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The antifungal activity of essential oils from some plants in Vietnam against the pathogenic fungi *Candida albicans* and *Aspergillus fumigatus*

Vu Xuan Tao[1*], Tran Bao Tram[1], Nguyen Thi Hien[1], Thai Hanh Dung[2], Tran Van Tuan[2]

[1]*Center of Experimental Biology, National Center for Technological Progress, Ministry of Science and Technology*
[2]*Faculty of Biology, University of Science, Vietnam National University, Hanoi*

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Abstract:

Vietnam possesses an abundant and highly diverse resource of plants. Therefore, the search and evaluation of bioactive compounds extracted from these plants are a potential research direction to support developing products that improve human health. The purpose of this study is to assess the antifungal activity of some essential oils produced by materials harvested in Vietnam such as King orange peel (*Citrus sinensis*), lemongrass (*Cymbopogon flexuosus*), peppermint (*Mentha arvensis*), and betel leaf (*Piper betle*). The antifungal effect of these essential oils was determined by the Kirby-Bauer disc diffusion technique. In addition, the antifungal properties of the essential oils were also assessed through their effects on the reproduction of *C. albicans* and the spore germination, mycelium elongation, and sporulation of *A. fumigatus*. The results demonstrated that all peppermint, lemongrass, and betel leaf essential oils showed antifungal activity against *C. albicans* and *A. fumigatus*. Especially, betel leaf essential oil could perform antifungal activity at a low dilution concentration of 10% and could also inhibit the reproduction of *C. albicans* and the spore germination, mycelium elongation, and sporulation of *A. fumigatus*. Meanwhile, orange peel essential oil did not exhibit any antifungal properties.

Keywords: **antifungal activity, *Aspergillus fumigatus*, *Candida albicans*, essential oil.**

Classification number: **3.5**

Introduction

Nowadays, pathogenic microorganisms causing diseases in humans are highly diverse and complex. Besides bacteria and viruses, which have been widely known as common pathogens, micro-fungi are also reported to be responsible for several dangerous diseases. Due to the improper use of antibiotics, overdose of immunosuppressive drugs, the increasing number of immunodeficiency diseases, and the poor living conditions, poor nutrition, and lack of hygiene, fungi now have more chances to spread and function as factors of infection. *Candida albicans* and *Aspergillus fumigatus* are two opportunistic pathogens in humans, especially in immunocompromised individuals. For instance, up to 90% of HIV patients are infected with *Candida* [1]. *C. albicans* is a common micro-fungus causing vaginal infection in women, which accounts for 85-90% of fungal infection causes. Severe illnesses can lead to complications such as endometritis, oophoritis, and infertility [1, 2]. *A. fumigatus* has been recorded as a fungus causing respiratory infection, especially in the lungs, and its function is affected by the immune status of the human body [3, 4].

Currently, the control of these pathogenic fungi mainly depends on antifungal chemicals. The use of natural active ingredients such as essential oils has not been extensively studied. Essential oils are secondary metabolites synthesized by various plant organs such as flowers, leaves, stems, and seeds and are often characterized by a specific odour. Vietnam is one of the countries possessing the most abundant and diverse plant resources. The study of active compounds extracted from medicinal materials is a potential research direction to support developing products to improve human health. A number of studies have proved that essential oil is resistant to many pathogenic microorganisms. The resistance to pathogenic fungi of essential oils greatly

[*]*Corresponding author: Email: taovx.tsa@gmail.com*

depends on their chemical composition, specifically two main components including terpenoids and phenolics [5]. This study was conducted with the aim to assess the antifungal activity of four types of essential oils extracted from the King orange peel (*Citrus sinensis*), lemongrass (*Cymbopogon flexuosus*), peppermint (*Mentha Arvensis*), and betel leaf (*Piper betle*) on the two human pathogenic fungi *C. albicans* and *A. fumigatus*.

Materials and methods

Materials

C. albicans JCM2070 was provided by the Japan Collection of Microorganisms and *A. fumigatus* VTCC1414 was provided by the Vietnam Type Culture Collection, Institute of Microbiology and Biotechnology (IMBT), Vietnam National University, Hanoi. These two strains of fungi were kept in the Genomics Unit, The Key Laboratory of Enzyme and Protein Technology, VNU University of Science, Vietnam National University, Hanoi.

Essential oils were collected by the method of steam distillation from raw materials collected in Vietnam. Essential oil samples of King orange peel (*Citrus sinensis*), lemongrass (*Cymbopogon flexuosus*), peppermint (*Mentha arvensis*), and betel leaf (*Piper betle*) were provided by the Center of Experimental Biology, National Center for Technological Progress, Ministry of Science and Technology.

Methods

Preparation of fungal spores: the *A. fumigatus* strain of VTCC1414 was cultured on PDA. After 5 d of cultivation at 28°C, sterile distilled water was added to the surface of the dish, then a clean sterile squeegee was used to remove the spores from the mycelium. The collected fluid was filtered through a Miracloth filter (Calbiochem, Germany) and centrifuged at a rate of 4000 rpm for 10 min and the clear supernatant was then discarded. The spores were washed twice with sterile distilled water. The sediment after centrifugation containing fungal spores was dissolved in sterile distilled water and adjusted to a concentration of 10^6 spores/ml using a Thoma counting chamber [6]. For the *C. albicans* JCM2070 strain, the strain was cultured on liquid Hansen medium. The cell density of the culture fluid was also determined by a Thoma cell counting chamber and was adjusted to a concentration of 10^7 cells/ml.

Evaluation of antifungal activity: the test to assess antifungal activity was conducted with the Kirby-Bauer disc diffusion technique [7]. A 30 µl spore suspension of *A. fumigatus* VTCC1414 and *C. albicans* JCM2070 were cultured on PDA and Hansen media, respectively. Each plate was placed with a sterile blotting paper (6 mm in diameter). A 20 µl drop of betel leaf oil was added dropwise onto a sterile paper disc and left for 60 s. Each essential oil was diluted with DMSO 5% to the concentrations of 5, 10, 20, 30, 40, and 50% [8]. The Petri dishes were then kept at 4°C for essential oil diffusion for 4 h. With *A. fumigatus* VTCC1414, all petri dishes were incubated at 28°C for 4-5 d. With *C. albicans* JCM2070, all petri dishes were incubated at 37°C for 1-2 d. The antifungal activity of each type was calculated according to the size of the inhibition zone.

Evaluation the effects of essential oil on the reproduction of C. albicans JCM2070: the medium used was liquid Hansen medium. Essential oils were added to the media to the concentrations of 0.025, 0.05, 0.075, and 0.1%. The initial cell density of *C. albicans* JCM2070 was 1.75×10^6 cells/ml. The strain was cultivated at 37°C at the rate of 200 rpm. Cell density monitoring was conducted after intervals of 2, 4, and 6 h by dilution and inoculation on Hansen medium.

Assessing the effects of essential oil on the development of A. fumigatus mycelium VTCC1414: the slide culture technique was used, and observations were made under an Olympus optical microscope [9]. Essential oils were mixed into the PDA medium to the concentrations of 0.025, 0.05, 0.075, and 0.1 %. PDA media containing essential oils were used to cultivate fungi. Samples were incubated at 28°C and observed after 24 and 48 h.

Results and discussion

The antifungal activity of essential oils against C. albicans and A. fumigatus

Four types of essential oils, including King orange peel, lemongrass, peppermint, and betel leaf, were evaluated for their ability to resist against *C. albicans* and *A. fumigatus* with the Kirby-Bauer disc diffusion technique. The results showed that the essential oil from King orange peel did not exhibit any antifungal activities. On the other hand, the three other essential oils including peppermint, lemongrass, and betel leaf all exhibited antifungal activities against *C. albicans* and *A. fumigatus* (see Tables 1, 2 and Figs. 1, 2). With *C. albicans*, the peppermint essential oil (undiluted) and lemongrass essential oil (undiluted and diluted to 50%) completely inhibited the fungal growth (i.e. the fungi did not grow over the whole agar plate medium). However, the peppermint essential oil showed weak antifungal activity against *C. albicans* at a 50% dilution and no longer performed antifungal activity at a 40% dilution. Similarly, the lemongrass essential oil diluted to

Table 1. Antifungal activities of several essential oils against *C. albicans*.

Essential oils	Inhibition zone (mm) at different concentration of essential oils (%)						
	100	*50*	*40*	*30*	*20*	*10*	*5*
King orange peel	-	-	-	-	-	-	-
Peppermint	No fungus growth	5.33±0.58	-	-	-	-	-
Lemongrass	No fungus growth	No fungus growth	18.33±1.15	11.66±1.53	2.33±0.58	-	-
Betel leaf	22.00±1.00	20.67±0.58	15.33±0.58	12.33±1.15	11.67±1.52	7.33±0.58	-

- no inhibition zone exhibited.

a concentration of 20% exhibited weak antifungal activity against *C. albicans* and no antifungal activity at the 10% concentration. Meanwhile, the betel leaf essential oil diluted to a concentration of 20% still showed strong antifungal activity against *C. albicans* and could still perform similarly at 10% concentration. Thus, the betel leaf and lemongrass essential oils can be used to control the spread of *C. albicans*. However, the betel leaf essential oil shows more advantages due to its function even at low concentrations (10%) against *C. albicans*.

The result was similar to that obtained from the evaluation of the resistance abilities of the essential oils mentioned above to *A. fumigatus*. The peppermint essential oil (undiluted) and lemongrass (diluted to 30%) completely inhibited the growth of *A. fumigatus*. The lemongrass essential oil showed strong antifungal activity against *A. fumigatus* up to the concentration of 30%, meanwhile it could not perform any antifungal activity below the concentration of 20%. This can be explained by the strong diffusion ability of the peppermint and lemongrass oils and their characteristics of volatility at room temperature. The essential oil vapour cannot escape out of the petri dish, therefore, it completely inhibited the fungi growth. When the concentration of essential oil vapour decreased, the amount of diffused essential oil in the petri dish was insufficient to inhibit the filamentous fungi. On the other hand, the betel leaf essential oil could perform antifungal activity to a dilution concentration as low as 10%. Interestingly, in this research, we determined that the betel leaf essential oil has potential to be applied to controlling both human pathogenic fungi *C. albicans* and *A. fumigatus*. The ability of antifungal activity of the essential oils depends on their chemical compositions [5]. Due to the difference in quantities and ingredients among the distinguished types of essential oils, their antifungal characteristics are not only affected by a specific mechanism but also by various ones [5]. The main mechanism is due to their hydrophobic features, where they can attack the cell membrane and disrupt it or affect the enzyme systems leading to respiratory depression and eventually cell death [10]. Peppermint, lemongrass, and betel leaf essential oils have been globally recognized to possess the ability of resistance to several types of micro-fungi [6, 8, 11]. This research claims that the mentioned

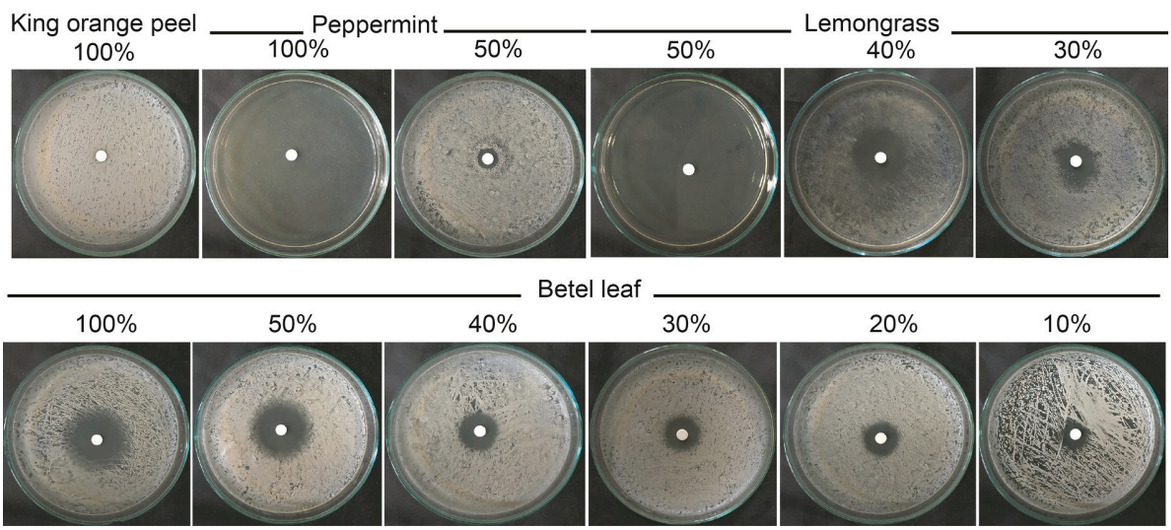

Fig. 1. Inhibition zones against *C. albicans* of some essential oils at different concentrations.

Table 2. Antifungal activities of several essential oils against *A. fumigatus*.

Essential oils	Inhibition zone (mm) at different concentration of essential oils (%)						
	100	*50*	*40*	*30*	*20*	*10*	*5*
King orange peel	-	-	-	-	-	-	-
Peppermint	No fungus growth	-	-	-	-	-	-
Lemongrass	No fungus growth	No fungus growth	No fungus growth	No fungus growth	-	-	-
Betel leaf	34.33±0.58	22.67±1.53	21.33±1.15	18.33±1.15	11.00±1.00	1.67±0.58	-

"-" no inhibition zone exhibited

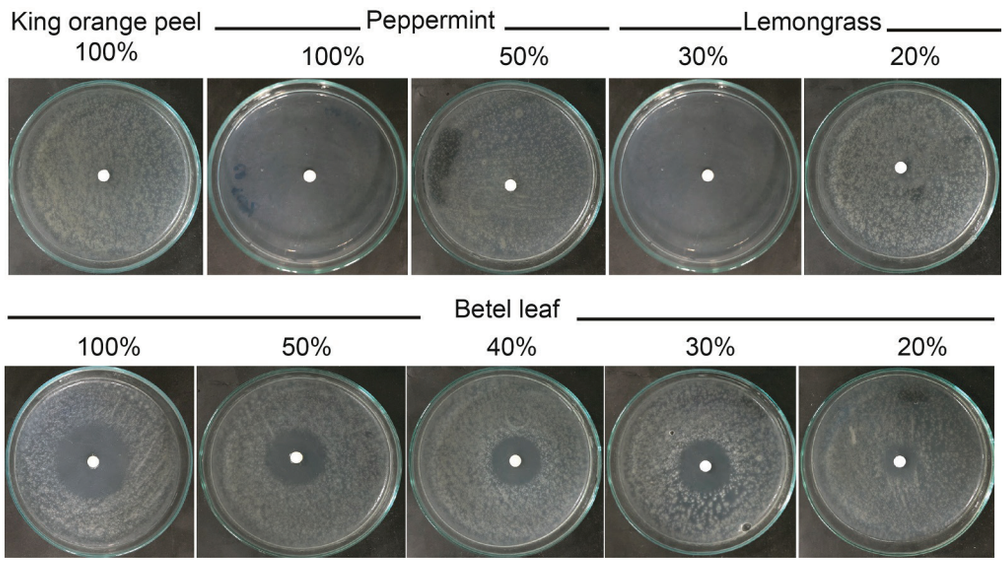

Fig. 2. Inhibition zones against *A. fumigatus* of some essential oils at different concentrations.

essential oils extracted from plants harvested in Vietnam also show strong antifungal activities and therefore have huge potential for being applied to the development of probiotics or medications for the diseases caused by the two fungi *C. albicans* and *A. fumigatus*.

While betel leaf essential oil could perform antifungal activity at a lower dilution concentration (10%), the lemongrass essential oil did not function below the dilution concentration of 20%. Thus, we believe that usage of betel leaf essential oil is a more economical and profitable material for the production of medical products. On the other hand, while the performance of lemongrass depends mostly on its dilution concentration, it showed a stronger inhibition towards fungus growth. Lemongrass essential oil could completely inhibit the growth of *C. albicans* at concentrations of 100% and 50% and completely inhibit the growth of *A. fumigatus* from the concentration of 30%, which was not exhibited by betel leaf essential oil. Therefore, betel leaf and lemongrass essential oils both possess certain advantages and disadvantages. As both are popular materials that can be easily grown and collected in Vietnam at low expense, we can consider their advantages and disadvantages in order to apply the appropriate type to mass industrial application.

Effects of betel leaf essential oil on the reproduction of the fungus C. albicans

In this research, the betel leaf essential oil was evaluated to have potential for controlling two human pathogenic fungi, *C. albicans* and *A. fumigatus*. Subsequently, we continued to evaluate the effects of the betel leaf essential oil on the reproduction of fungus *C. albicans*. The results showed the inhibition of the reproduction process in every medium containing the essential oil (Fig. 3). With the media containing the betel leaf essential oil at the concentration of 0.025-0.075%, the quantity of *C. albicans* cells after 2-6 h of cultivation did not demonstrate a significant change compared to the original quantity. Meanwhile, with the medium containing the betel leaf essential oil at a concentration of 0.1%, the quantity of fungi cells showed a tendency to gradually decrease. This could be due to the

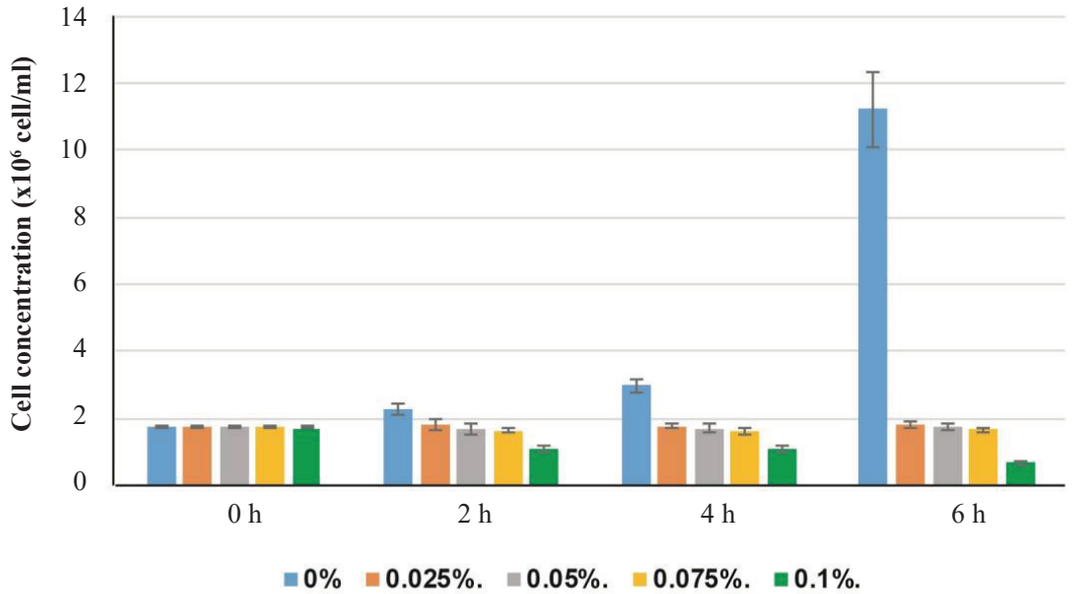

Fig. 3. Effects of betel leaf essential oil on the reproduction of *C. albicans.*

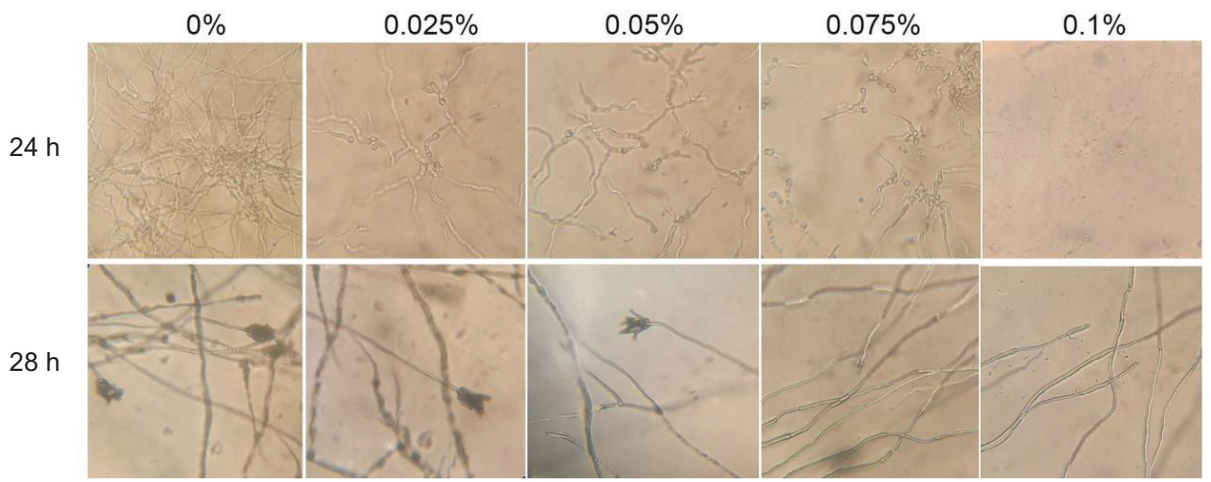

Fig. 4. Effects of betel leaf essential oil on the mycelium elongation of *A. fumigatus.*

start of fungal cell death by the betel leaf essential oil at a concentration of 0.1%, however, the process was dependent on the time. A previous study claimed that all parts of the betel plant showed strong antimicrobial activity by releasing their secondary metabolites [12]. Hydroxychavicol in the betel leaf was also proven to be resistant to the fungi *Candida* spp. by disrupting its cell membrane [13]. This research added a controlling role of the betel leaf essential oil on the fungus *Candida* through supressing its reproduction.

Effects of the betel leaf essential oil on the mycelium elongation of A. fumigatus

The betel leaf essential oil was proven to be resistant to several filamentous fungi of the genus *Aspergillus* such as *A. flavus*, *A. fumigatus*, *A. niger*, and *A. parasiticus* [13]. However, the majority of the research was only aimed at defining the minimum inhibitory concentration (MIC) [14]. A few other works mentioned that the betel leaf essential oil did not affect the cell wall of microorganisms [15]. This research was conducted to evaluate the effect of betel leaf essential oil on the mycelium elongation of *A. fumigatus*. The result showed that betel leaf essential oil inhibited the spore germination, mycelium elongation, and sporulation of *A. fumigatus* at every dilution concentration (Fig. 4). After 24 h of observation, the media containing betel leaf essential oil at concentrations of 0.025-0.1% delayed the process of spore germination and mycelium elongation. Especially in the medium containing betel essential oil at

the concentration of 0.1%, there was no spore germination even after 24 h. After 48 h of cultivation, in the media containing betel leaf essential oils at concentrations of 0.025% and 0.05%, there was a formation of conidiophores, however with a lower quantity compared to the medium not containing the essential oil. With the media containing essential oils at concentrations of 0.075% and 0.1%, there was recognition of the mycelium elongation but no formation of conidiophores. The inhibition of the spore germination, mycelium elongation, and sporulation of *A. fumigatus* could be recognized as a mechanism of controlling this pathogenic fungus by the betel leaf essential oil.

Conclusions

Peppermint, lemongrass, and betel leaf essential oils all showed antifungal activities towards *C. albicans* and *A. fumigatus*; especially betel leaf and lemongrass essential oils, which showed strong antifungal activities. Meanwhile, the King orange peel did not show any fungi resistance abilities.

The betel leaf essential oil was recognized to inhibit the reproduction of *C. albicans* and the spore germination, mycelium elongation, and sporulation of *A. fumigatus*.

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The authors declare that there is no conflict of interest regarding the publication of this article.

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