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Synergic effect of essential oils from Vietnamese Cinnamomum cassia and *Alpinia coriandriodora* on *Malassezia* species

Tran, B. T.[1], Vu, X. T.[1], Dao, N. A.[1,2], Do, T. K. T.[1], Nguyen, T. T. M.[1], Hoang, Q. C.[1], Vu. H. H[3,4], Dinh, T. M.[5], Soytong, K.[5,6], Tran, V.T.[2], Nguyen, Q. H.[2*]

[1]Center for Experimental Biology, National Center for Technological Progress, Ministry of Science and Technology, C6 Thanh Xuan Bac, Thanh Xuan, Hanoi, Vietnam; [2]Faculty of Biology, University of Science, Vietnam National University, Hanoi (VNU), 334 Nguyen Trai, Thanh Xuan, Hanoi, Vietnam; [3] Institute of Molecular Biology, Mainz, Germany; [4] Faculty of Biology, Johannes Gutenberg University, Mainz, Germany. [5]Bio-Agriculture Company, Thuong Mo, Dan Phuong, Hanoi, Vietnam; [6]Department of Plant Production Technology, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Landkrabang, Bangkok Thailand.

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Abstract *Malassezia* is known as yeast which colonizes on healthy skin, and pathogenic potential under appropriate conditions. Malassezia-associated disease can lead to serious disorders, especially in immune-incompetent or immune-compromised patients. Although several antifungal agents are available for treatment of the diseases, drug resistance of the fungi and toxicity of the agents are raising concerns in terms of public health. Several essential oils from plants are able to cure *Malassezia*-associated disorders. In this study, essential oils extracted from Vietnamese *Cinnamomum cassia* and *Alpinia coriandriodora* exhibited strongly antifungal activity of three *Malassezia* species at MIC of 0.25 µl/ml and 1 µl/ml, respectively. GC/MS analysis showed the composition of essential oils with many antifungal compounds. Essential oil of *C. cassia* contains 7 compounds with trans-Cinnamaldehyde as the major components (88.09%). Decenal <2E-> occupies 60.42% which being the main component among 26 compounds in *A. coriandriodora* essential oil. Notably, combination of two essential oils showed the synergic effect against *Malassezia* reducing MIC of two essential oils at 0.025 µl/ml (*C. cassia*), and 0.25 µl/ml (*A. coriandriodora*). The killing-time assay indicated that the combination showed strongest effect in first 5 minutes of treatment, reaching a peak after 20 minutes. The obtained results suggested to be anti-*malassezia* potentials of *C. cassia* and *A. coriandriodora* essential oils. This activity can be enhanced by combining two essential oils providing a cue for formulating new medicines and daily products acting against *Malassezia*-associated diseases. The antifungal efficiency is enhanced in combination of EOs compounds which provideed the new medicines and daily products effectively treated *Malassezia*-associated disease.

Keywords: *Cinnamomum cassia, Alpinia coriandriodora, Malassezia*-associated diseases, Essential oils, Synergic effects

* **Corresponding Author**: Nguyen, Q. H.; **Email:** huy_nq@hus.edu.vn

Malassezia, commensal yeasts are commonly found on skin of warm-blooded animals including human (Ashbee, 2007). However, disturbances of these universal species have been reported to be skin disorders such as pityriasis versicolor, malassezia folliculitis, seborrheic dermatitis, and atopic dermatitis (Difonzo *et al*., 2013). Besides, *Malassezia* species are related to catheter-related fungemia, sepsis, and serious infections, especially in people with weakened immune systems (Tragiannidis *et al*., 2010).

Class *Malasseziomycetes* includes three clusters, Cluster A consists of *M. furfur*, *M. japonica*, *M. obtusa*, and *M. yamatoensis*; Subcluster B1 includes *M. globosa* and *M. restricta* which can be found on human skin; subcluster B2 includes *M. sympodialis*, *M. dermatis*, *M. caprae*, *M. equina*, *M. nana*, and *M. pachydermatis*; and *M. cuniculi* and *M. slooffia* are sorted into cluster C (Wu *et al*., 2015). *M. globosa* and *M. restricta* can be found on human skin regardless of whether the skin is healthy or in bad condition (AA and Casa ño, 1999). Other species are related to human skin disease are *M. sympodialis* and *M. furfur* (Jagielski *et al*., 2014). *Malassezia* species respond differently to antifungal agents. For instance, *M. sympodialis* is highly sensitive to terbinafine and fluconazole, whereas *M. globosa* is resistant to both compounds (Gupta *et al*., 2000; Rojas *et al*., 2014). Ketoconazole can effectively kill *M. furfur*, but sensitivity of the fungus to econazole and miconazole is much lower (Hammer *et al*., 2000). Although several antifungal agents are available to control *Malassezia*-associated disorders, improper application of the agents which regarding to toxicity to the host and resistance to synthetic antimicrobial substances.

Several plants are able to synthesis aromatic oily liquids known as essential oils (EOs). Chemically, EOs are composed of different compounds from various chemical classes. Characteristics of EOs are generally represented by main compounds which could attribute to the major biological activities of the EOs (Baser and Buchbauer, 2009; Franz, 2010). The main components can act as an additive or synergistic manner, and eliminate pathogen by affecting different organelles of the microbial cells in combination of several mechanisms. Accordingly, EOs may be more affected than chemically synthesized agents in eradicating pathogens, preventing the development of strain resistance, and lessening user's toxicity. It may be expressed the best alternation or addition to the use of conventional antimicrobials (Asadim, 2008; Adorjan and Buchbauer, 2010; Franz, 2010; Santomauro *et al*., 2016).

EOs have been used for medicinal and health purposes and generally considered as safe agents. It has been shown to effectively suppress numerous human pathogens, including antibiotic-resistant *Staphylococcus aureus* and *Candida albicans*(Asadim, 2008; Adorjan and Buchbauer, 2010; Franz, 2010; Santomauro *et al*., 2016). EOs and their derived products have also been reported to be effective in eradication of *Melassezia* in both

preclinical and clinical studies (Donato *et al*., 2020). For example, Pooja and co-workers demonstrated that EOs from *Cinnamomun zeylanicum* and *Malaleuca alternifolia* can eliminate *M. furfur* at the MIC of 32 µg/mL and the combination of *C. zeylanicum, M. leucadendrum* and *Ocimum kilimandscharicum* can synergistically eradicate *Melassizia* species (Arun and Maninder 2013). Bohmova *et al*. (2019) described the synergism of clotrimazole with *Melaleuca alternifolia, Mentha piperita and Origanum vulgare* EOs against *M. pachidermatis* (Bohmova *et al*., 2019*)* while Vinciguerra *et al*. (2018) described that EOs from *O. vulgare* and *Thymus vulgaris* strains resistant to fluconazole (Vinciguerra *et al*., 2019). Furthermore, clinical trials reported that EO-derived shampoo, cream or lotion can effectively treat *Malassezia*-associated dandruff and *P. versicolor* (Carmo *et al*., 2013; Oliveira *et al*., 2018). Previously, we documented that the combination EOs from *Piper betle* and *Mentha arvensis* which synergistically deactivated *M. globose* and *M. furfur in vitro* (Vu *et al*., 2021). In an attempt to identify the most effective combination of EOs for simultaneously eradicating *Melassezia* specifies, we would like to characterize chemical compositions of EOs from *Cinnamomum cassia* barks and *Alpinia coriandriodora* rhizomes, and to evaluate their anti-*melassezia* synergism.

C. cassia is a member of the *Lauraceae* family, which is widely grown in Southeast Asia. *C. cassia* bark is a natural spice which widely used in food and traditional medicine to treat several diseases such as gastritis, blood circulation disturbances, and inflammatory diseases (Tang and Eisenbrand, 1992). EO from *C. cassia* possesses antioxidant (Lin *et al*., 2003), antifungal (Giordani *et al*., 2006), and antibacterial (Chaudhry and Tariq, 2006) properties. *C. cassia* bark and leaf EOs are recorded to be safe as food additive agent in the State (Barceloux, 2009). In Vietnam, *C. cassia* are cultivated in many provinces from the north to the south and primarily consumed locally (Barceloux, 2009). Trinh *et al.* (2015) showed that EO from Vietnamese *C. cassia* leaves contain 3 main components including trans-Cinnamaldehyde (90%), trans-Cinnamylacetate (4.69%), and Coumarin (1.2%). They reported that the EO was more active than its main component, trans-Cinnamaldehyde, in disruption of bacteria membranes, suggesting that several compounds in the EO cooperate to disrupt the cell membrane (Trinh *et al*., 2015). EO composition from Vietnamese *C. cassia* barks and their anti-microbial activities have not reported yet.

A. coriandriodora is also known as sweet ginger is a member of the Zingiberaceae, whose fleshy rhizome is widely utilized in cooking. *A. coriandriodora* is essential oil-rich with iron salt and other substrates, which can be used in fresh, marinated, or processed. Traditionally, *A. coriandriodora* has been used to treat certain disorders such as sweating, antiemetic, and expectorant. Although *A. coriandriodora* EO is widely used in traditional medicine, few published data about chemical compositions

and pharmacological properties are available. Thus, the characterization of *A. coriandriodora* EO cultivated in Vietnam and its anti-microbial properties, especially anti-malassezia, will provide useful information for better insights of *A. coriandriodora* EO application.

In this study, chemical compositions of EOs from the Vietnamese *C. cassia* barks and *A. coriandriodora* rhizomes were investigated. Gas chromatography–mass spectrometry (GC-MS) investigated the major compositions of the EOs for trans-Cinnamaldehyde and Decenal <2E->. The anti-*malassezia* activity of two EOs was investigated by different methods to show the potential application of the EOs in *Malassezia* treatments.

Materials and methods

Plant materials

Fresh *C. cassia* barks and *A. coriandriodora* rhizome were collected from Lang Son province. After being harvested, the samples were cleaned with tap water followed by washing with sterilized distilled water.

Fungal strains

Malassezia furfur VNF01 and *Malassezia globosa* VNG02 strains were obtained from the Center of Experimental Biology - National Center for Technological Progress. The *Malassezia furfur* ATCC 14521 strain was purchased from ATCC.

EO extraction

100 g of materials were grinded to the size of 0.5 cm to 1 cm before subjecting to hydro distillation. The samples were kept in a conventional Clevenger type apparatus with 300 ml of distilled water, and the EOs were obtained after 3 hours at 100^{o}C. The EOs were dehydrated using anhydrous sodium sulfate. Samples were then stored at 4^{o}C for subsequent experiments.

GC/MS analyses

GC/MS were performed as same method described by Sparkman (2005). GC/MS-QP2020-Shimadzu mass spectrometer instrument using a SH-Rxi-5SilMS column (30 m x 0,25 mm x 0,25 µm) was used to obtain GC/MS data. Helium was used as carrier gas; temperature was set at 60°C in 2 min, rising to 240°C at the rate of 5°C/min and maintain at 240°C for 5

min. The experiment was conducted twice. The mean data of GC analysis was calculated for quantitative results.

Agar diffusion assay

The method for agar diffusions assays were modified from the method of Hadacek and Harald (2000) and (Hadacek and Greger, 2000). The mDixon agar plates contained malt extract 36 g/l, desiccated oxbile 20 g/l, tween 40 10 ml/l, peptone 6 g/l, glycerol 2 ml/l, oleic acid 2ml/l, pH 6, were used for spreading *Malassezia* strains (50 µl at 10^6 cells/ml). The EOs were diluted by serial dilution before being added to 9 mm wells at the middle of plates. EOs were diluted in DMSO, the same volume of DMSO (Sigma-Aldrich, cat # 67-68-5) was used as the negative control. After keeping at 4^oC in 4 hours, the plates then were transferred to 30^oC. Antifungal activity was determined by measuring diameter of inhibition zones (plate circles without visible fungi) which measured after 72 hours of incubation. Experiments were repeated three times.

Agar dilution assay

The methods demonstrated in research of Lambert and Pearson (2000) were applied to the experiment. A volume of serial dilluted EOs (75 µl) was added to plates containing same amount of mDixon agar media (7.5 ml). The negative control plate contained DMSO. A same volume of different cell concentrations (10^6, 10^5, 10^4 cells/ml) were pipeted on each plate. The visible growth was determined after three day of incubation at 30^oC. Each experiment was repeated 3 times.

The fractional inhibitory concentration index (FICI) represents the interactions between two substances. Two substances are considered to act synergistically when FICI ≤ 0.5, additively if 0.5 <FICI<1 , indifferently if 1 < FICI < 4, and antagonistically when FICI > 4 (Iten *et al*., 2009). The formular for FICI is described below:

$$FICI = \frac{MIC\ of\ the\ first\ essential\ oil\ in\ combination}{MIC\ of\ the\ first\ essential\ oil\ separately} + \frac{MIC\ of\ the\ second\ essential\ oil\ in\ combination}{MIC\ of\ the\ second\ essential\ oil\ separately}$$

Killing-time analyses

The killing-time curve assay was developed from the method of Joray *et al*. (2011). DMSO or a mixture of *C. cassia* (0.025 µl/ml) and *A. coriandriodora* (0.25 µl/ml) were added to the media containing 10^6 yeast cells/ml. Samples from the test culture were collected at different time points (5, 10, and 20 minutes of incubation). The samples were diluted and

grown on the agar plates. Incubation temperature was 30^{o}C, and CFU were counted after 72 hours. Percent of cell death were calculated by 100% - % of living cells. Percentage of living cells is calculated the ratio of cell concentrations at 5, 10, or 20 minutes to concentration of cells at zero time point.

Results

Anti-melassezia activity of C. cassia and A. coriandriodora EOs

M. furfur ATCC 14521 and VNF01 strains, and *M. globosa* VNG02 strain were used for agar diffusion assays. The fungi were able to grow all over the plate with DMSO (negative controls) after three-day incubation (Figure 1A - top left) but partially inhibited by 2% ketoconazole (Figure 1A - bottom left). In contrast, no visible growth was observed on both plates with *A. coriandriodora* (Figure 1A - top right) and *C. cassia* 100% EO (Figure 1A - bottom right). These data suggested that both *A. coriandriodora* rhizome and *C. cassia* bark EOs had strong anti-*malassezia* properties.

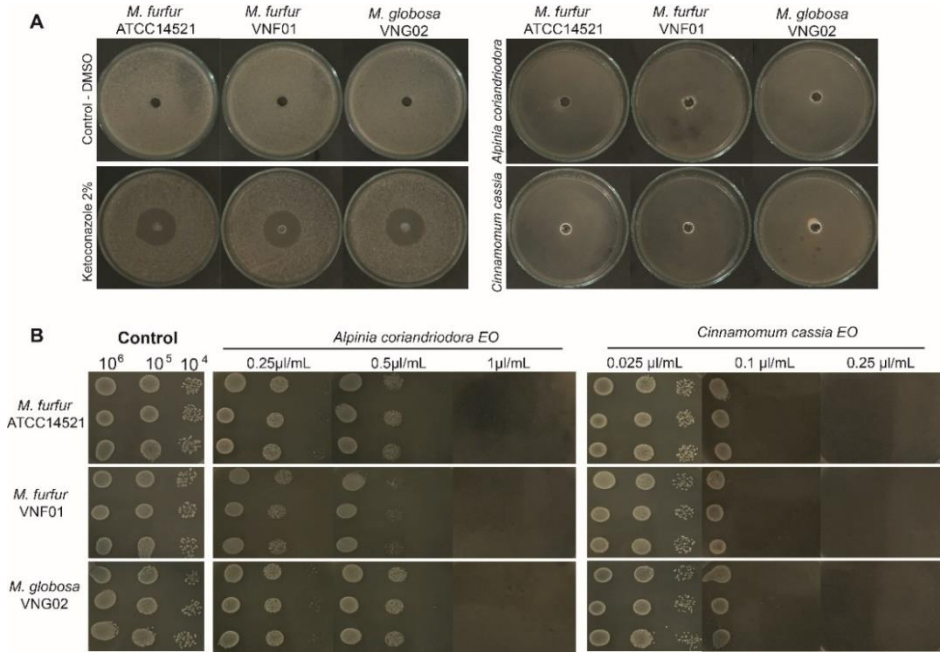

Figure 1. Anti-*malassezia* activity and MIC of *A. coriandriodora* and *C. cassia* EOs. **(A)** Anti-*malassezia* activity against 10^6/ml of *M. furfur* (ATCC 14521 and VNF01), and *M. globosa* (VNG02) cells. **(B)** MIC of *A. coriandriodora* and *C. cassia* EOs. Concentrations of 1 µl/ml *A. coriandriodora* EO and 0.25 µl/ml *C. cassia* EO completely inhibits visible growth of tested fungal strains

The minimum inhibitory concentrations (MIC) of *A. coriandriodora* rhizome or *C. cassia* bark EOs were identified by agar dilution assays. The visible growth of *M. furfur* ATCC 14521 and VNF01 strains, and *M. globosa* VNG02 strain was completely defected on plate containing *A. coriandriodora* rhizome EO at the concentration of 1 µl/ml (Figure 1B). The fungi still grow at EO concentration of 0.5 µl/ml suggesting that MIC of *A. coriandriodora* rhizome EO was 1 µl/ml. MIC value of *C. cassia EO* was identified with the same method at the concentration of 0.25 µl/ml (Figure 1B).

Table 1. Chemical composition of *Cinnamomum cassia* EO

Compound	Relative composition ratio, %	Compound	Relative composition ratio, %
Benzaldehyde	3.68	Copaene	3.08
Dihydro cinnamaldehyde	1.71	Coumarin	1.00
***cis*-Cinnamaldehyde**	0.76	Cinnamyl acetate	1.68
***trans*-Cinnamaldehyde**	**88.09**		

Table 2. Chemical composition of *Alpinia coriandriodora* D. Fang EO

Compound	Relative composition ratio, %	Compound	Relative composition ratio, %
Mycrene	0.1	Copaene <a->	0.47
Octanal <n->	0.26	Decenoic acid <2E->	0.40
Cymene <o->	2.01	Elemene <cis-b->	0.39
Limonene	0.11	Decenyl Acetate<2E>	4.34
Ocimene <(E)-b->	0.24	Dodecanal	0.12
Octenal <2->	1.37	Caryophyllene <E->	0.46
Linalool	2.10	Dodecanal <2E>	1.57
Nonanal	0.11	Humulene <a->	1.60
Decenal <4Z->	0.36	Selinene <b->	3.67
Decanal	1.41	Selinene <a->	2.3
Decenal <2E->	**60.42**	Cadinene <d->	0.12
Decen-1-ol <2E->	1.42	Nerolidol <E->	0.90
Decanol <n->	0.34	Caryophyllene oxide	0.39

Synergic effect of A. coriandriodora rhizome and C. cassia EOs on Malassezia species

Combinations of two EOs at different concentration were added to agar plates for evaluating the synergic effects of *A. coriandriodora* rhizome and *C. cassia* EOs on *M. furfur* ATCC 14521 and VNF01 strains, *M. globosa* VNG02 strain. Out of 20 combinations, 14 mixtures completely inhibited visible growth of all three fungal strains (Figure 2). The fractional inhibitory concentration index (FICI) of these 14 mixtures revealed the interactions between these two EOs. The smallest FICI of *A. coriandriodora*

rhizome (0.25 µl/ml) and *C. cassia* (0.025 µl/ml) EOs was 0.35, indicating a synergistic action of these two EOs against *Malassezia* species.

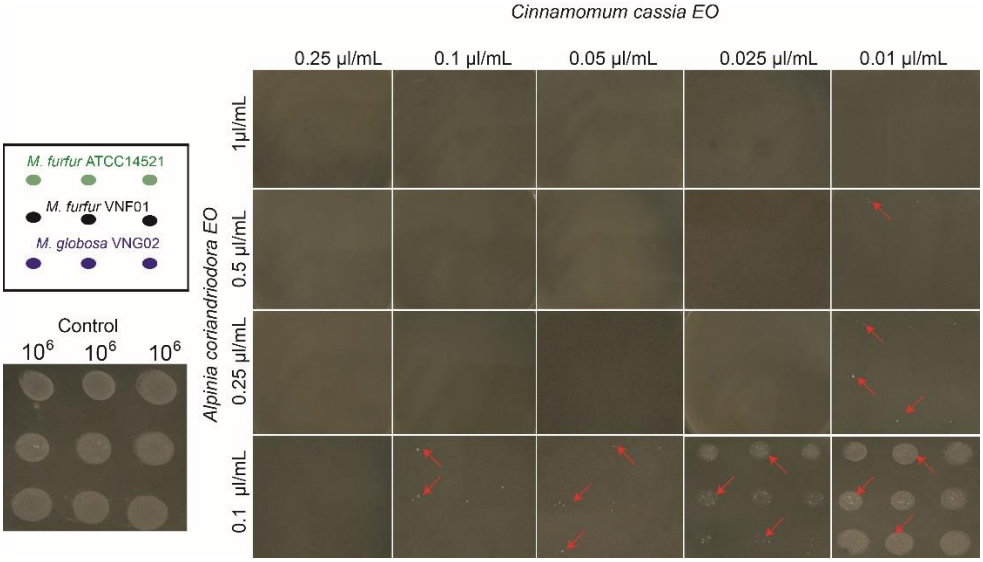

Figure 2. Synergic effect of *A. coriandriodora* rhizome and *C. cassia* EOs on *malassezia* species. Combination of 0.25 µl/ml of *A. coriandriodora* EO and 0.025 µl/ml of *C. cassia* EO completely inhibit visible growth of three fungal strains

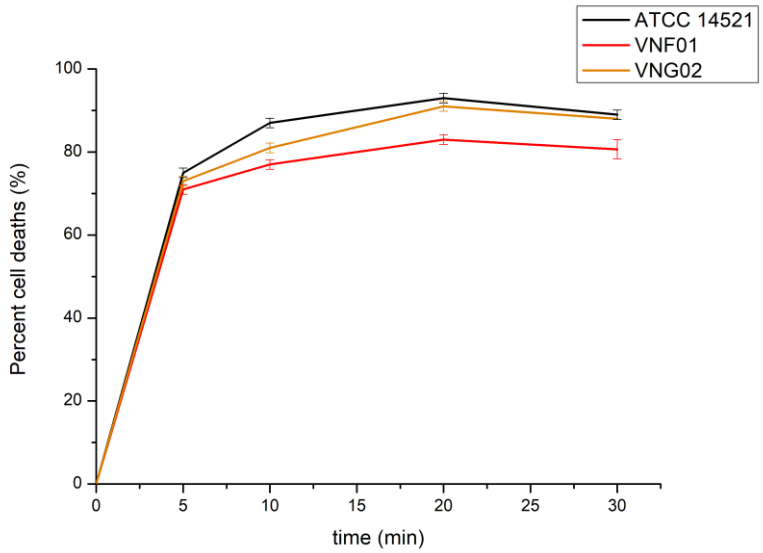

Figure 3. Killing-time curves of *A. coriandriodora* rhizome and *C. cassia* EOs against 10^6 CFU/ml *M. furfur* (ATCC 14521, VNF01) and *M. globosa* (VNG02) yeast cells. Strongest effect was recorded after 20 minutes of treatment. After reaching a peak, antifungal activity slightly reduced after 30 minutes

The anti-*Malassezia* activity of the combination of *A. coriandriodora* rhizome and *C. cassia* EOs were recorded over 30 minutes. The number of living cells in negative control (treated with DMSO) remained the same after 30 minutes. However, in the samples with the mixture of 0.25 µl/ml *A. coriandriodora* rhizome and 0.025 µl/ml *C. cassia* EOs, the cell deaths percentage was increased over the time and reached a peak after 20 minutes. However, the cell death proportion slightly decreased after 30 minutes. After 20 minutes of incubation, the cell death percentage of *M. furfur* ATCC 14521 strain was highest (93%), while those of *M. globosa* VNG02 and *M. furfur* VNF 01 strains were slightly lower at 91% and 83%, respectively (Figure 3).

Discussion

Pure essential oils contains various components which can be categorized into two classes: volatile fraction and nonvolatile residue (Hanif *et al*., 2019). In most case, the biophysical and biological features of the essential oils are similar as those of main components (Ipek *et al*., 2005). However, there is a possibility that other minor molecules can elevate activity of major components (Hoet *et al*., 2006). For decades, several components of EOs from different plants has been documented to have antimicrobial activity (Morris *et al*., 1979; El-Keltawi *et al*., 1980; Hili *et al*., 1997). One of the most striking features of EOs is containing hydrophobic molecules that allow EOs insert into lipid layers of cell membrane leading to increase in permeable and cell disruption (Sikkema *et al*., 1994). EOs also can negatively affect function of mitochondria, biofilm formation, and mycotoxin synthesis (Nazzaro *et al*., 2017). In Vietnam, *C. cassia* and *A. coriandriodora* have been used to treat different disorders in hundred years. However, antimicrobial activity of essential oils extracted from Vietnamese *C. cassia* barks and *A. coriandriodora* rhizomes has not been documented. In this project, we proposed the components of EOs from those objectives as well as their antimicrobial activity against *Malassezia* species.

The research finding, both EOs extracted from *C. cassia* barks and *A. coriandriodora* rhizomes showed strongly anti-*Malassezia* activity with MIC of 0.25 µl/ml and 1 µl/ml, respectively. The EOs chemical profiles revealed the major component of *C. cassia* EO is trans-Cinnamaldehyde occupied 88.09% of the bark EO, while Decenal <2E-> takes up 60.42% of *A. coriandriodora* EO. Cinnamaldehyde is capable of inhibit microorganisms by inhibiting ATPases, cell wall synthesis as well as

interfering membrane structure (Bang *et al*., 2000; Usta *et al*., 2003; Xie *et al*., 2004). Besides, (2E)-decenal (C10) was proven as an effective fungicide by targeting fluidity of the lipid bilayer (Kubo *et al*., 2003). The strong antimicrobial activity of these two compounds might explain the why EOs from *C. cassia* and *A. coriandriodora* completely inhibited the growth of *Malassezia* species. EOs of *C. cassia* and *A. coriandrodora* exhibited synergistic action against *Malassezia* species. The combination of ten-fold lower dose of MIC value of *C. cassia* EO and four-fold lower dose of MIC value of *A. coriandriodora* EO leaded to growth inhibition of the fungi. The enhancement of antifungal activity resulted to be synergistic effects of EOs components on the fungi by different mechanisms. Since the lead compounds of both EOs target the cell membrane, EOs mixture can rapidly kill fungal cells by cell membrane disruption. Insertion of hydrophobic molecules to cell membrane also increase permeability enhancing the uptake of other fungicides (Pei *et al*., 2009). Other components of EOs also can be involved in microorganism inhibition for instance: Benzaldehyde (3.68% in *C. cassia* EO) disrupting cellular antioxidation systems of microorganism, or terpene compounds which found in *A. coriandriodora* EO reducing cellular respiration rate (Kim *et al*., 2011, Mahizan *et al*., 2019)

The killing time assays using mixture of *C. cassia* and *A. coriandriodora* EOs against *Malassezisa* species revealed that the EOs rapidly kill cells in first five minutes of treatment. The antifungal activity reaches a peak after 20 minutes resulting in 93% cell death of *M. furtur* ATCC 14521, 91% and 83% cell death of *M. globosa* VNG02 and *M. furtur* VNF 01 strains, respectively. Therefore, antifungal activities of EOs resulted to be highest during 20 minutes of treatment. The slight decrease in cell deaths after 30 minutes resulted from evaporation of volatile compounds in EOs.

In summary, EOs extracted from Vietnamese *C. cassia* bark and *A. coriandriodora* rhizomes are shown to be promising antifungal properties against *Malassezia* species. The combination of two EO extracts significantly increased anti-*Malassezia* activity suggesting synergistic effect of the EOs. Results provided the evidence of application of *C. cassia* and *A. coriandriodora* EOs in *Malassezia* treatment. The enhancement in antifungal efficiency under combination of EOs or compounds from these EOs is possible to provide the cues for new medicines or daily products which effectively treat *Malassezia*-associated disease.

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