyclopentadecane (1.58%), γ-sitosterol (6.25%), α-terpineol (6.97%), cyclotetracosane (1.24%), and cyclooctacosane (2.17%), etc.

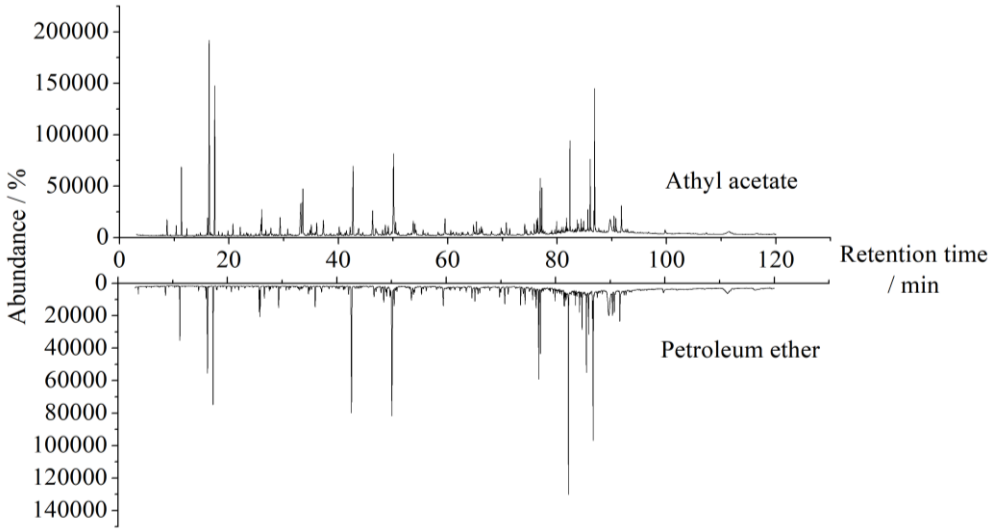

Fig. 2: Total ion chromatogram of the xylem ethyl acetate and petroleum ether extracts of C. camphora.

Tab. 2: Volatile components and their relative area of peak extracted by ethyl acetate and petroleum solvent extraction.

No.	Compounds	Ethyl acetate			Petroleum ether		
		Retention time (min)	Relative content (%)	Matching rate (%)	Retention time (min)	Relative content (%)	Matching rate (%)
1	Eucalyptol	8.682	1.18	99	-	-	-
2	Linalool	11.321	3.45	96	11.309	2.8	97
3	Terpinen-4-ol	16.108	1.02	97	-	-	-
4	(1r,4r)-(+)-Camphor	16.393	13.85	98	16.367	6.6	98
5	1,3,3-Trimethyl-2-oxabicyclo[2.2.2]octan-6-ol	20.74	0.9	97	-	-	-
6	1,7-Dimethyl-7-(4-methyl-3-pentenyl)-tricyclo[2.2.1.0(2,6)]heptane	25.843	0.77	99	25.831	1.69	99
7	Safrole	25.992	1.48	98	25.986	1.8	98
8	(-)-b-Santalene	29.362	1.41	94	29.375	1.64	96
9	d-Cadinene	36.057	1.03	96	36.051	1.88	96
10	Tetradecanal	42.701	5.32	99	42.701	10.49	99
11	2-Isopropyl-5-methyl-9-methylene-bicyclo[4.4.0]dec-1-ene	48.607	0.82	93	48.594	1.36	94
12	Myristic acid	53.717	1.53	99	-	-	-
13	Pentadecanoic acid	59.526	1.49	99	59.493	1.82	99
14	Palmitic acid	64.733	0.83	99	-	-	-
15	Palmitic acid vinyl ester	65.263	0.73	91	-	-	-
16	Cyclopentadecane	70.729	1.19	94	70.73	1.58	91
17	Linoleic acid	75.833	0.68	99	-	-	-

18	Di-2-ethylhexyl phthalate	86.888	6.04	98	-	-	-
19	γ-Sitosterol	89.715	3.18	99	89.76	6.25	97
20	α-Terpineol	-	-	-	17.389	6.97	87
21	Cyclotetracosane	-	-	-	86.746	1.24	96
22	1-Triacontanol,1-acetate	91.772	1.82	95	-	-	-
23	Cyclooctacosane	-	-	-	91.766	2.17	94

*Identification was achieved using computer matching of the mass spectra with the NIST library.

The results of GC/MS identification and analysis of *C. camphora* xylem extracts extracted by four solvents showed that the main compounds of the extracts extracted by water and acetone were similar to those of essential oil extracted from *C. camphora* leaves extracts in Australia by Stubbs et al. (2004), which were made up of camphor and various phenols. The main compounds of the extracts extracted by ethyl acetate and petroleum ether were similar to compounds of essential oil extracted from *C. camphora* leaves extracts by Guo et al. (2016), but the content was different. Pragadheesh et al. (2013) identified and classified the chemical components of essential oil obtained from *C. camphora* by steam distillation, and identified the components with certain biological activities, including linalool, safrole, camphor, α-pinene, β-phellandrene, 1,8-cineole, eugenol, etc., and concluded that *C. camphora* essential oil had strong fumigation and contact toxicity. The compounds were similar to the compounds of extracts in this experiment, which could indicate that *C. camphora* essential oil and active ingredients had the potential as nature fumigants and insecticide.

Classification analysis of extracts

It could be seen from Fig. 3 that the four extraction solvents had different effects on the extraction of various compounds in the *C. camphora* xylem (Chen et al. 2020).

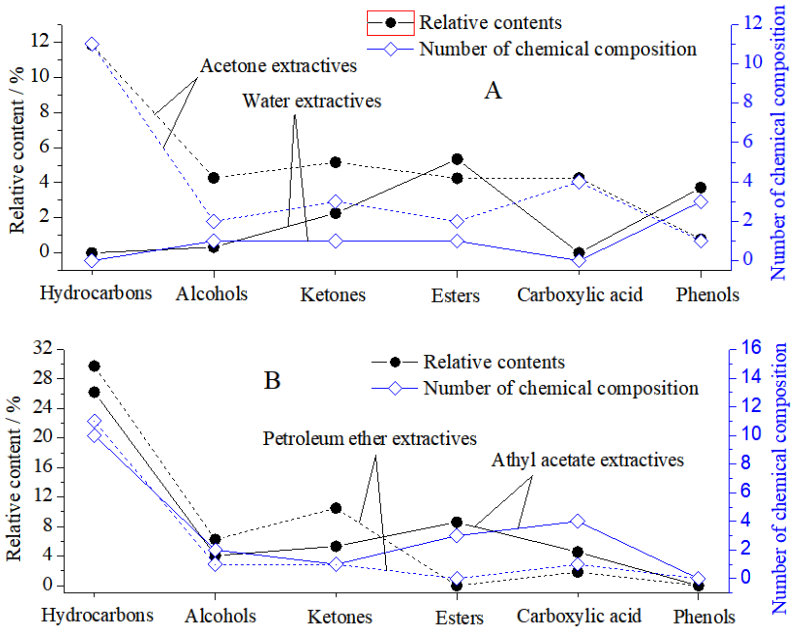

Fig. 3: Relative contents and number of chemical composition of extracts from the of C. camphora xylem by different solvent: a) water and acetone, b) ethyl acetate and petroleum ether.

The relative contents of category was shown in Fig. 3a. Water had a good effect on the extraction of esters and phenols, but it had a poor effect on hydrocarbons and carboxylic acids. The extracts mainly included alcohol (0.33%), aldehyde and ketones (2.26%), esters (5.34%), and phenols (3.72%). Using acetone as solvent to extract camphor xylem could obtain hydrocarbons (11.84%), alcohols (4.27%), ketones (5.16%), esters (4.25%), carboxylic acids (4.26%) and phenols (0.78%). The relative content could be seen from Fig. 3b. Ethyl acetate had the best effect on the extraction of hydrocarbons, while had the worst effect on ketones and phenols. Petroleum ether had a good effect on the extraction hydrocarbons and ketones, but had a poor effect on extraction of esters and phenols.

A comprehensive comparison of Fig. 3, Tab. 1 and Tab. 2 on the classification of *C. camphora* xylem extract can be found. Although water as a solvent was less effective than ethyl acetate and petroleum ether in extracting camphor, its extract had a relatively high phenolic content. Phenolic compounds are easily combined with wood rot fungal proteins at low concentrations to denature them, and can cause protein precipitation at high concentrations (Maciejewicz et al. 1999). Therefore, it can achieve a good pharmacologic effects of anti-fungi. Acetone had a good effect on the extraction of hydrocarbons and ketones, but it had a poor effect on phenols. The main hydrocarbons were terpenes, accounting for 7.46%, including camphor (5.18%), linalool (0.91%), terpinen-4-ol (0.27%), and d-cadinene (0.61%), 1,8-epoxy-p-menthan-2-ol (0.49%), and other terpenoids. Part of the chemical compounds have insecticidal, antipruritic, antibacterial and antiseptic effects (Lee et al. 2006). Ethyl acetate and petroleum ether both can be used as solvent to extract linalool, camphor, safrole, (-)-b-santalene, d-cadinene, myristic acid, pentadecanoic acid, cyclopentadecane, γ-sitosterol and other substances from *C. camphora* xylem. The most abundant component was camphor which has antibacterial and anti-inflammatory effects (Li et al. 2013). Myristic acid is used to prepare food flavors such as fat and milk, and γ-sitosterol can be used to treat diabetes (Balamurugan et al. 2011).

The inhibitory effect of the extracts against wood rot fungi

It could be seen from Fig. 4 of the growth inhibition ration results that the ethyl acetate extracts of *C. camphora* showed different degrees of inhibitory effects on the four wood rot fungi. At a concentration of 2.5 $g \cdot L^{-1}$, the growth inhibition rates of *C. camphora* extractives on PP, CV, TV and GT were 52.24%, 52.51%, 43.26%, and 54.63%, respectively. From the overall trend, the inhibitory effect of ethyl acetate extracts of *C. camphora* against wood rot fungi was positively correlated with the concentration, and the growth inhibition ration became larger as the concentration increases (Breda et al. 2016).

The toxic regression equation of ethyl acetate extracts of *C. camphora* on the growth of wood rot fungal mycelium was shown in Tab. 3. The concentration for 50% of maximal effect (EC_{50} value) of ethyl acetate extracts of *C. camphora* to wood rot fungus were 2.07-4.72 $g \cdot L^{-1}$. According to the EC_{50} value, the inhibitory order were GT > PP > CV > TV. It could be seen that the inhibitory effect of ethyl acetate extracts of *C. camphora* against brown rot fungi was better than that of on white rot fungi (Li et al. 2014, Zhang et al. 2020).

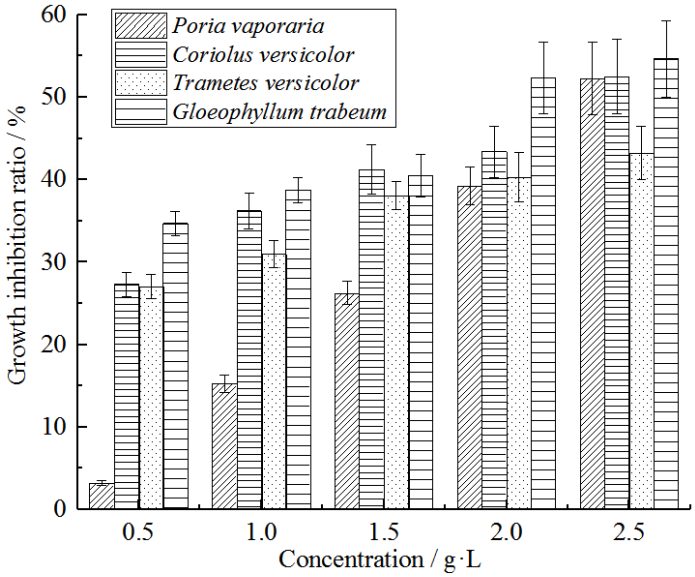

Fig. 4: Inhibitory activity of C. camphora extractives against wood rot fungi.

Tab. 3: Toxicity of C. camphora extracts against wood rot fungi.

Fungi	[a]Toxic regression equation	R^2	[a]EC_{50} /g·L^{-1}
Poria vaporaria	y = 2.6697x + 3.9474	0.9971	2.48
Coriolus versicolor	y = 0.8693x + 4.644	0.9544	2.57
Trametes versicolor	y = 0.6534x + 4.5598	0.9674	4.72
Gloeophyllum trabeum	y = 0.7414x + 4.7657	0.8331	2.07

Note: [a] Average of three replications.

CONCLUSIONS

There were some differences in the total number of compounds and relative contents of each solvent extract from *C. camphora* xylem. Compared with the *C. camphora* extracts all over the world, it was found that the origin of the *C. camphora* had little effect on its main compounds, but had a large effect on the proportion of its compounds. GC/MS analysis of the four solvent extracts of *C. camphora* xylem identified terpenoids and phenols with strong insect repellent, bacteriostatic and antiseptic effects. There were higher effect for the brow-rot fungi (GT and PP) as for the white-rot fungi (CV and TV). The research provides guidance for further development of biotic wood preservatives.

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