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THE PHYSIOLOGICAL AND BIOCHEMICAL MECHANISMS OF *CINNAMOMUM CAMPHORA* XYLEM EXTRACTS INHIBIT WOOD-DECAY FUNGI

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ABSTRACT

The present study investigated the physiological and biochemical mechanism of extracts derived from *Cinnamomum camphora* (L.) Presl. The methanol and chloroform extracts of *C. camphora* xylem exhibited inhibitory activity against oxygen consumption in *Coriolus versicolor* and *Gloeophyllum trabeum*. The inhibitory effect of cellulose secreted by *G. trabeum* was concentration-dependent. The application of the ethyl acetate extracts of *C. camphora* xylem on the *G. trabeum* hyphae resulted in an improvement in electric conductivity, which followed a concentration-dependent fashion. Protein permeability increased with higher concentrations of the ethyl acetate extracts of *C. camphora* xylem. This research provided theoretical basis for understanding of the physiological and biochemical mechanisms of *C. camphora* extracts inhibit wood-decay fungi and the development of natural extracts as wood preservatives.

KEYWORDS: *Coriolus versicolor*, *Gloeophyllum trabeum*, *Cinnamomum camphora*, oxygen consumption, electric conductivity, protein permeability.

INTRODUCTION

Wood decay caused by fungal infection severely reduces the quality of timber including its main physical and mechanical properties, as well as influences the turnout rate and service

life of wood products, thereby resulting in huge economic losses every year (Kahl et al. 2017). Traditional wood preservatives such alkaline copper quaternary (ACQ) and chromium copper arsenic (CCA) contain toxic compounds that may cause environment pollution (Janin et al. 2011, Mehmet et al. 2009, Palanti et al. 2009), therefore, new types of wood preservatives are currently in high demand. Recent studies have focused on anti-bacterial and anti-corrosion performance of plant extracts because these are generally environment friendly (Syehee et al. 2010), efficient broad-spectrum (Suhua et al. 1973), and targeted antimicrobial therapy (Schubert et al. 2008, Susi et al. 2011). *Cinnamomum camphora* (L.) Presl. (*C. camphora*) is one of the most important hardwood species indigenous to China (Roszaini et al. 2013). *C. camphora* is a strong decay resistance wood species. It has decay resistance performance due to its extracts has a significant anti-fungal activity and has been applied in the antiseptic field. More and more studies have shown that the extractives of *C. camphora* has anti-bacterial, anti-inflammatory, desinsectization and other effects (Li et al. 2014, Liu et al. 2018). It has good prospect in development and application for the extracts from the xylem of *C. camphora*.

The research and development of *C. camphora* extractives usually focuse on evaluating the decay resistance performance of *C. camphora* extractives by wood preservation test, as well as their anti-microbial and insecticidal performance (Guo et al. 2016, Lee et al. 2006, Li et al. 2018). Reporting the volatile ingredients isolated from a fresh tree of *C. camphora* (camphor tree) and 98.68% of the constituents consisting 24 components from the tree (Miyazawa et al. 2001). However, the mechanism of the *C. camphora* xylem extractives inhibits *Gloeophyllum trabeum* (*G. trabeum*) and *Coriolus versicolor* (*C. versicolor*) is unknown up to now (Kerem et al. 1999, Zhang et al. 2016). In order to reveal the relation between secondary metabolite of *C. camphora* and wood-decaying fungus. Author think that it is necessary to comprehensively investigate the physiological and biochemical mechanisms of *C. camphora* extractives in wood preservatives from the reality needs of wood preservation and protection (Bhardwaj et al. 2012, Kumari et al. 2015).

In this study, the effect of *C. camphora* xylem extracts on *G. trabeum* and *C. versicolor* oxygen consumption, cellulase activity (Leskinen et al. 2015, Matsubara and Kuroda 2008), electrical conductivity, and leakage protein content was investigated to explore the physiological and biochemical mechanisms of *C. camphora* extracts against wood-decay fungus and their possible targets (Li et al. 2016, Voda et al. 2003). This paper provided theoretical basis for understanding of the anti-fungal mechanism of *C. camphora* xylem extractives against wood-decaying fungus and the development of natural extracts as wood preservatives (Schultz et al. 2007).

MATERIAL AND METHODS

Materials

The xylem of *C. camphora* was pulverized and 100 g of the pulverized powder was placed at the bottom of a round-bottom flask and used in heat reflux extraction. Hot water, methanol, acetone, ethyl acetate, and chloroform were used as solvents, respectively. The extraction temperature of each solvent was their respective boiling point temperature. The raw materials were extracted for twice, first heated for 5 h at the ratio of 1 : 10 ($g \cdot m^{-1}L^{-1}$), then heated for 3 h at the ratio of 1 : 5 ($g \cdot m^{-1}L^{-1}$). The products of the two extractions were mixed and filtered to obtain a liquid extract. The solvents were retrieved by reduced pressure distillation, and the *C. camphora* extracts were dissolved in the respective solvents at desired concentrations and stored at 4°C for uses.

Sample preparation

Brown-rot fungus (*G. trabeum*) was provided by the College of Life Science of Fujian Agriculture and Forestry University; White-rot fungus (*C. versicolor*) was provided by China Forestry Culture Collection Center. PS (potato and sucrose) medium: potato, 200 g; sucrose, 20 g; and distilled water, 1,000 mL.

Experiment reagents

Coomassie brilliant blue (CBB) G-250 staining, bovine serum albumin (FBS) medium, DNS medium, 3,5-dinitrosalicylic acid medium, acetic acid (HAc), sodium acetate, HAc-sodium acetate buffer solution, glucose standard solution, and 1% hydroxymethyl cellulose (CMC) solution. These solutions and reagents were all produced by China National Pharmaceutical Group (Sinopharm).

Main instruments and devices

Laminar flow cabinet: HT-840.U, Suzhou Antai Airtech Co., Ltd. (Suzhou, China). Incubator: HHS.250B, Nanjing Hengyu Instrument Equipment Manufacture Co., Ltd. (Nanjing, China). Magnetic stirrer: 79HW-1, Jintan Ronghua Instrument Manufacture Co., Ltd. (Nanjing, China). Bacteria shaker: ZHWY-211B, Shanghai Zhicheng Instrument Manufacture Co., Ltd. (Shanghai, China). Micro-barometer: DMPY-2C, institute of applied physics of Nanjing University (Nanjing, China). UV spectrophotometer: UV-1100, Shanghai Mapade Instruments Co., Ltd. (Shanghai, China). Conductometer: 3173-COND, Shanghai Jenco Co., Ltd. (Shanghai, China). Oxygen consumption measurement instrument (homemade: magnetic stirrer, DP-AW micro-barometer, and water bath).

Wood-decay fungus aerobic respiration measurement

NaOH can absorb the CO_2 generated by wood-decay fungus during respiration. In a closed environment and under constant volume and temperature, changes in pressure are indicative of CO_2 generation or consumption, and thus alterations in pressure can be used to calculate the respiration rate of wood-decay fungus. The instrument used for measuring oxygen consumption is shown in Fig. 1.

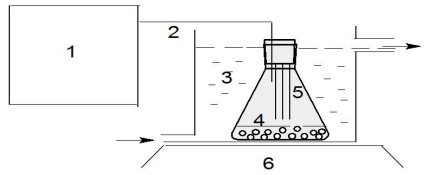

Fig. 1: Device of respiration determination: 1. Micro-pressure difference, 2. Gas catheter, 3. Constant temperature bath, 4. Mycelium suspension, 5. Gauze (soaked with 10% NaOH), 6. Magnetic stirrer.

A sterile culture medium was prepared in a 100-mL Erlenmeyer flask and five different concentrations were included: 0.2 $g{\cdot}L^{-1}$, 0.4 $g{\cdot}L^{-1}$, 0.6 $g{\cdot}L^{-1}$, 0.8 $g{\cdot}L^{-1}$, and 1 $g{\cdot}L^{-1}$. In each culture flask, five pieces of a 7 mm fungus cake were inoculated. A piece of gauze soaked with 10% NaOH was placed at the opening part of the flask to absorb CO2. The flasks were incubated at 28°C in a water bath and placed on a magnetic stirrer (stirring for 15 min). Then, the flasks were connected to a microbarometer. Once the reaction was stable, the measuring instrument was reset to zero. The pressure inside of the flask was recorded every 10 min for a total of 160 min. During

the measurement, the same flask and microbarometer were used to calculate the respiration rate under different medium concentrations.

Cellulase activity measurement

Under alkaline conditions, 3,5-dinitrosalicylic acid (DNS) reacts with reducing sugars to form 3-amino-5-nitrosalicylic acid. 3-amino-5-nitrosalicylic acid presents a red-brown color under boiling conditions, and the color is proportional to the amount of reducing sugars. Therefore, a colormetric method can be used to measure reducing sugar content. Moreover, DNS reacts with a broad range of reducing sugars, not only with specific groups. Thus, DNS is suitable for measuring the amount of sugar that is reduced from a polysaccharide (cellulose, hemicellulose, and starch) hydrolysis reaction (Dashtban et al. 2010).

G. trabeum hyphae were incubated in the stove until the fast growing stage (about 15 days, wherein cellulose secretion is at the highest level). The collected hyphae were vacuum filtrated and the obtained filtrate was used for enzyme activity measurement.

G. trabeum leakage protein content measurement

Coomassie brilliant blue (CBB) was used to measure *G. trabeum* leakage protein content after treatment with the *C. camphora* xylem extracts. CBB G-250 is red when in an unbound state and has a maximal absorption value at a wavelength of 488 nm, its bonds with proteins and forms a complex. The complex presents bright blue color and has a maximal absorption value at a wavelength of 595 nm. Moreover, the absorption value is proportional to the amount of proteins, thus CBB G-250 can be used for protein content measurement. *G. trabeum* was treated with ethyl acetate extracts of *C. camphora* xylem for 24 h. Different concentrations of ethyl acetate extracts were included: 0.2 $g{\cdot}L^{-1}$, 0.4 $g{\cdot}L^{-1}$, 0.6 $g{\cdot}L^{-1}$, 0.8 $g{\cdot}L^{-1}$, and 1 $g{\cdot}L^{-1}$. After ethyl acetate extracts of *C. camphora* xylem treatment, *G. trabeum* solutions were filtered, and the filtrates were kept. A 1-mL aliquot of the filtrate was mixed with 5 mL of the CBB solution, and the mixture was incubated for 10 min. Its absorption was measured at a wavelength of 595 nm, and each treatment was repeated thrice. Protein content was calculated using the standard curve generated using albumin from bovine serum (BSA).

To generate the standard curve, albumin from bovine serum was dissolved in Milli-Q at a concentration of 100 $mg{\cdot}L^{-1}$. BSA was added to seven glass tubes (numbered from 0 – 6) and diluted to the final concentration as described in Tab. 1.

Tab. 1: The preparation table of bovine serum albumin for test.

Tube number	0	1	2	3	4	5	6
100 $mg{\cdot}L^{-1}$ BSA solution (mL)	0.0	0.1	0.2	0.3	0.4	0.5	0.6
Coomassie brilliant blue solution (mL)	5	5	5	5	5	5	5

Tube 0 was used as the blank control. The mixture was incubated for 10 min and its absorption was measured at a wavelength of 595 nm. The standard curve was generated by plotting the protein concentration as the X axis and absorption as the Y axis. Based on the standard curve, a regression equation was calculated.

After adding BSA to each tube, the reaction was incubated for 10 min, and absorption was measured. All obtained values subscribed the absorption value from tube 0. A standard curve was generated by plotting the protein concentration as the X axis and the subscribed absorption value as the Y axis. Based on the standard curve, a regression equation was calculated.

G. trabeum conductivity measurement

Cell membrane permeability of *C. camphora* xylem extract-treated wood-decay fungi was quantified by measuring the conductivity of the released electrolytes from cells and into the solutions. The higher the conductivity of the hyphae after treatment, the higher the cell membrane permeability. It is indicated that more electrolytes were released by the cells and into the solution (Tsujimoto et al. 2006). M_1 is the conductivity of hyphae after treatment. M_2 is the conductivity of the untreated hyphae.

G. trabeum was inoculated in a PS medium and incubated at 28°C for 6 days. The hyphae were vacuum-dried and washed with sterile water for several times. Then, 1 g of the hyphae was added to 30 mL of different concentrations of ethyl acetate extracts (1 $g{\cdot}L^{-1}$, 2 $g{\cdot}L^{-1}$, 3 $g{\cdot}L^{-1}$, 4 $g{\cdot}L^{-1}$, and 5 $g{\cdot}L^{-1}$). The hyphae conductivity of different samples was measured over time, and the sterile Milli-Q water group was used as the control group. The corrected conductivity was calculated using the following Eq. 1:

$$\text{Corrected conductivity} = M_1 - M_2 \quad (1)$$

RESULTS AND DISCUSSION

The effect of *C. camphora* xylem extracts on *G. trabeum* and *C. versicolor* aerobic respiration

Respiration is a central step for energy conversion and provides energy for various biological activities (Pedersen et al. 2012). In this study, the effect of *C. camphora* xylem extracts on *G. trabeum* and *C. versicolor* aerobic respiration was investigated. *C. camphora* extract-treated wood-decay fungi were kept in a sealed instrument that was kept under constant volume and temperature. CO_2 generated from aerobic respiration was absorbed by NaOH and the resulting changes in pressure were recorded. Therefore, aerobic respiration rate was calculated. The results provide additional insights on fungal hyphae physiological conditions when the cellular membrane structure and function are altered.

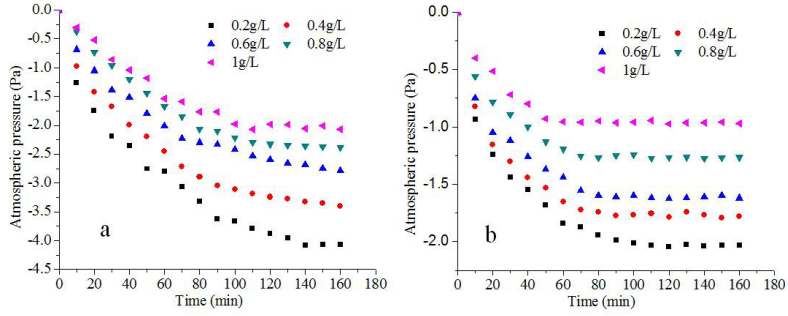

Fig. 2: Effect of G. trabeum on oxygen consumption of extracts of C. camphora: a) chloroform extracts of C. camphora, b) methanol extracts of C. camphora.

The methanol and chloroform extracts of *C. camphora* xylem inhibited *G. trabeum*i aerobic respiration (Fig. 2). With increased extract concentration, alterations in pressure decreased and reached a plateau, indicating a decrease in the aerobic respiration rate of *G. trabeum*i. Therefore,

the higher the concentration of extracts, the stronger the inhibitory effects. At the highest extract concentration (1 g·L^{-1}), the respiration of chloroform extract-treated *G. trabeum*i was arrested 100 min after treatment; however, the respiration of methanol extract-treated *G. trabeum*i was arrested 50 min after treatment. The methanol extracts of *C. camphora* xylem showed better inhibitory effects on *G. trabeum*i respiration than the chloroform extracts of *C. camphora* xylem (when the same concentration was applied) because methanol extract-treated *G. trabeum*i showed a lower level of respiratory and pressure changes.

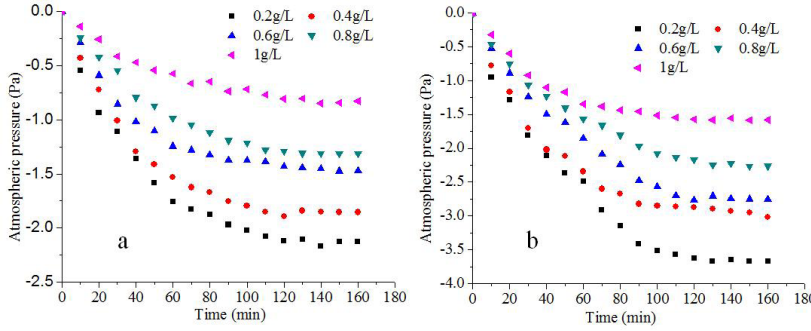

Fig. 3: Effect of C. versicolor on oxygen consumption of extracts of C. camphora: a) chloroform extracts of C. camphora, b) methanol extracts of C. camphora.

The methanol and chloroform extracts of *C. camphora* xylem also inhibited *C. versicolor* aerobic respiration (Fig. 3). When *C. versicolor* was treated with 0.2 g·L^{-1} of chloroform extracts of *C. camphora* xylem, the difference in pressure increased and reached a plateau 110 min after treatment. When the concentration was increased to 1 g·L^{-1}, the chloroform extracts of *C. camphora* xylem showed stronger inhibitory effects, and the difference in pressure was smaller and reached a plateau at 90 min after treatment. With increased concentration of the respective extract, pressure decreased at each time point and respiration was arrested at earlier time points. When the same concentration and treatment time were applied, the chloroform extracts of *C. camphora* xylem showed better inhibitory effects on *C. versicolor* respiration than the methanol extracts of *C. camphora* xylem because chloroform extract-treated *C. versicolor* showed a lower degree of change in pressure (Liu et al. 2003).

The results showed that the respiration of *G. trabeum*i and *C. versicolor* were significantly inhibited under all concentrations. With increased extract concentrations, the pressure decreased and respiration was completely arrested above certain concentrations. The results also showed that *G. trabeum*i was most sensitive to methanol extracts of *C. camphora* xylem; however, *C. versicolor* was most sensitive to chloroform extracts of *C. camphora* xylem. Both extracts significantly inhibited wood-decay fungal respiration.

The effect of ethyl acetate extracts of *C. camphora* xylem on *G. trabeum* cellulase activity

Different volumes (0 mL, 0.2 mL, 0.4 mL, 0.6 mL, 0.8 mL, and 1.0 mL) of standard glucose solution (1 mg·m^{-1}L^{-1}) were added to a 25 mL tube and distilled water was added to a final volume of 1.0 mL. In each tube, 1.5 mL of the DNS reagent was added. The tubes were first incubated for 5 min in a water bath and then cooled in running water. Distilled water was added to a

final volume of 20 mL. The absorption of each tube was measured at a wavelength of 540 nm. A standard curve was generated and is shown in Fig. 4.

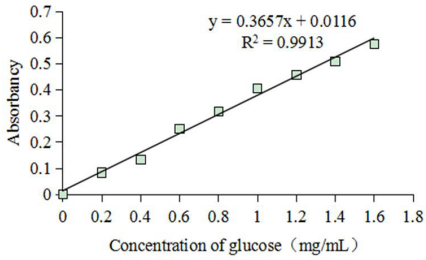

Fig. 4: Standard curve of glucose.

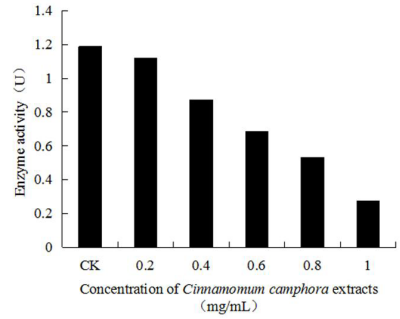

Fig. 5: The effect of extracts on the enzyme activity of cellulase of G. trabeum.

With increased concentration of ethyl acetate extracts of *C. camphora* xylem, cellulase activity of *G. trabeum*i decreased (Fig. 5). Starting from 0.2 $mg{\cdot}m^{-1}L^{-1}$, ethyl acetate extracts of *C. camphora* xylem showed significant inhibition of *G. trabeum*i cellulase activity. When the concentration reached 1 $mg{\cdot}m^{-1}L^{-1}$, the extracts showed strongest inhibition, showing > 50% inhibition of enzyme activity. These findings indicate that ethyl acetate extracts of *C. camphora* xylem can significantly inhibit *G. trabeum*i cellulase activity, which is the main functional enzyme for *G. trabeum*i to decay woods (Yu et al. 2018).

The effect of ethyl acetate extracts of *C. camphora* xylem on *G. trabeum* conductivity

Changes in the conductivity of *C. camphora* ethyl acetate extract-treated *G. trabeum* hyphae are shown in Fig. 6.

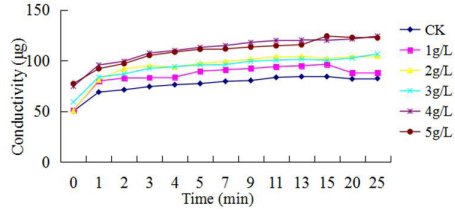

Fig.6: The effect of extracts on the cells conductivity of G. trabeum.

Compared to the control group (CK), all five concentrations of the ethyl acetate extracts of *C. camphora* xylem induced an increase in *G. trabeum* hyphae conductivity. The higher the concentration of ethyl acetate extracts of *C. camphora* xylem, the higher the *G. trabeum* hyphae conductivity. When treated with 4 $g{\cdot}L^{-1}$ or 5 $g{\cdot}L^{-1}$ of ethyl acetate extract of *C. camphora* xylem, the conductivity significantly increased. After ethyl acetate extract treatment, the intracellular contents *G. trabeum* hyphae were released, thereby resulting in an increase in conductivity; between 1 min and 7 min after treatment, the conductivity rapidly increased and reached the highest level at 11 min after treatment. However, in the control group, the conductivity slowly increased and reached the highest level at 15 min after treatment. Using same amount of hyphae, changes in conductivity reflect the total amount of intracellular electrolytes, thus indicating changes in cell membrane permeability (Xiushu et al. 2018).

The effect of ethyl acetate extracts of *C. camphora* xylem on *G. trabeum* leakage protein content

After treatment with different concentrations of ethyl acetate extracts of *C. camphora* xylem for 24 h, the amount of leakage protein from *G. trabeum* hyphae was measured (Fig. 7).

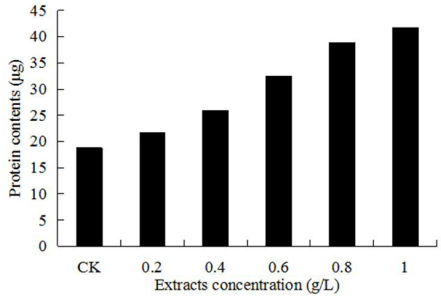

Fig. 7: The effect of extracts on the leakage of protein from the cells of G. trabeum.

With increased concentration of ethyl acetate extracts of *C. camphora* xylem, the amount of leaked protein also increased. When the concentration reached 1 $g \cdot L^{-1}$, the highest amount of leaked protein was observed. Leakage protein content further indicated that ethyl acetate extracts of *C. camphora* xylem can change *G. trabeum* cell membrane permeability, thus releasing more proteins (Tsujimoto and Shimizu 2007). The results obtained in this experiment, combined with conductivity experiment, demonstrate that ethyl acetate extracts of *C. camphora* xylem can increase *G. trabeum* cell membrane permeability.

CONCLUSIONS

A large number of chemical compounds in the *C. camphora* xylem extracts has a variety of biological activity and certain application value. Methanol extracts of *C. camphora* xylem showed stronger inhibitory effects on *G. trabeum* aerobic respiration and chloroform extracts of *C. camphora* xylem showed stronger inhibitory effects on *C. versicolor* aerobic respiration. Ethyl acetate extracts of *C. camphora* xylem exhibited good inhibitory effect on to *G. trabeum* cellulase activity. The experimental results of conductivity show that the ethyl acetate extract of *C. camphora* xylem could increase *G. trabeum* cellular membrane permeability. The leakage of protein from the cells of *G. trabeum* reflects that ethyl acetate extracts of *C. camphora* xylem can change *G. trabeum* cell membrane permeability.

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