IJPSR (2017), Vol. 8, Issue 5 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 13 October, 2016; received in revised form, 06 December, 2016; accepted, 16 December, 2016; published 01 May, 2017

EFFICACY OF MASSOIA OIL IN COMBINATION WITH SOME INDONESIAN MEDICINAL PLANTS OILS AS ANTI-BIOFILM AGENT TOWARDS CANDIDA ALBICANS

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Keywords:

Essential oils, Biofilm, *Candida albicans*, Checkerboard method, Synergism

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ABSTRACT: Microbial resistance to antibiotics is often caused by biofilm formation of the microbial pathogen. One strategy used to combat this recalcitrance mechanism is by using the combination of antimicrobial drugs. Essential oils have evoked interest as sources of natural products and represent an alternative approach in combating microbial pathogen. The aim of the present work was to investigate the possible synergistic activity of *Massoia aromatica* oil in combination with *Cinnamomum burmanii*, *Ocimum basilicum*, *Citrus hystrix* and *Piper betle* oils against *Candida albicans* biofilm. Biofilm formation inhibition assay and biofilmdegradation assay of essential oils were determined using microtiter broth method. The Fractional inhibitory concentration indices (FICI) of essential oils in combinations were calculated from the checkerboard assay. The synergistic activity was found in combination of *M. aromatica* oil with all the essential oil tested in inhibit the formation of intermediate stage of *C. albicans* bio-film. The interaction of *M. aromatica* oil with all the essential oil tested in inhibit the formation of mature stage of *C. albicans* biofilm were varied from synergistic to neutral, however, the activity of essential oils combination in breaking down the established biofilm were found to be less active. The results obtained clearly indicate that combinations of these oils are potential for enhancing their anti biofilm properties. This research could contribute to the development of new strategies to prevent and treat *C. albicans* biofilm infections.

INTRODUCTION: Opportunistic fungal infection is the leading cause of death in patients with immune-suppression such as cancer and HIV. The high rate resistance of the fungal pathogen to antifungal drugs also reduce the success of antifungal therapy. *Candida albicans* is one of the few species of the Candida genus that cause local and systemic infections in humans, and is responsible for the majority of nosocomial infections and for 50-90% of all cases of invasive candidiasis in humans [1, 2].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.8(5).2013-25

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.8 (5).2013-25

The ability of *C. albicans* in causing the disease is closely related to its ability to grow as a biofilm community. The ability of Candida to attach and colonize a variety number of surfaces, including surfaces of medical devices such as IUDs (intrauterine devices), implants or prosthesis and form a biofilm makes Candida resistant to antifungal drugs [3].

Biofilm itself is a natural condition where microbes tend to live in colonies instead of planktonic (drifting free, individualistic, and quickly divide), attached to a surface and secrete an extracellular polymeric substance (EPS). EPS binds multiple layers of microbial cells and produce biofilm matrix, and serves as a protection against external factors that might harm microbial growth such as antimicrobials and immune systems.

Microorganisms in biofilm are generally more resistant to antimicrobial compounds and the host's defense mechanisms than planktonic cells. This fact makes biofilm a source of progressive infections. It is difficult for antimicrobial compounds to provide the intended effect on microbes because of the EPS barrier, and also because microbes grow in biofilms have a slower growth rate compared to when these microbes are in planktonic state [4]. In the human body biofilm commonly encountered in dental plaque, in the lungs of patients cystic fibrosis, and in the medical devices implanted as implants in the body such as contact lens, artificial joint in the joints, pacemakers, catheters, IUD, etc. [3].

Nowadays biofilm infection is become a major problem in health, since microbial communities within the biofilm matrix is highly resistant to antimicrobial agents. The National Institutes of Health (NIH) states that biofilms are the cause of the majority of infections in humans [5]. In many cases, the treatment of biofilm infection caused by microbial biofilms on implants has to be done by removing the implant and replace it with the new one, and this procedure is time consuming, high cost and often life-threatening. To date there are very few chemotherapeutic agent that has the ability to kill microbes in a biofilm phase [6].

Although the mechanism of resistance against Candida biofilms antifungal drugs is still poorly understood, anti-fungal action likely prevented by its ability to penetrate the biofilm matrix [7]. The ability of Candida biofilm to grow well on the surface of implants such as catheters, prosthetic heart valves, contact lenses, artificial joints and the intra-urine (IUD) which can cause bloodstream infections are a major cause of morbidity and mortality among hospitalized patients [8, 9].

Natural compounds derived from medicinal plants are known to have contribution in the development of modern medicine and in the formulation of herbal preparations in traditional medicine systems. Essential oil is one of the natural compounds that are considered quite promising for the discovery of new antimicrobial agents [10]. The combination use of potent antifungal compounds is one of the strategies to improve the efficacy of antifungal drugs. The mechanism of this combination is expected to reduce the efficacious dose of antifungal drugs, minimize its side effects, and provide solutions to overcome the problem of fungal resistance [11].

In this study we have analyzed the possibility of synergistic activity of *Massoia aromatic* oil in combination with other essential oils against *Candida albicans* biofilm, which could increase antifungal drug effectiveness, thus are expected to be able to reduce the level of Candida resistance. Considering the fact that some essential oils such as massoia oil are quite irritating, it is expected that by combining essential oils could reduce the irritant properties without reducing its effectiveness in inhibiting and / or breakdown the *C. albicans* biofilm. In our previous study [12] we explored the activity of *M. aromatica* oil against *P. aeruginosa* and *S. aureus* biofilm, however, to our knowledge, up to date this is the first study that was carried out to evaluate the synergy of *M. aromatica* oil with *Piper betle, Cinnamomum burmanii, Ocimum basilicum* and *Citrus hystrix* oils against *Candida albicans.* In this study, the minimum inhibitory concentration of the essential oils tested, both in single and combinations, towards the growth of *C. albicans* planktonic and biofilm is discussed. TLC and GCMS were also performed to determine the content of essential oil compounds tested.

MATERIALS AND METHODS:

Fungal strain and growth condition: A standard strain of *Candida albicans* ATCC 10231 was cultured in Sabouraud planktonic Dextrose Broth (SDB) medium and incubated at 37°C with agitation (120 rpm) for 24 h. Following incubation, cells were sedimented by centrifugation (5000 x *g* for 15 min at 4˚C), washed twice with 5 mL of sterile PBS (phosphate buffered saline) buffer pH 7.2, and finally suspended to 10^7 cells/mL by adjusting the optical density of the suspension to 0.38 at 520 nm[13, 14].

Medicinal plants and essential oil extraction: A list of the plants studied, including the botanical name and voucher specimen are listed in **Table 1**. The plants were collected from Yogyakarta, Indonesia and its surroundings on the basis of ethno pharmacological information. The species were

identified, authenticated, and voucher specimens were preserved in Department of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia for further reference. Plant essential oils were obtained by water-steam distillation process. Oil samples obtained were dried over anhydrous sodium sulphate (Na_2SO_4), filtered using a whatman filter paper no. 40, and stored in sealed dark glass vial at 4°C for further use.

Essential oil's chemical composition analysis: Gas chromatography mass spectrometry (GC-MS) was used to identify the main constituents of essential oils tested according to the method of Wu *et al*. [15] on a GC-2010 gas chromatography (Shimadzu, Japan) equipped with a GC-MS-QP2010 Plus mass spectrometer (Shimadzu, Japan). An Rxi-5MS capillary column (30 m length, 0.25 mm diameter, 0.25 µm film thickness, Shimadzu, Japan) was used for separation. A split injector was used and diluted samples (1/100 in ethyl acetate, v/v) of 1.0 mL were injected by an auto sampler in the split mode (1/153). The oven temperature was programmed from 60°C to 290°C at a rate of 10°C ml^{-1}. Helium was used as the carrier gas. Qualitative identification of compounds was performed by comparisons of their relative retention times and mass spectra with those recorded in the National Institute of Standards and Technology (NIST) database. Quantitative analysis of each essential oil component (expressed as area percentage) was carried out by peak area normalization measurement.

Determination of minimum inhibitory concentration (MIC) of plant oils: Minimum Inhibitory concentration (MIC) of plant essential oils against *C. albicans* planktonic cells (PMIC) were determined using microdilution method measured by optical density. The PMIC assays were performed in triplicate in microtiter plates and used an inoculums of 1 x 10^3 CFU/mL in SDB. The final volume including SDB, cells and test compound in each well was 200 µl. The assay of plant essential oils involved in serial, twofold dilutions using SDB, starting at 1% v/v and ending at 0.06 % v/v in methanol. As positive control, Nystatin with concentration of 500 µg/mL was used, and as vehicle control, wells received SDB, Methanol (MeOH) and *C. albicans* inoculums. Negative control wells received only SDB and *C. albicans* inoculums. Inhibition was monitored based on OD_{595} of treated vs control after 48 hours of incubation at 37°C [16]. $PMIC_{50}$ was calculated using probit analysis.

Effect of plant essential oils on *C. albicans* biofilm formation inhibition and breakdown: The susceptibility study of *C. albicans* biofilm to essential oils tested, alone and in combinations was carried out using micro broth dilution method [17]. Biofilms were formed on polystyrene flat bottom 96-well microtiter plates (Iwaki). Briefly, 100 µL of a standardized cell suspension (10^7 cells/mL) on RPMI 1640 medium without sodium bicarbonate supplemented with L-glutamine (Sigma) was transferred into each well of a microtiter plate, and the plate was incubated for 90 minutes at 37 ˚C of adhesion phase. RPMI 1640 medium was used because this medium capable to induce hyphal formation in *C. albicans*. For media control, wells should be unseeded, and in negative control, biofilms were not exposed to antifungal agent. Following the adhesion phase, the cell suspension were aspirated and each wells was washed twice with 150 µL of PBS to remove loosely adhered cells. A total of 100 µl RPMI media containing various concentration of plant oils alone and in combinations (in ratios 1;1) were added to the washed wells. Nystation concentration of 500 µg/mL was usedas a positive control in this study, and methanol (MeOH) was used for vehicle control. The plates were then incubated at 37˚C for 24 hours for intermediate phase biofilm, and 48 hours for mature phase biofilm. Quantification of biofilm formed was done using XTT reduction assay using microtiter plates reader (Bio-Rad 680 XR) at 495 nm. Testing was performed in triplicate. Plant oils found to reduce at least 50% biofilm formation were considered as biofilm preventive, $MBIC_{50}$ [18].

To determine the effects of single and combined essential oils on *C. albicans* pre-formed biofilms, *C. albicans* biofilms were grown for 24, and 48 h at 37˚C on the wells of microtiter plates using the protocol described by Yu *et al.* [18]. Briefly, 100 µL of a standardized cell suspension (10^7 cells/mL) on RPMI 1640 medium without sodium bicarbonate supplemented with L-glutamine (Sigma) was transferred into each well of a microtiter plate, and

the plate was incubated for 24 and 48 hours at 37˚C. Following biofilm formation, the medium was aspirated and non adherent cells were removed by washing the biofilms three times in 150 µL sterile PBS per well. After each wash, residual PBS was remove by blotting the microtiter plates in an inverted position with paper towels. Various concentrations of plant essential oils (in RPMI 1640 medium), alone and in combinations, were then added to the washed wells and the plates were incubated at 37˚C for another 24 and 48 h. The capability of plant essential oils to breakdown *C. albicans* established biofilms were estimated using the XTT reduction assay as describes below.

XTT reduction assay: XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl) - 2H- tetrazolium -5 carboxa nilide) (Sigma) solution (0.5 g liter^{-1} PBS) was prepared, filter-sterilized using a 22-µm pore size filter, aliquot into 10 mL working volumes, and stored at -70˚C. A stock solution of 10 mM menadione (Sigma) in 100% acetone was prepared, filter sterilized, aliquot into smaller volume (about 50 µL) and stored at -70˚C. Prior to each assay, tubes containing 10 mL XTT solution were thawed and 1 µL of the stock solution of menadione was added to each tube of XTT solution to achieve a final menadione concentration of 1 µM.

The biofilm were first washed three times with 200 µl PBS, and then 100 µL of the XTT-menadione solution were added to each of the prewashed wells. The microtiter plate was then covered with aluminium foil and incubated in the dark for 2-3 h at 37˚C. Following incubation, 75-80 µL of the resulting colored supernatant from each wells was transferred to a new microtiter plate and the color change in the solution was measured with a microtiter plate reader at 495 nm. The absorbance values for the media controls were subtracted from the values for the test wells to calculate the minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC). $MBIC_{50}$ and $MBEC_{50}$ is the antifungal concentrations at which a 50% decrease in absorbance is detected in comparison with the control biofilms formed by the fungal isolate in the absence of antifungal drug [19]. Dilutions of Massoia oil alone and its mixtures with other oils tested were prepared in a checkerboard format as per standard methodology. In order to assess results of MBIC and MBEC of oil in mixtures, the fractional inhibitory concentrations indexes (FICI) were calculated as: ΣFIC = FICa + FICb, where FICa is a MIC of massoia oil in combination / MIC of massoia oil alone; and FICb is a MIC of second oil in combination / MIC of second oil alone. Second oil is the oil which was tested in combination with massoia oil. FICI were interpreted as follow: synergism FIC $\leq$ 0.5; indifference 0.5<FIC$\leq$4; antagonism FIC>4 [20].

Statistical methods: Statistical significance of the data was determined using ANOVA, followed by Dunnett's test. Differences were considered significant with *P* values of 0.05 or less.

RESULTS AND DISCUSSION:

Essential oil yields: Oil yields of the plant samples is presented in **Table 1**. Most plants had oil yield around 0.25-2.5 % w/w, and largest quantities were obtained from *Citrus hystrix* oil (20 % w/w).

TABLE 1: ESSENTIAL OIL YIELDS

Family	Binomial name	Local name	Voucher number	Volume oil obtained (mL)	Sample fresh weight (Kg)	Yield (% v/w)[a]
Lauraceae	*Massoia aromatica* Becc.	Masoyi	STP053	3	3	2
	Cinnamomum burmanii Nees ex Bl.	Manis jangan	STP051	20	3	6.67
Piperaceae	*Piper betle*	Sirih	STP099	10	5	2
Rutaceae	*Citrus hystrix* DC.	Jeruk purut	STP031	5	3	1.67
Labiatae	*Ocimum basilicum* L	Kemangi	STP046	2.5	5	0.5

GC-MS analysis: The principal components of the oils were determined using gas chromatography and identified by comparing the mass spectra of chemical compounds in essential oils with library mass spectra from NIST02 (www.nist.gov/index.html). In this study, the analysis by GC-MS showed that the major component of *C. burmanii* oil was cinnamic aldehyde (92.0 %). Alpha-copaene and 3-Phenyl-2-prophenyl acetate were present in small amount (4.10 % and 2.07 %, respectively), whereas massoia lactone (92.1 %) was the main constituent

of *M. aromatica* essential oil, and benzyl benzoate was found in small amount (2.67%). Massoia lactone has a simple molecular structure with 10, 12 and 14 carbon chain components so that each one is called the C-10, C-12 and C-14 massoia lactone. This lactone is alleged to have role in the antimicrobial activity of Massoia oil [21, 12].

TABLE 2: MAJOR CHEMICAL CONSTITUENTS OF *C. BURMANNII* AND *M. AROMATICA* ESSENTIAL OIL AS IDENTIFIED BY GC-MS

Essential oil sample	Peak	Retention time (RT)	Area	Area (%)	Similarity index (SI)	Chemical Component
C. burmannii	3	19.296	164665734	92.02	95	Cinnamaldehyde
	5	21.630	7342238	4.10	93	Alpha-Copaene
	7	23.472	3708184	2.07	91	3-Phenyl-2-prophenyl acetate
M. aromatica	3	25.346	156590945	92.05	94	5-Hydroxy-2-Decenoic acid lactone (Massoialactone)
	4	29.961	10522375	6.19	86	5-Hydroxy-2-Decenoic acid lactone (Massoialactone)*
	5	37.473	1835119	1.08	80	Benzoic acid
O. basilicum	1	11.877	6622297	6.56	96	6-Methyl-5-hepten-2-one
	2	15.188	9535731	9.44	97	L-linalool
	3	17.305	33293823	32.96	93	Z-citral
	4	17.959	47262357	46.79	94	Geranial
C. hystrix	2	9.117	2839381	11.86	93	Limonene
	3	15.581	8004641	32.03	93	Z-citral
	4	16.497	13273920	53.12	93	E-citral
P. betle	3	11.496	11878056	40.01	96	Sabinene
	5	12.142	3567823	12.02	97	Beta-Myrcene
	7	16.823	2739801	9.23	97	Linalool
	9	23.513	3072053	10.35	96	Anethole
	10	29.397	1040838	7.26	96	Beta-Caryophylene

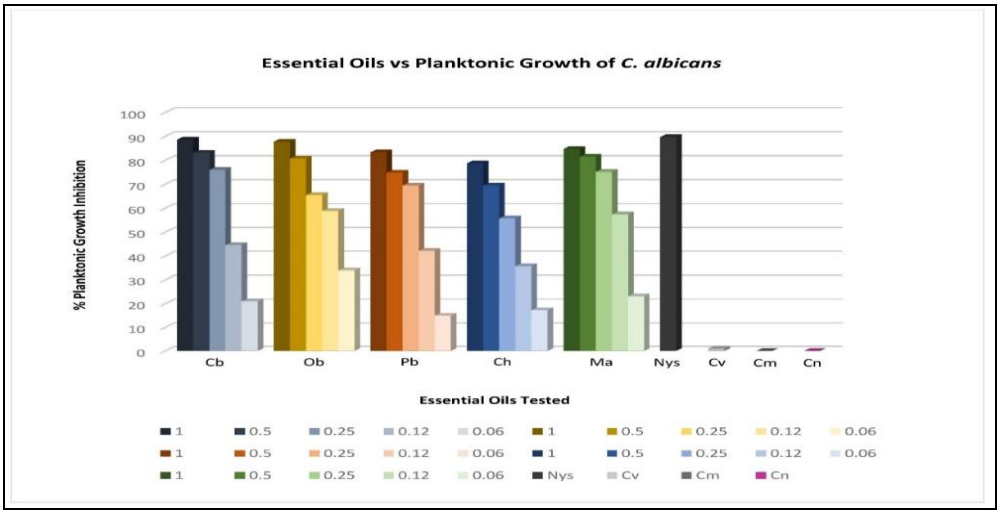

FIG. 1 : PERCENTAGE OF *C. ALBICANS* PLANKTONIC GROWTH INHIBITION BY *C. BURMANII* (CB), *O. BASILICUM* (OB), *P. BETLE* (PB), *C. HYSTRIX* (CH), *M. AROMATICA* (MA) ESSENTIAL OIL AT CONCENTRATION RANGING FROM 1 – 0.06 % V/V. CONTROLS WERE: CV (VEHICLE CONTROL, METHANOL 1% v/v), CM (MEDIA, SDB), CN NEGATIVE CONTROL), NYS (NYSTATIN, POSITIVE CONTROL) 500 µG/ML.

From GCMS result we found out that the major chemical composition of the volatile oils from *O. basilicum* was Z-citral (32.03%), and Geranial (46.79%). The major components of *C. hystrix* oil were limonene (11.86%), Z-citral (32.03%), and E-citral (53.12%) citronelal (54.63%) and 3-Tetradecanol (30.31%), whereas the main constituents of *P. betle* were Sabinene (40.01%),

Beta-Myrcene (12.02%) Linalool (9.23%), Anethole (10.35%), and Beta-Caryophylene (7.26%) **(Table 2)**.

Screening and determination of MIC plant oils for Candida planktonic growth: The screening for anti candida activity and determination of PMIC of plant oils obtained by microdilution method are shown in **Table 2** and **Fig. 1**. Majority of oils tested were found to have anti-Candida activity and only three of the oils (*P. cubeba, S. oleosa* and *C. odoratum*) were found failed to inhibit *C. albicans* growth at the highest concentration tested which was 1 % v/v. The oil from *C. burmanii, M. aromatica, O. basilicum* and the seeds of *L. cubeba* at lowest concentration tested (0.06 % v/v) were effective at inhibiting partial (50%) growth ($PMIC_{50}$) of *C. albicans*.

Effect of plant essential oils on *C. albicans* biofilm formation: *Candida albicans* biofilm formation is proceeding in intermediate phase of development (12 – 30 h) [22]. Plant essential oils at sub MIC concentration of 0.25% - 0.01% v/v was tested against *C. albicans* adherent cells populations at different stages of biofilm development. *C. albicans* biofilm formation occurs in three phases. The early phase is characterized by budding-yeast cell attachment to surfaces. The attached cells are proliferate, form micro colonies and deposit an extracellular matrix. Furthermore, Candida yeast cells transition into mycelia (dimorphism), and micro colonies are interconnected with the elongated hyphae, forming a confluent monolayer. The complexity of the biofilm increase after 24 hours, and *C. albicans* biofilm is composed of a mixture of yeast cells immersed among threads of pseudo-hyphae and true hyphae [22].

Using the MTT method, we have found that the inhibition of *C. albicans* biofilm formation by essential oils was dose dependent. The result of this experiment is shown in **Table 3** and **Fig. 2**, demonstrated that partial (50%) inhibition of *C. albicans* intermediate biofilms occurred in the presence of all oils tested. *C. burmanii* oil showed $MBIC_{50}$ at concentration of 0.02% v/v. At concentration of 0.03 % v/v this oil inhibited as much as 58.83 ± 0.03 % of *C. albicans* biofilm formation whereas at concentration of 0.01 % v/v gave as much as 33.97±0.00 % *C. albicans* biofilm formation.

The oils from the bark of *M. aromatic* exhibited the $MBIC_{50}$ at concentration of 0.04 % v/v in order to inhibit the intermediate phase of *C. albicans* biofilm. Compare to *C. burmani* and *M. aromatica* oils, the oil obtained from the leaves of *O. basilicum*, *P. betle,* and *C. hystrix* showed less potent activity in inhibit intermediate and mature phase of *C. albicans* biofilm. Higher concentration (0.05% v/v, 0.09 % v/v, and 0, 19% v/v) were needed by these oils to inhibit intermediate phase of *C. albicans* biofilm **(Fig. 2, Table 3)**.

TABLE 3: EFFECTS OF DIFFERENT ESSENTIAL OILS ON PLANKTONIC GROWTH AND BIOFILM FORMATION OF *CANDIDA ALBICANS* ATCC 10231. THE $PMIC_{50}$ FOR GROWTH WAS TESTED IN THE RANGE OF 1–0.06 % V/V, WHEREAS THE $MBIC_{50}$ FOR BIOFILM FORMATION AND $MBEC_{50}$ FOR BIOFILM BREAKDOWN ACTIVITY WERE TESTED AT SUB-PMIC

Oil Sample	$PMIC_{50}$ (% v/v)	$MBIC_{50}$ oil in % v/v against 24 h Candida biofilm formation	$MBIC_{50}$ oil in % v/v against 48 h Candida biofilm formation	$MBEC_{50}$ oil in % v/v against 24 h established Candida biofilm	$MBEC_{50}$ oil in % v/v against 48 h established Candida biofilm
Cinnamomum burmanii Nees ex Bl.	0.22	0.02	0.09	0.01	0.05
Citrus hystrix DC	0.19	0.19	0.57	0.50	0.51
Piper betle L.	0.21	0.09	0.09	0.10	0.50
Ocimum basilicum L.	0.08	0.05	0.10	0.06	0.11
M. aromatica Becc	0.09	0.04	0.11	0.10	0.11

Higher concentration of oil was needed by all of the oils tested to eradicate the established biofilm. Oils from the bark of *M. aromatica* and *P. betle* showed capability in partial disrupting (50%) the intermediate formed Candida biofilm at concentration of 0.10% v/v **(Fig. 3, Table 3)**,

whereas lower concetration (0.01 % v/v and 0.04 % v/v) was needed by *O. Basilicum* and *C. hystrix* oil to be able to disrupt established *C. albicans* intermediate biofilm. However, higher concentrations were needed by all oils to be able to breakdown *C. albicans* mature biofilm **(Fig. 3, Table 3)**.

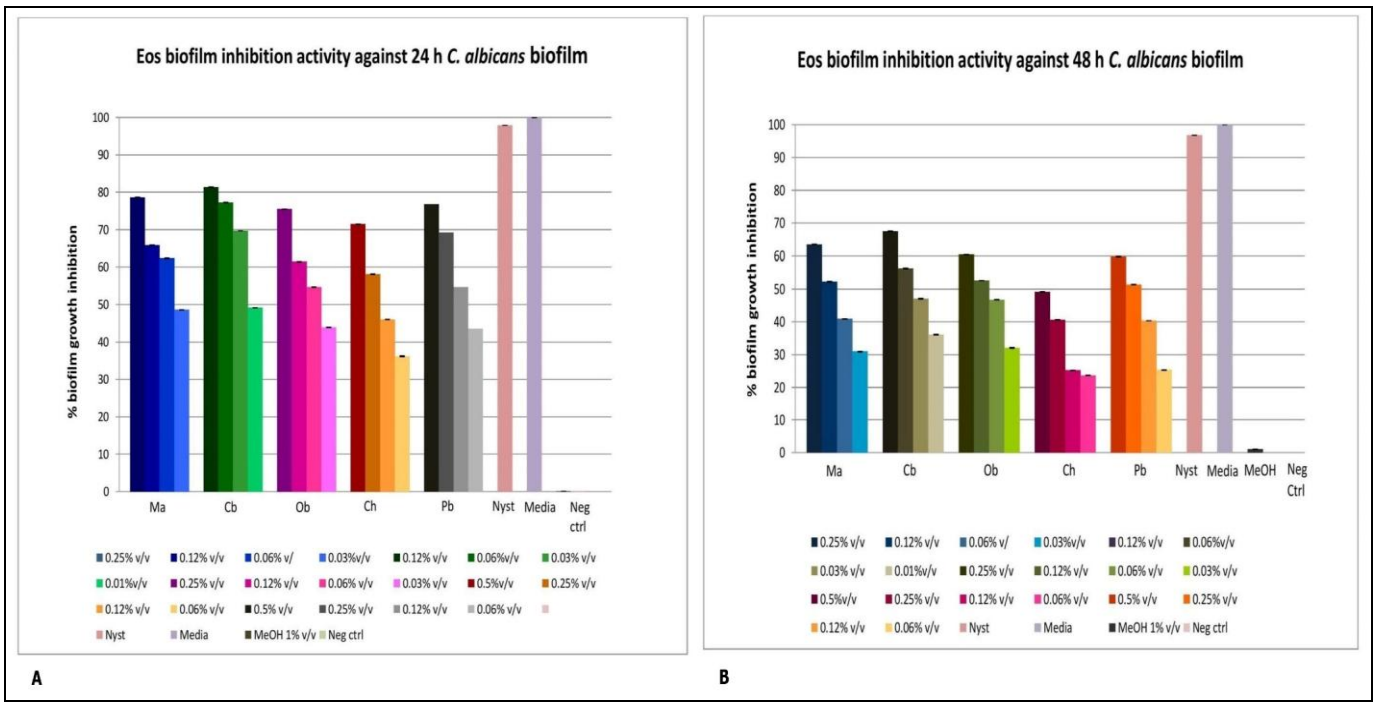

FIG. 2: THE PERCENTAGE ACTIVITY OF *C. BURMANII* (CB), *O. BASILICUM* (OB), *P. BETLE* (PB), *C. HYSTRIX* (CH), *M. AROMATICA* (MA) ESSENTIAL OIL AT CONCENTRATION RANGING FROM 1 – 0.06 % V/VININHIBIT *CANDIDA ALBICANS* BIOFILM FORMATION AT INTERMEDIATE PHASE (A), AND AT MATURE PHASE (B). THE BARS INDICATED THE STANDARD DEVIATIONS OF THE MEANS.

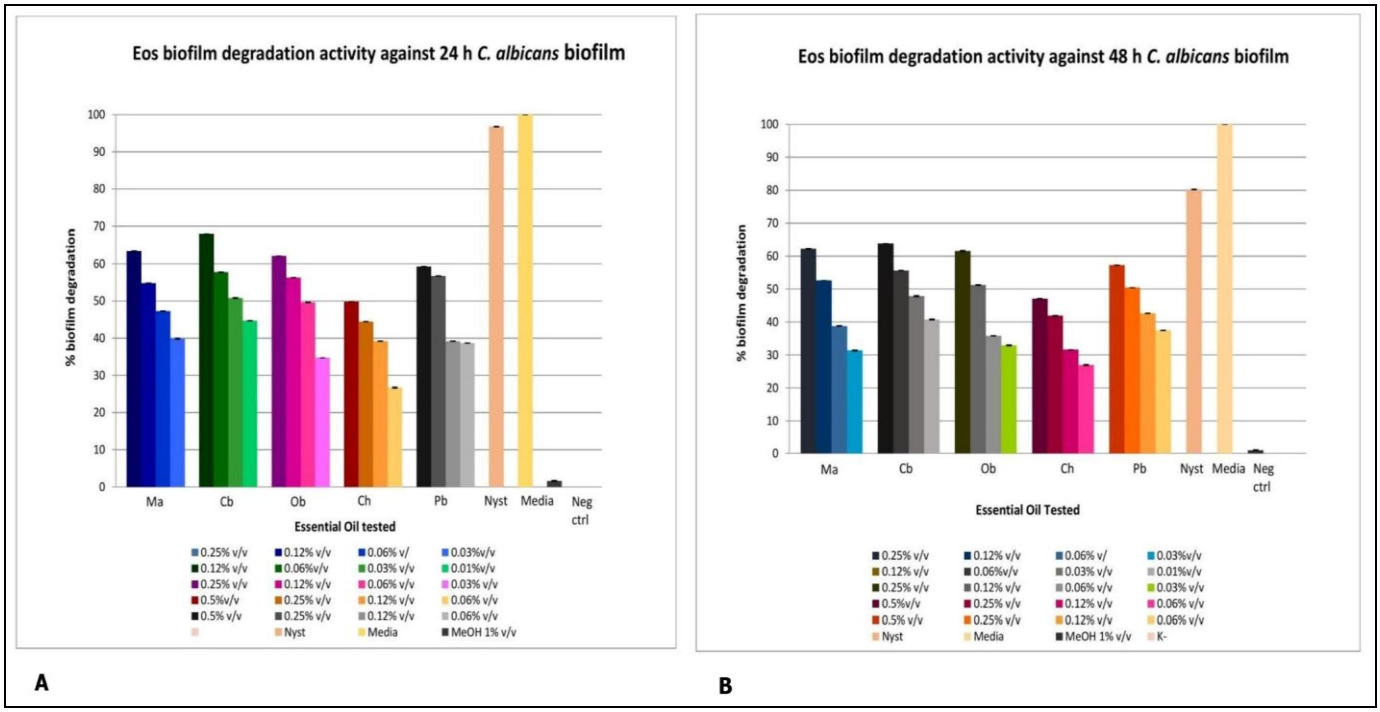

FIG. 3: THE PERCENTAGE ACTIVITY OF *C. BURMANII* (CB), *O. BASILICUM* (OB), *P. BETLE* (PB), *C. HYSTRIX* (CH), *M. AROMATICA* (MA) ESSENTIAL OIL AT CONCENTRATION RANGING FROM 1 – 0.06 % V/VINBREAKINGDOWN ESTABLISHEDINTERMEDIATE PHASE OF *CANDIDA ALBICANS* BIOFILM (A), AND MATURE PHASE OF *CANDIDA ALBICANS* BIOFILM (B). THE BARS INDICATED THE STANDARD DEVIATIONS OF THE MEANS.

Effect of Massoia Oil in Combinations towards *C. albicans* biofilm: Anti-biofilm activity of essential oils tested in combination against intermediate phase and mature phase of *C. albicans* biofilm was determined using the microdilution method on microtiterplate polystyrene flat-bottom 96 wells in accordance with the test protocols by Coffey and Anderson [19].

Data obtained from different concentrations of oil tested ($MBIC_{50}$ and sub $MBIC_{50}$) mediated inhibition and disruption of preformed intermediate phase and mature phase of *C. albicans* biofilm, and Massoia oil in combination with other oils at concentration of $MBIC_{50}$ and $subMBIC_{50}$ showed higher activity in inhibit and breakdown preformed intermediate phase and mature phase of *C. albicans* biofilm. The result showed that combination of Massoia oil with *C. burmanii* oil, both at $MBIC_{50}$ concentration (0.04 % v/v) could inhibit 86.70±0.78 % of *C. albicans* intermediate biofilm, whereas 50% of biofilm inhibition was obtained from the combination of Massoia oil at concentration of $PMIC_{50}$ with *C. burmanii* at $subPMIC_{50}$ concentration (0.008 % v/v), which in turn showed a synergistic effect of both oil in inhibit the growth of *C. albicans* biofilm. *C. albicans* intermediate phase of biofilm development was also partially (50%) inhibited by the mixture of Massoia oil at $MBIC_{50}$ concentration with *O. basilicum* concentration of 0.01% v/v.

The mixture of these two oils also showed a synergistic activity towards *C. albicans* biofilm development. Moreover, the combination of $MBIC_{50}$ concentration of Massoia oil with *C. hystrix* oil and *P. betle* oil at $subPMIC_{50}$ concentrations (0.05 % v/v and 0.03 % v/v, respectively) also give partial inhibition towards *C. albicans* intermediate phase development, although the combinations activity of those oils were found to be indifference (**Table 4, Fig. 4)**.

TABLE 4: FIC AND FICI VALUES DETERMINATION OF MASSOIA OIL IN COMBINATION AGAINST INTERMEDIATE PHASE OF *C. ALBICANS* BIOFILM.

Eos	$MBIC_{50}$ (% v/v) Oil alone	$MBIC_{50}$ of oil in mix with Massoia oil	FIC_{50}	$FICI_{50}$	Interpretation
M. aromatica	0.04	-	-	-	
C. burmanii	0.04	0.008	0.20	0.24	Synergy
O. basilicum	0.05	0.01	0.20	0.24	Synergy
C. hystrix	0.09	0.05	0.55	0.59	Indifference
P. betle	0.05	0.03	0.6	0.64	Indifference

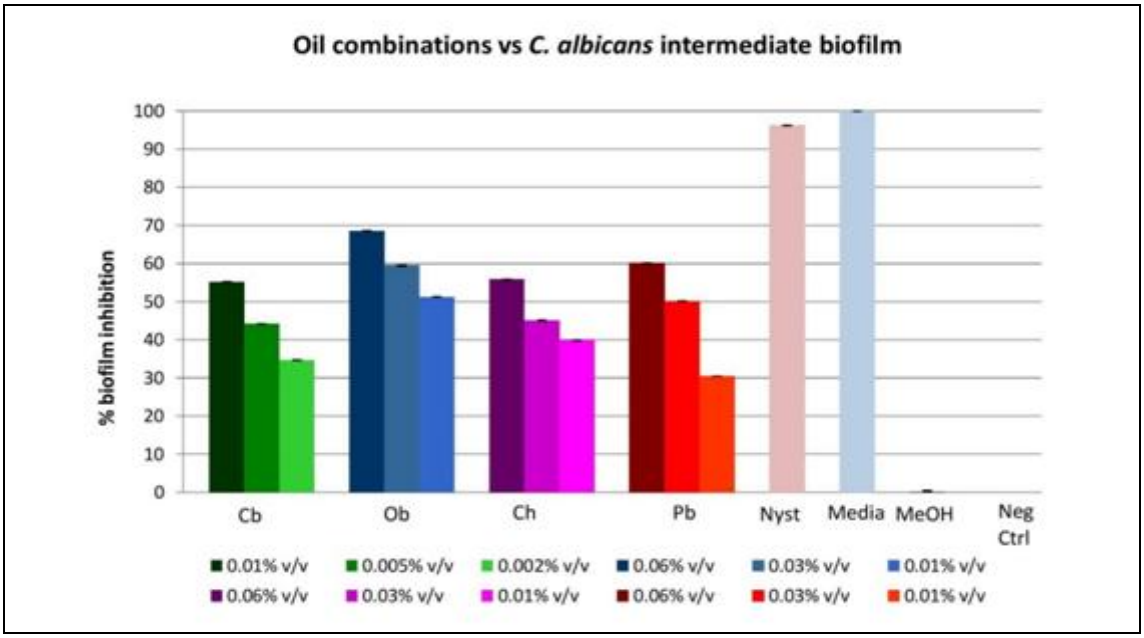

FIG. 4: INHIBITORY ACTIVITY OF ESSENTIAL OILS IN COMBINATION AT $MBIC_{50}$ CONCENTRATIONS TOWARDSS THE INTERMEDIATE PHASE OF *C. ALBICANS* BIOFILM. NYSTATIN (500 µG/ML) WAS USED AS POSITIVE CONTROL. MEDIA = MEDIA CONTROL, NEG CTRL = NEGATIVE CONTROL, AND MEOH (METHANOL) AS CONTROL SOLVENTS. CB: *CINNAMOMUMBURMANII*, OB: *OCIMUMBASILICUM*, PB: *PIPER BETLE*, CH: *CITRUS HYSTRIX*, MA: *MASSOIA AROMATICA*

TABLE 5: FIC AND FICI VALUES DETERMINATION OF MASSOIA OIL IN COMBINATION AGAINST MATURE PHASE OF *C. ALBICANS* BIOFILM.

Eos	$MBIC_{50}$ (% v/v) Oil alone	$MBIC_{50}$ of oil in mix with Massoia oil	FIC_{50}	$FICI_{50}$	Interpretation
M. aromatica	0.11	-	-	-	
C. burmanii	0.09	0.02	0.22	0.33	Synergy
O. basilicum	0.10	0.11	1.1	1.21	Indifference
C. hystrix	0.57	0.15	0.26	0.37	Synergy
P. betle	0.09	0.11	1.22	1.33	Indifference

Similar result was also found towards mature phase of *C. albicans* biofilm. The combination of Massoia oil at $PMIC_{50}$ concentration (0.11 % v/v) with sub MBIC50 of *C. burmanii* oil (0.02 % v/v) and with *C. hystrix* oil at sub MBIC50 concentration (0.25 % v/v) could inhibit 50% of *C. albicans* mature phase biofilm, and give synergistic activity against mature phase of *C. albicans* biofilm. However, to be able to give 50% of *C. albicans* biofilm inhibition in combination with $MBIC_{50}$ concentration of Massoia oil, higher concentration of $MBIC_{50}$ was needed by *O. basilicum* oil and *P. betle* oil, i.e. 0.11% v/v. These combinations of oil also showed indifference activity compare to their activity alone towards mature phase of *C. albicans* biofilm **(Table 5, Fig. 5)**.

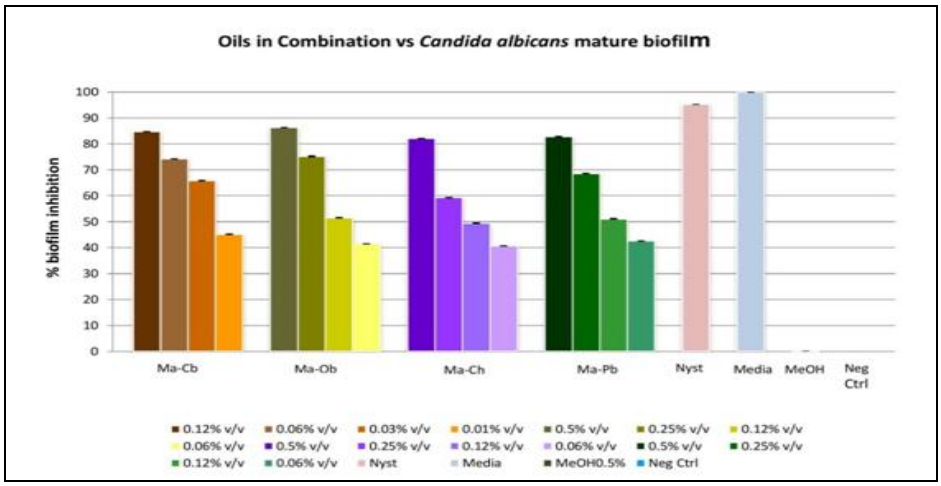

FIG. 5: INHIBITORY ACTIVITY OF ESSENTIAL OILS IN COMBINATION AT $MBIC_{50}$ CONCENTRATIONS TOWARDSS THE MATURE PHASE OF *C. ALBICANS* BIOFILM. NYSTATIN (500 µG/ML) WAS USED AS POSITIVE CONTROL. MEDIA = MEDIA CONTROL, NEG CTRL = NEGATIVE CONTROL, AND MEOH (METHANOL) AS CONTROL SOLVENTS. CB: *CINNAMOMUMBURMANII*, OB: *OCIMUMBASILICUM*, PB: *PIPER BETLE*, CH: *CITRUS HYSTRIX*, MA: *MASSOIAAROMATICA*.

TABLE 6: FIC AND FICI VALUES DETERMINATION OF MASSOIA OIL IN COMBINATION AGAINST INTERMEDIATE PHASE OF PREFORMED *C. ALBICANS* BIOFILM.

Eos	$MBEC_{50}$ (% v/v) Oil alone	$MBEC_{50}$ of oil in mix with Massoia oil	FIC_{50}	$FICI_{50}$	Interpretation
M. aromatica	0.01	-	-	-	
C. burmanii	0.50	0.06	0.12	0.13	Synergy
O. basilicum	0.10	0.23	2.3	2.31	Indifference
C. hystrix	0.06	0.26	4.33	4.34	Antagonism
P. betle	0.10	0.25	2.5	2.51	Indifference

Up to the highest levels of the test (0.5% v / v), essential oil of *O. basilicum*, *C. hystrix*, and *P. betle* combined with Massoia oil failed to degrade the mature phase of *C. albicans* biofilm. Masoia oil in combination with *C. burmanii oil* produces antagonism effect. This is likely due to the architecture of mature phase *C. albicans* biofilm which is composed of a dense network of yeast cells, and intertwined hyphae and pseudohyphae, embedded in anexopolimeric matrix substance. The structural complexity of *C. albicans* biofilm makes biofilm more resistant to antimicrobial agents compare to its planktonic counterpart.

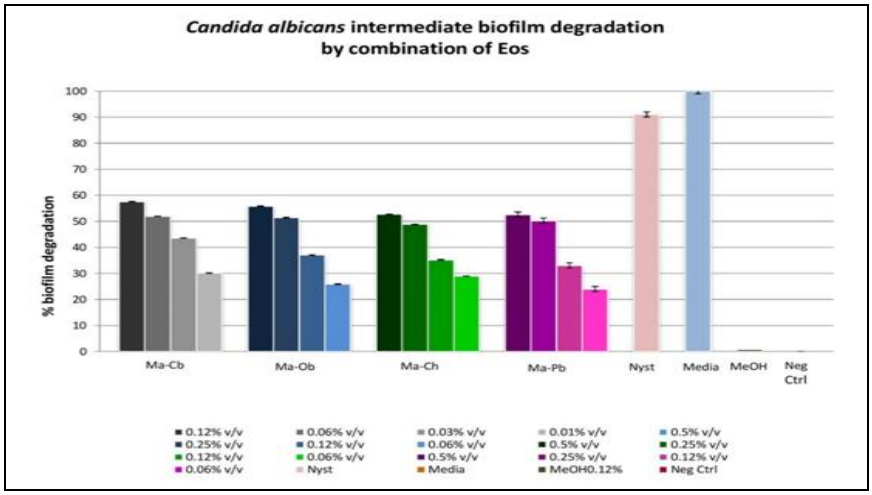

FIG. 6: BREAKDOWN ACTIVITY OF ESSENTIAL OILS IN COMBINATION AT $MBEC_{50}$ CONCENTRATIONS TOWARDSS THE INTERMEDIATE PHASE OF PREFORMED *C. ALBICANS* BIOFILM. NYSTATIN (500 µG/ML) WAS USED AS POSITIVE CONTROL. MEDIA = MEDIA CONTROL, NEG CTRL = NEGATIVE CONTROL, AND MEOH (METHANOL) AS CONTROL SOLVENTS. CB: *CINNAMOMUMBURMANII*, OB: *OCIMUMBASILICUM*, PB: *PIPER BETLE*, CH: *CITRUS HYSTRIX*, MA: *MASSOIAAROMATICA*.

TABLE 7: FIC AND FICI VALUES DETERMINATION OF MASSOIA OIL IN COMBINATION AGAINST MATURE PHASE OF PREFORMED *C. ALBICANS* BIOFILM.

Eos	$MBEC_{50}$ (% v/v) Oil alone	$MBEC_{50}$ of oil in mix with Massoia oil	FIC_{50}	$FICI_{50}$	Interpretation
M. aromatica	0.05	-	-	-	
C. burmanii	0.51	0.12	0.23	0.28	Synergy
O. basilicum	0.50	0.47	0.94	0.99	Synergy
C. hystrix	0.11	0.45	4.09	4.14	Antagonism
P. betle	0.11	0.27	2.45	2.50	Indifference

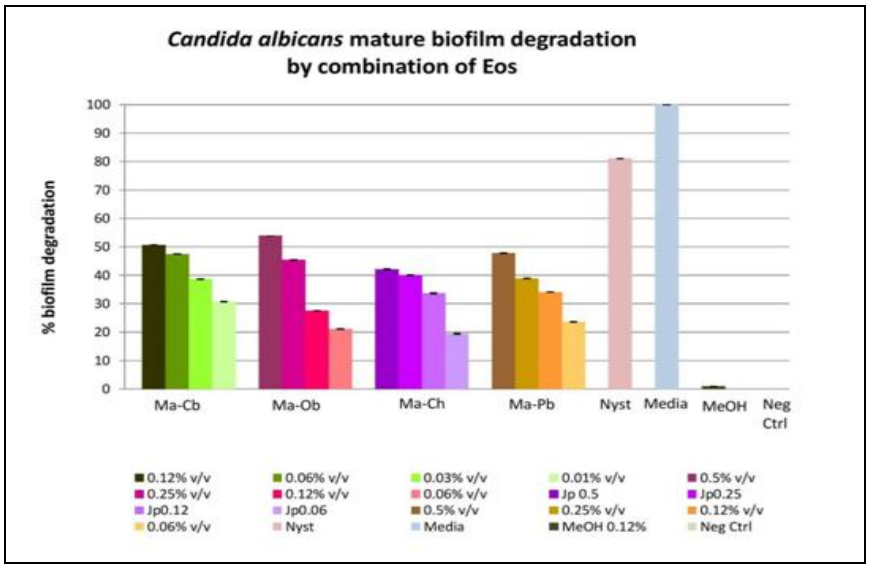

FIG. 7: BREAKDOWN ACTIVITY OF ESSENTIAL OILS IN COMBINATION AT $MBEC_{50}$ CONCENTRATIONS TOWARDSS THE MATURE PHASE OF PREFORMED *C. ALBICANS* BIOFILM. NYSTATIN (500 µG/ML) WAS USED AS POSITIVE CONTROL. MEDIA = MEDIA CONTROL, NEG CTRL = NEGATIVE CONTROL, AND MEOH (METHANOL) AS CONTROL SOLVENTS. CB: *CINNAMOMUMBURMANII*, OB: *OCIMUMBASILICUM*, PB: *PIPER BETLE*, CH: *CITRUS HYSTRIX*, MA: *MASSOIA AROMATICA*.

Therefore, higher concentration of antimicrobial compounds needed to be able to penetrate the biofilm's exopolysaccharide matrix and kill microbial cells inside it. The discovery of new anti-biofilm compounds are indispensable as a therapeutic option for biofilm related infections *C.albicans*, and currently focused to natural products with anti biofilm activity.

A 1, 8-cineole, main component of eucalyptus oil for instance, showed a high anti biofilm activity against *C. albicans* biofilm [23]. Terpenes, which is the main component of essential oil, known by its antifungal activity. Terpene derivatives such as carvacrol, geraniol and thymol are also known to exhibit potent activity against biofilm [24, 25]. *C. burmanii* essential oil has cinnamaldehyde (44.2%) and eugenol (90.2%) as its main compounds [26]. Both cinnamaldehyde and eugenol have antifungal activity against *C. albicans* [27]. According to Narong [28], the main component of *P. betle* essential oil is eugenol (63.39%) and acetyleugenol (14:05%).

Biofilm associated *C. albicans* infections were reported by various studies to have tolerance towards the commonly antifungal drugs. Therefore, combination of antifungal agents might be a good strategy to overcome this problem [29]. From the result above, combinatorial approach of essential oils against planktonic form of *C. albicans* was found quite promising. However only limited studies are available on bio-films, and result obtained from studies using planktonic form may not always work in biofilm setup. Biofilm was found more resistant towards essential oils tested compare to the planktonic form. Higher concentration of oils, in single or in combinations, was needed to be able to inhibit the formation of biofilm as well as to disrupt the established biofilm of *C. albicans* compare to planktonic form. However, in mixture, lower concentration of essential oils might be needed to inhibit and to breakdown *C. albicans* biofilm as a result of synergistic activity of essential oils mixture.

According to Kon and Rai [30], effects of interactions between essential oils which provide the synergistism activity depends on the interactions of the essential oils components. Essential oils with phenolic compound such as eugenol, possess high antimicrobial activity. Hydroxyl group of eugenol may react with proteins and inhibit enzyme activity. Eugenol also capable to destroy the cytoplasmic membrane, alter membrane fluidity and integrity, which will eventually lead to the cell wall damage and causes the release of intracellular materials [31]. Cynnamaldehyde which is a main component in *C. burmanii* oil, also known to have high antimicrobial activity by inhibiting microbial's energy metabolism, and interaction with cell membrane leading in disruption of cell membrane and leakage of the cell content. Antifungal activity of cinnamaldehyde is influenced by its ability to inhibit the synthesis of β- (1, 3)-glucan synthase in the cell walls of fungi, which affect the structure of the fungal cell wall [32]. Synergy between massoia lactone with cynamaldehyde, citral and geranial may play a key role in the synergism effect between massoia oil, cinnamomum oil, and citrus oil against *C. albicans* bio-film. Essential oils important characteristics are their hydrophobicity which enable them to breakdown lipid component of fungal membrane, disturbing cell structure resulted in cell death from the leakage of fungal cell [32].

Although Khan and Ahmad [27] have reported that cinnamaldehyde and eugenol, the main component of *C. burmanii* and *P. betle* oil have anti biofilm activity, and Dagli *et al*. [33] also reported that *C. burmanii* could be used for the treatment of oral candidiasis, the activity of Massoia oil in combination of with other oils tested against *C. albicans* biofilm has never studied. The effectiveness of essential oils in combinations against *C. albicans* biofilm is an interesting thing to enhance the role of essential oils as anti biofilm compounds, which can be used in the development of new strategies for treating infectious diseases caused by microbial bio-films.

ACKNOWLEDGEMENT: The authors are gratefully acknowledge the funds support of this research by Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia. The authors also thankful to Yogaswara Tawang Gumbara and Rahajeng Elok Hapsari for the technical assistance in biofilm assay.

CONFLICT OF INTEREST: The authors declare that they are no conflict of interest regarding this manuscript.

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How to cite this article:
Pratiwi SUT and Hertiani T: Efficacy of massoia oil in combination with some indonesian medicinal plants oils as anti-biofilm agent towards *Candida albicans*. Int J Pharm Sci Res 2017; 8(5): 2013-25.doi: 10.13040/IJPSR.0975-8232.8(5).2013-25.