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Chemical compositions and Anti-*malassezia* properties of Vietnamese *Mentha arvensis* and *Piper betle* essential oils

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Abstract *Malassezia* species causes serious diseases in immuno-incompetent or immuno-compromised hosts. Synthetic antifungal agents can treat *Malassezia*-associate disorders. However, the hypersensitivity, toxicity, and resistance to the synthetic drugs due to their improper use are raising public concerns. Thus, the safer antifungal agents are actively seeking. Plant essential oils are natural defenders which protect their hosts from both biotic and abiotic attacks. Several essential oils have been shown to inactivate *Malassezia* spp. *in vitro* and to fight *Malassezia*-associated diseases *in vivo*. Essential oils from *Mentha arvensis* and *Piper betle* plants have been widely used in Vietnam and demonstrated to kill a variety of bacteria and fungi. Nevertheless, their inhibitory effects against *Malassezia* species have not been evidenced. In this study, essential oils yielded from *M. arvensis* and *P. betle* plants cultivated in Vietnam were 1% (0.9% - 1.09%) and 0.25% (0.2% - 0.3%), respectively. *M. arvensis* oil contained 11 compounds, of which menthol (68.19%) and menthone (22.77%) were the two major molecules. Eugenol acetate (38.66%), and m-eugenol (30.28%) were the most abundant compounds in *P. betle* essential oil. Agar diffusion method showed that *M. arvensis* and *P. betle* essential oils inhibited the growth of *Malassezia. furfur* (ATCC 14521 and VNF01) and *Malassezia globosa* (VNG02) by ~ 100% and ~ 40%, respectively. Agar dilution assays identified 2.5 µl/ml and 1 µl/ml as minimum inhibitory concentration of *M. arvensis* and *P. betle* essential oils, respectively. Furthermore, different combinations of *M. arvenis* and *P. betle* essential oils showed an additive effect on eliminating the fungal growth *in vitro*, probably by attacking distinct organelles of the yeast cells. Finally, kill-time analyses indicated that 80% - 90% of the tested strains were eliminated after 20 minutes of treatment with a combination of 1 µl/ml of *M. arvensis* and 0.5 µl/ml of *P. betle* essential oils. These data suggested that *M. arvensis* and *P. betle* essential oils (Eos) can be potential agents for formulating shampoo, cream or lotion to treat *Malassezia*-associated disease.

Keywords: *Mentha arvensis*, *Piper betle*, Essential oils, *Malassezia*, Antifungi

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Introduction

Malassezia spp. is a genus of lipid-dependent yeasts, which lacks the ability to synthesize lipid, and hence, depends on lipid for their survivals. *Malassezia* is the most abundance commensal yeasts of human and warm-blood animal skins (Ashbee, 2007). Nonetheless, the yeasts can become pathogenic agents under certain circumstances. For example, *malassezia* yeasts have been found in various dermatological afflictions, including pityriasis versicolor, malassezia folliculitis, seborrheic dermatitis, atopic dermatitis, and psoriasis in immunologically competent hosts (Difonzo *et al*., 2013). Additionally, the species have also associated with catheter-related fungemia, sepsis, and a variety of deeply invasive infections in immunocompromised patients, including patients with acquired immune-deficiency syndrome (AIDS), immunohematological and oncological patients, as well as solid organ and bone marrow transplant recipients (Tragiannidis *et al*., 2010).

Malassezia genus is classified as *Malasseziomycetes* (Wang *et al*., 2014), which is further divided into three clusters; Cluster A consists of *M. furfur, M. japonica, M. obtusa,* and *M. yamatoensis*; subcluster B1 contains *M. globosa* and *M. restricta,* the most abundantly occurring human skin inhabitants; subcluster B2 includes *M. sympodialis, M. dermatis, M. caprae, M. equina, M. nana,* and *M. pachydermatis*; and cluster C embraces *M. cuniculi* and *M. slooffiae* (Wu *et al*., 2015). *M. globosa* and *M. restricta* are the species most commonly found on healthy and diseased human skin (Crespo Erchiga *et al*., 1999). Nevertheless, other species, such as *M. sympodialis* and *M. furfur*, have also linked to many human skin disorders (Jagielski *et al*., 2014).

In vitro studies demonstrated that different *Malassezia* species respond dissimilarly to various antifungal agents. For instance, *M. furfur, M. globosa,* and *M. obtusa* are more tolerant to terbinafine than other *Malassezia* spp., whereas *M. sympodialis* is highly susceptible (Gupta *et al*., 2000). Fluconazole is highly active against *M. sympodialis* and *M. slooffiae*, but inactive against *M. globosa* and *M. restricta* (Rojas *et al*., 2014). Itraconazole had high activity against *M. globosa* while ketoconazole is more active against *M. furfur* than econazole and miconazole (Hammer *et al*., 2000). Although these antifungal agents either alone or in combination can be used for treating *malassezia*-associated disorders, the hypersensitivity, toxicity, resistance to synthetic antimicrobials due to their improper application concerned public health. Hence, identification of antifungal agents with safer properties has been actively seeking.

Plant essential oils (EO) are complex mixtures of natural compounds, which have antiseptic and medicinal properties. EOs have been used as folk medicine and agro-food preservers since ancient times and have been shown to possess antimicrobial and antioxidant activities. Although

chemical compositions of EOs differ among species, the main compounds identified in EOs often belong to the family of terpenes, which are highly lipophilic and low molecular weight, and thus, capable of disrupting the cell membrane, causing cell death or inhibiting the sporulation and germination of fungi. Chemical components of EOs are affected by factors such as the geographical location, environment, the stage of maturity and method of extraction. This chemical difference is directly correlated to dissimilarity in biological activities. EOs can inactivate fungi by disrupting structure and function of cell wall/membrane, mitochondria, biofilm, and mycotoxin synthesis (Nazzaro *et al*., 2017). EOs and their components, therefore, has been shown to inhibit the development of a variety of fungi (Karpiński, 2020; Abd-Rashed *et al*., 2021). For instance, EOs from *Thymus, Artemisia, Malaleuca, Cinnamomun, Ocimum, Zataria, Rosmarinus, Origanum, Syzigium, Foenicolum, Thapsia, Tachyspermum, Myrtus* have been demonstrated to eliminate the growth of *malassezia* genus. Furthermore, EOs from *Cymbopogon citratus* and *C. flexuosus* have been formulated in shampoo, cream or lotion for the successful treatment of dandruff and pityriasis versicolor in two clinical trials (Donato *et al.*, 2020). EOs from *Mentha arvensis* (*M. arvensis*) and *Piper betle* (*P. betle*) plants are widely used in Vietnam, and capable of killing several fungi and bacteria. However, the chemical compounds and inhibitory effects of these two EOs on the development of *malassezia* spp. have not been characterized.

In this study, chemical compositions of the essential oils (Eos) from *M. arvensis* and *P. betle* was analyzed by Gas chromatography–mass spectrometry (GC-MS) while inhibitory effects and minimum inhibitory concentrations of the single or combined EOs against *M. globosa* and *M. furfur* species were identified by agar diffusion and dilution assays. The anti-*malassezia* kinetics curves of *M. arvensis* and *P. betle* EOs were determined by kill-time analyses.

Materials and methods

Plant materials

P. betle Leaves and whole *M. arvensis* plants (except roots) with flowers were collected at the Hanam and Bacninh province. The materials were washed thoroughly with tap water, and then, with sterilized distilled water before EO isolation.

EO isolation

Fresh materials (100 g each) were grinded to 0.5 mm to 1.5 mm and subjected to hydro distillation in a conventional Clevenger type apparatus

containing 300 ml of distilled water for 3 hours at 100°C. The EOs were dried over anhydrous sodium sulfate and kept in sterile tubes at 4°C until use.

GC/MS analyses

GC/MS were analyzed according to the method described by Sparkman (2005). Briefly, GC/MS data were obtained on an Agilent Technologies HP 6890 mass spectrometer instrument using a HP-5MS column (0.25 µm x 60 m x 0.25 mm). The carrier gas was helium; temperature programming, 1 min at 40°C, rising at 3°C/min to 230°C and maintain at 230°C for 10 min. Duplicate analysis was performed. Quantitative results are mean data derived from GC analysis.

Fungal strains

Malassezia furfur VNF01 and *Malassezia globosa* VNG02 were provided by the Center of Experimental Biology - National Center for Technological Progress while the *Malassezia furfur* ATCC 14521 were purchased from ATCC.

Agar diffusion assay

Agar diffusions assays were conducted as described by Hadacek and Harald (2000). Briefly, 50 µl of each *Malassezia* strain (10^6 cells/ml) were spread on mDixon agar plate (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). A 9 mm well was generated at the middle of each plate. 50 µl of a serial dilluted EO was added to each well. 50 µl of DMSO (Sigma-Aldrich, cat # 67-68-5) was added to the well of the negative control plate because EOs was dilluted in DMSO (Makkar *et al*., 2018). The plates were kept at 4°C for 4 hours, and then transferred to 30°C. The inhibition zones (plate circles without visible fungi) were measured after 72 hours of incubation at 30°C. Each experiment was repeated 3 times, and included 3 replicates for each condition.

Agar dilution assay

Agar dilution methods were performed as demonstrated by Lambert and Pearson (2000). Briefly, 75 µl of a serial dilluted EO (either alone or in combination) was added to each plates containing 7.5 ml of mDixon agar media. 75 µl of DMSO was added to the negative control plate. 5 µl of each cell concentration (10^6, 10^5, 10^4 cells/ml) were dropped on each plate with 3 technical replecates. The plates were incubated at 30°C. The visible growth was accessed at the third day. Each experiment was repeated 3 times.

Kill-time analyses

The kill-time curve assay was modified from Joray *et al*. (2011). Briefly, broth media with 10^6 yeast cells/ml were supplemented with either DMSO or a mixture of *M. arvensis* (1 µl/ml) and *P. betle* (0.5 µl/ml) and incubated at 5, 10, 20, and 30 minutes. At selected time intervals, samples from the test culture were taken, serially diluted in the same broth media, and plated in the agar plate media. All plates were then incubated at 30°C, and CFU were counted after 72 hours of incubation. Percent of cell death were calculated by 100% - % of living cells. % of living cells = (concentration of cells at 5, 10, or 20 minutes divided to concentration of cells at zero time point) x 100.

Results

Chemical profiles of M. arvensis and P. betle oils

The averaged EO yields from the *M. arvensis* and *P. betle* were 1.09% and 0.25%, respectively (data not shown). The components of the two EOs. GC-MS detected 11 compounds, accounting for 99.87% of *M. arvensis* EO, while GC-MS analyses of *P. betle* EO identified to be 4 compounds, which composed of 78.43% of the *P. betle* EO. Menthol and menthone, the two major compounds of *M. arvensis* EO, comprised 68.19% and 22.77% of the EO content, respectively while eugenol acetate and m-eugenol, the two most abundant molecules in *P. betle* EO, constituted 38.66% and 30.28% of the EO, respectively (Tables 1 and 2).

Table 1. Chemical composition of *M. arvensis* EO

Compound	Relative composition ratio, %	Compound	Relative composition ratio, %
α-pinene	1.45	α - bourbonene	0.12
α-Myrcene	1.35	Camphene	0.04
D-Limonene	1.73	Eucalyptol	0.53
I-menthone	22.77	α -santalol	0.33
dI-menthol	68.19	Bis(2-ethylhexyl)phthalate	0.55
Menthyl Acetate	2.81	**Total**	**99.87**

Table 2. Chemical composition of *P. betle* EO

Compound	Relative composition ratio, %	Compound	Relative composition ratio, %
Chavicol	3.71%	Eugenol acetate	38.66%
m-Eugenol	30.28%	Chavicol acetate	5.78%
	Total	**78.43%**	

Inhibitory effects of M. arvensis and P. betle oils

The anti-*malassezia* activities of of *M. arvensis* or *P. betle* EOs was evaluated against *M. furfur* ATCC 14521 and VNF01 strains, and *M. globosa* VNG02 strain by using disc diffusion assays. *M. arvensis* EO showed stronger inhibition than *P. betle* one (Figure 1). The fungi fully covered the discs with 50 µl of DMSO (negative controls) after culturing for three days (bottom panel). On other hand, no visible yeast cells were appeared in the discs with 50 µl of *M. arvensis* 100% EO (top panel) while the fungal growth were inhibited by 30-43% in the discs containing 50 µl of *P. betle* 100% EO (middle panel) in the same experimental conditions as the negative control.

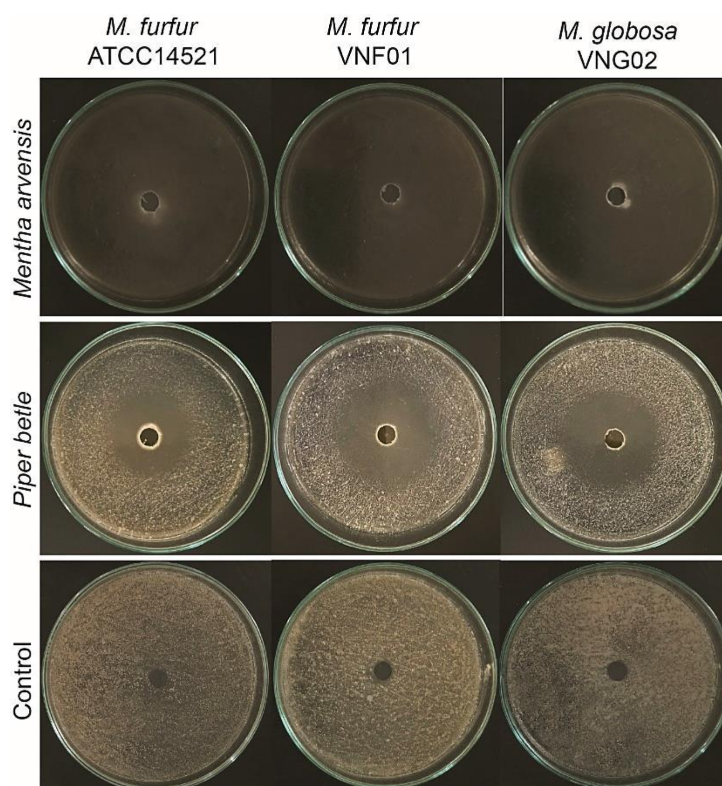

Figure 1. Anti-*malassezia* properties of *M. arvensis* and *P. betle* EOs against 10^6/ml of *M. furfur* (ATCC 14521 and VNF01), and *M. globosa* (VNG02) cells

Minimum inhibitory concentration of M. arvensis and P. betle oils

The minimum inhibitory concentrations (MIC) of single or combination of *M. arvensis* and *P. betle* EOs were identified by agar dilution assays. As shown in Figure 2A, the visible growth of *M. furfur*

ATCC 14521 and VNF01 strains, and *M. globosa* VNG02 strain was completely absent when cultured with *M. arvensis* oil at the concentration of 5 µl/ml and 2.5 µl/ml, but the colonies were visible at 1 µl/ml, suggesting that MIC of *M. arvensis* EO was 2.5 µl/ml. Similarly, MIC of *P. betle* oils was identified at the concentration of 1 µl/ml (Figure 2B).

To evaluate the additive/synergic effects of *M. arvensis* and *P. betle* EOs on the development of *M. furfur* ATCC 14521 and VNF01 strains, and *M. globosa* VNG02 strain, different combinations of these two EOs were added to agar medium plates. Among 9 combinations, the mixtures of *M. arvensis* and *P. betle* essential oils with the ratios of 2.5 + 1 µl/ml; 2.5 + 0.5 µl/ml; 2.5 + 0.25 µl/ml; 1 + 1 µl/ml; 1 + 0.5 µl/ml; 0.5 + 1 µl/ml, respectively; completely suppressed visible extension of all three fungal strains (Figure 2C).

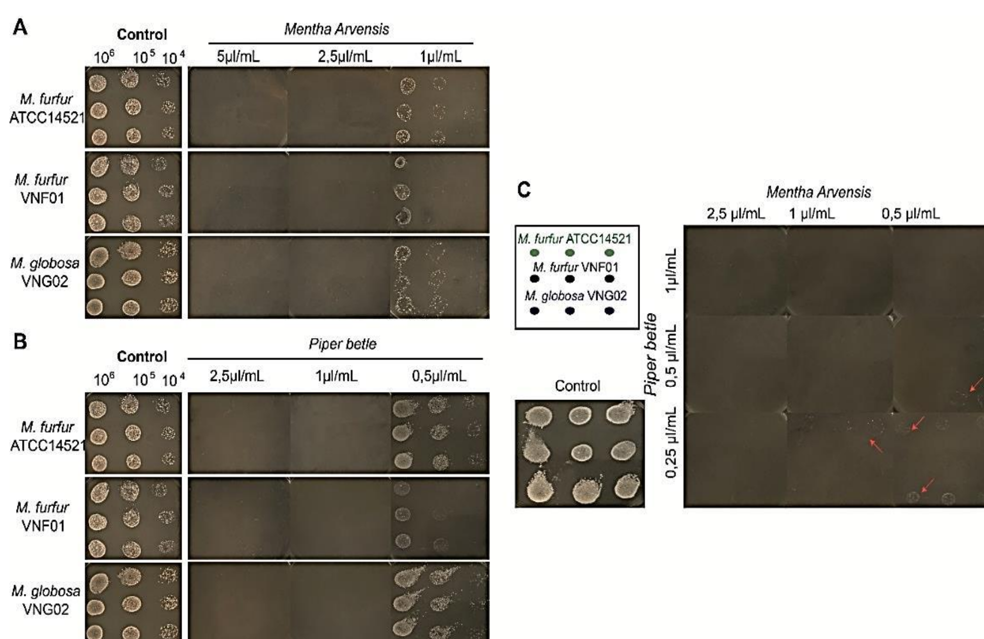

Figure 2. Minimum inhibitory concentrations of single or combination of *M. arvensis* and *P. betle* EO. Inhibitory growth by *M. arvensis* EO (A), *P. betle* EO (B), and their combination (C) at different dilution

Anti-*malassezia* kinetics of *M. arvensis* and *P. betle* EO

The anti-*massezia* kinetics curves of *M. arvensis* and *P. betle* EOs were presented in Figure 3. The number of cells treated with DMSO did not vary over 30 minutes of incubation. However, the fungi inoculated with a mixture of 1 µl/ml *M. arvensis* and 0.5 µl/ml *P. betle* EOs increased proportions of cell deaths gradually and was peaked at 20 minutes. The cell death ratios started to drop after 30 minutes of incubation. After 20 minutes

of treatment, the *M. furfur* VNF01 strain had the highest percent of cell deaths (90%), while those of *M. furfur* ATCC 14521 and *M. globosa* VNG02 strains were 78% and 80%, respectively. However, the number of cell deaths were slightly decreased after 30 minutes of incubation.

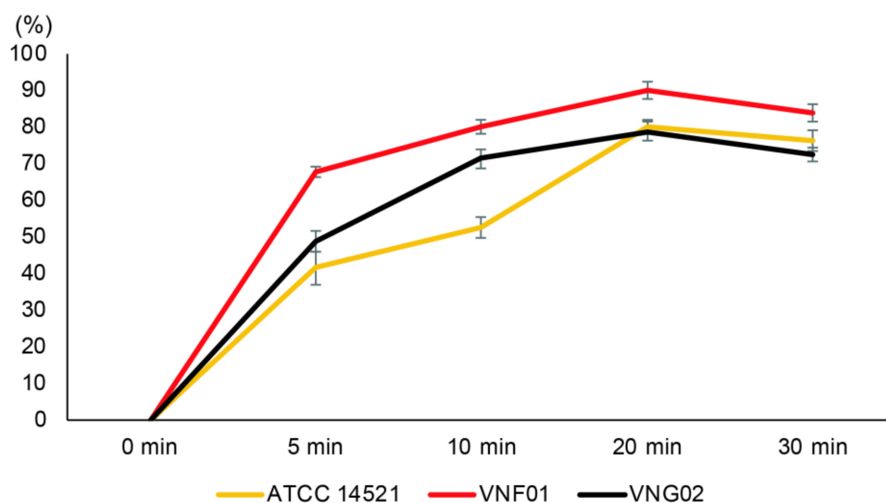

Figure 3. Anti-*malassezia* kinetics linears of *M. arvensis* and *P. betle* EOs against 10^6 CFU/ml *M. furfur* (ATCC 14521, VNF01) and *M. globosa* (VNG02) yeast cells

Discussion

EOs are complex mixtures of chemically distinct molecules. EOs mainly contain teroenoidic compounds, but nitrogenous derivatives, glucosinolates or isothiocyanate constituents, coumarins or furocoumarins and short-chain aliphatic molecules also present in EOs of certain plants (Friedrich, 1976). EOs usually compose of 2 – 3 main components, which could be ascribed to their major biological activities. Nevertheless, the additive or synergistic actions have also been evidenced (Franz, 2010). Chemical components of EOs are affected by factors such as the geographical location, environment, the stage of maturity and method of extraction. This chemical difference is directly correlated to dissimilarity in biological activities of different samples. EOs can inactivate fungi by disrupting structure and function of cell wall/membrane, mitochondria, biofilm, and mycotoxin synthesis (Nazzaro *et al.*, 2017). EOs from *M. arvensis* and *P. betle* plants are widely used in Vietnam, and capable of killing several fungi and bacteria. However, their chemical components and anti-*malassezia* properties have not been characterized. Here, we present the chemical compositions and anti-*malassezia* attributes of EOs from *M. arvensis* and *P. betle* cultivated in Vietnam.

M. arvensis leaves contain 0.4 - 0.8% of EO, which is usually dominated by menthol (above 60%) and menthone (4 - 18%) (Kalemba and Agnieszka, 2020). Average EO yield from *M. arvensis* grown in Bacninh, Vietnam were ranged from 0.9 – 1.09%, which were slightly higher than that reviewed in Kalemba and Agnieszka, 2020. Menthol (68.19%) identified in this study was in the range of literature review whereas menthone (22.77%) were faintly elevated. These data indicate that the yield and lead compound proportions of EO from *M. arvensis* cultured in Bacninh, Vietnam were similar to that reported in literatures. Consistently, the Vietnamese *M. arvensis* EO has the MIC values of 2.5 µl/ml for *M. furfur* and *M. globose* species, which is in the MIC ranges (0.25 to 3 µl/ml) of *M. arvensis* EO against a variety of bacteria and fungi (Kalemba and Agnieszka, 2020).

P. betle plants consist of 0.1% to 0.15% of EO, whose chemical components belong to the classes of monoterpenes, sesquiterpenes, phenylpropanoids, and aldehydes. Chemical constituents of *P. betle* EO vary depend on botanical origin, plant ages, and harvesting time, but eugenol, carvacrol, chavicol, chavibetol (m-eugenol) are usually major molecules identified in *P. betle* leaf EO (Nayaka *et al*., 2021). Our *P. betle* plants contains 0.2% - 0.3% of EO, whose main components were eugenol acetate, chavibetol, chavicol, chavicol acetate. Of these compounds, eugenol acetate and chavibetol (m-eugenol) were the two dominant compounds and accounted for 38.66% and 30.28%. Again, our EO yield was higher than, but contains similar compound as that reported in literature. Accordingly, our agar dilution assays showed that MIC of *P. betle* EOs were 1 µl/ml, which is slightly higher than MIC values (0.3 µl/ml to 0.7 µl/ml) against 14 fungal species identified in (Prakash *et al*., 2010).

This study also revealed the additive effects of *M. arvensis* and *P. betle* EOs against two *malassezia* species, that is a combination of a half dose of the MIC value of each EOs can inhibit visible growth of all fungi tested. Even though the mechanism(s) underlying this phenomenon remains to be clarified, the additive property may depend on the combined actions of the EOs' main components. Eugenol has been reported to change or disrupt fungal cell wall structure, causing cell deaths due to dysfunction of membrane fluidity and permeability. Furthermore, eugenol acetate and chavicol acetate were predicted to strongly interact to amino acid constructing fungal protein structures, and therefore may reduce fungal survivability (Nayaka *et al*., 2021). *M. arvensis* EO and its lead compounds have been shown to kill *Candida* species by inhibiting ergosterol biosynthesis and PM-ATPase activity (Samber *et al*., 2015). Altogether, these data suggest that the combination of *M. arvensis* and *P. betle* EOs may enhance killing capacity by targeting different cell organelles. Moreover, kill-time assays revealed the mixture of *M. arvensis* and *P. betle* EOs can

eliminate up to 90% of *M. furfur* and 80% of *M. globosa* after 20 minutes of treatment.

This is the first study to demonstrate that *M. arvensis* and *P. betle* EOs can inhibit the development of members of *malassezia* genus with the MIC of 2.5 µl/ml and 1 µl/ml, respectively. This work also showed that *M. arvensis* and *P. betle* EOs additively eliminated *M. furfur* and *M. globosa* species, probably by disrupting structures and/or functions of different organelles. Furthermore, *M. arvensis* and *P. betle* EOs were capable of killing up to 90% of *M. furfur* and 80% of *M. globose* species after 20 minutes of treatment. Altogether, this study suggested that *M. arvensis* and *P. betle* EOs can be potential agents for formulating shampoo, cream or lotion to treat *malassezia*-associated disease.

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