Anti-microbial and anti-biofilm compounds from Indonesian medicinal plants

Pratiwi, S.U.T.

Chapter 6

Efficacy of Massoia lactone as an Anti-biofilm Agent

Pratiwi, S.U.T.[1,2], Hertiani, T.[2], Lagendjik, E.L.[1], de Weert, S.[1,3], Van den Hondel, C.A.M.J.J[1*]

1. Section of Molecular Microbiology and Biotechnology, Institute Biology Leiden, Leiden University, Sylviusweg 72, 2333 BE Leiden, The Netherlands
2. Department of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University, Sekip Utara, Yogyakarta 55281, Indonesia
3. Koppert Biological Systems, Veilingweg 14, 2650 AD Berkel en Rodenrijs, The Netherlands. sdweert@koppert.nl

*Corresponding author.

Email address : c.a.m.van.den.hondel@biology.leidenuniv.nl

ABSTRACT

Massoia lactone ($C_{10}H_{16}O_2$), also known as *(R)*-5,6-dihydro-6-penthyl-2H-Pyrane-2-one was isolated from *Massoia aromatica* bark essential oil. Preparative Thin Layer Chromatography (TLC), followed by Gas Chromatography-Mass Spectrometry (GC-MS) and 1D- ^{1}H-Nuclear Magnetic Resonance (NMR) analysis were used to isolate and elucidate this compound. The effect of massoia lactone on microbial biofilm was evaluated. The result showed that this compound exhibit anti-biofilm activity against *Candida albicans* ATCC 10231, *Pseudomonas aeruginosa* PAO1 and *Staphylococcus aureus* Cowan I ($MBIC_{50}$ at concentration of 25, 50 and 100 µg/mL). The anti-biofilm activity of massoia lactone also found to be three times greater when in combinations with anti-fungal/anti-bacterial. These result indicated that massoia lactone displayed potent activity against microbial biofilms *in vitro* and therefore has potential therapeutic implication for biofilm-associated microbial infections.

Keywords : massoia lactone, TLC, GC-MS, NMR, biofilm

INTRODUCTION

Massoia aromatica (synonym : *Cryptocarya massoy* (Oken) Kosterm) is an evergreen tree belongs to the family of Lauraceae. It is a medium-sized tree, growing up to 15 m tall, with optimal growth in rainforests between 400 m and 1000 m altitude. This plant is a rare plant species that grow in Indonesia, distributed mostly in Maluku and West Papua (Gunawan, 1999).

Local people common used massoia woods as building materials, and the bark is used as a mixture of red dye in batik making. In the islands of Seram, Maluku Indonesia, fishermen are commonly utilize masoyi leaves to fill pillows, and used to warm their heads when they go to sea (Guenther, 1972). Javanese and Balinese women are commonly use massoia bark powder to prepare a warming ointment, called bobory, which is used to lubricate the body, especially on days cold and rainy. The result makes the body feels warm, and it is believed to help alleviate complaints to various diseases. Massoia tree bark has a pleasant, sweet coconut-like aroma and is steam distilled to yield massoia bark oil that is used as the fragrance materials in incense manufacture (Hapsari and Simanjuntak, 2010).

The pleasant smell of massoia oil is believed to be capable of stimulating the emergence of sexual desire. In some areas in Indonesia, this plant is used as a medicinal plant to treat asthma, for a carminative (a herb or preparation that either prevents formation of gas in the gastrointestinal tract or facilitates the expulsion of said gas, thereby combatting flatulence), diaphoretic (herbs that induce prespiration, sweating and in return it will help to reduce fever, cool the body and speed the elimination of toxins from the system) (Heyne, 1987).

Previous studies mentioned that massoia oil was traditionally used to treat diarrhea, abdominal cramps, fever, tuberculosis, muscle pain, headache and chronic constipation, vaginal discharge, fever, anti-inflammatory agent, and as herbal postpartum. It is also believed that massoia oil has antiinsect and antifungal activity, since canoe or boat made of wood masoyi known to be able to resist insects and fungi (Lily, 1980; Gunawan, 1999; Bustanussalam *et al*, 2012).

Up to now, not much scientific research on the efficacy of massoia oil for health treatment that has been done. Thus, the mechanism underlying the efficacy of oil masoyi in modern medicine has not been widely known. From several studies it is known that massoia wood could act as a counter irritant (moisten capillaries) and aromaticum (fragrance materials, used as a mixture of fragrant incense). A study by Widowati and Pudjiastuti (1999) noted that infuse (water decoction) of massoia bark has properties of analgesics (pain relievers). A research by Sa'roni and Adjirni (1999) revealed that massoia bark also has activity as an anti-inflammatory.

Our previous study showed that beside the capability of inhibition of planktonic growth and biofilm formation, *M. aromatica* essential oil also showed activity in breaking the biofilm of *Pseudomonas aeruginosa* PAO1, *Staphylococcus aureus* Cowan I and *Candida albicans* ATCC 10231. The oil also showed activity in inhibit quorum sensing (QS) using QS reporter strain *Chromobacterium violaceum* CV026 and *P. aeruginosa* PAO1. In the experiments reported here, the active compound found in *M. aromatica* oil was isolated and screened for antibiofilm activity against *P. aeruginosa* PAO1, *S. aureus* Cowan I and *C. albicans* ATCC 10231.

MATERIALS AND METHODS

Plant Material

As much as five kilograms of *M. aromatica* bark were obtained from Nabire district, West Papua, Indonesia. A voucer specimen was identified and deposited at Department of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University, Indonesia. The essential oil was obtained by steam-hydrodistillation process. The oil sample was dried over anhydrous sodium sulphate (Na_2SO_4), stored in sealed dark glass vial, and kept at 4°C.

Planar chromatographic separation and analysis

Chromatography was performed on 10 × 20 cm silica gel 60 F_{254} aluminium sheet Thin Layer Chromatography (TLC) plates (Merck, Germany). Before use, the plates were preconditioned by heating at 120°C for 3 h. Without this procedure adsorbent layers became partly detached when soaked. Pure samples of eugenol, cynamaldehyde, and 1,8-cineole used as standards were obtained from Sigma Aldrich, dissolved in toluene to give solutions containing 1 µL/30 µL, and 3 µL was applied to the 10 x 20 cm TLC silica gel F_{254} type plates, using Minicaps capillary pipettes (West Germany). For the solution of the essential oils 100 µL was dissolved in 1 mL ethanol, and 5 µL of it was applied to the 10 x 20 cm TLC silica gel F_{254} type plates with Minicaps capillary pipettes. The position of the starting line was 1.0 cm from the bottom and 1.0 cm from the left side. The standards of the main components of the essential oils were applied to the TLC plates next to the spots of the oils. After the sample application, the TLC plates were developed with the previously optimized mobile phase. For the separation of essential oils, toluene:ethyl acetate, 93:7, as a mobile phase is recommended (Wagner and Bladt, 1996). The development mode was ascendant chromatography in a saturated twin-trough chamber (Camag, Switzerland). All TLC separations were performed at room temperature (20°C). For the bioautographic assay, solvents applied in TLC developing systems were also tested.

After chromatographic separation, the absorbent layers were dried in an oven at 90°C for 5 min to remove the solvent completely. An alcoholic vanillin–sulphuric acid reagent was used to visualize the separated compounds. The developed layers were dipped into this reagent and heated for 5 min at 100°C. Detection of the separated compounds was performed according to R_f (retardation factor) value: the ratio of the distance traveled by the center of a spot to the distance traveled by the solvent front; and colour of the standards. The TLC plate for bioautography was processed in parallel without final development with reagent (Wagner and Bladt, 1996; Horváth *et. al*., 2010).

Bioautography analysis

Agar diffusion or contact bioautography was used for the bioautography analysis. *P. aeruginosa* PAO1, *S. aureus* Cowan I and *C. violaceum* ATCC 31532 (wild type strain) were grown overnight in 100 mL Luria Bertani (LB) broth, at 37°C for *S. aureus* and 28°C for *P. aeruginosa* and *C. violaceum* in a shaker incubator for 24 h to an optical density (600 nm) of 1.0 or greater. The bacterial suspension was diluted with fresh LB to OD_{600} = 0.1, which corresponds to approximately 1×10^8 colony-forming units (CFU)/mL. *C. albicans* ATCC 10231 was cultured in Sabouraud Dextrose Broth (SDB) and incubated for 24 h at 30^{o}C with agitation (250 rpm). Following incubation, cells were finally resuspended to a concentration of 10^7 CFU/mL by adjusting the optical density of the suspension to 0.38 at 520 nm.

A 100 µL sample of the microbial culture was spreaded onto 15 mL of LB Agar for bacteria and Sabouraud Dextrose Agar (SDA) for yeast in petri dishes. After the agar solidified, the chromatogram plate developed was placed face down onto the inoculated agar layer and left for 24 hour at 4^{o}C to enable diffusion. Following the incubation, the chromatogram plate was removed and the agar layer was incubated at 37°C for 24 hours. The inhibition zone was observed on the agar surface in the places where the spots of compounds are diffuses into the agar. The presence of anti-bacterial activity within a sample was indicated by a clear halo on the agar surface were the separated compounds are present. The Rf value of the inhibition zone was measured by the distance of the substance over the distance moved by the solvent (Horváth *et. al*., 2010).

Isolation of *M. aromatica* Oil Active Compound

A Preparative TLC separation of *M. aromatica* oil was performed on 10 × 20 cm silica gel 60 F_{254} aluminium sheet TLC plates (Merck, Germany) as described above by spotting the essential oil over the whole width of the starting line (streaking the plate). UV_{254} instead of stain reagent was used for

the visualization. The area (band) which is showed the same Rf value as the Rf of the inhibition zone was marked, and carefully rubbed out off using a spatula. The loosened silica then transferred onto a glass funnel where glass wool is present and ethylacetate as mobile phase is poured over them as a solvent. The eluted fraction that has been collected, dried using SpeedVac, and dissolved in ethanol was further analysed by TLC and visualized under UV_{254}. GC-MS analysis also performed to obtain information about the chemical composition of the eluted fraction.

Gas Chromatography-Mass Spectometry (GC-MS) analysis

The isolated fraction of *M. aromatica* essential oil was analysed by GC-MS according to the method of Wu *et al.* (2008) on a GC-2010 gas chromatography (Shimadzu, Japan) equipped with a GC-MS-QP2010 Plus mass spectrometer (Shimadzu, Japan). An Agilent J&W DB-1 column (30 m length, 0.25 mm diameter, 0.25 µm film thickness, Agilent) was used for separation and also a s split injector. Diluted samples (1/100 in ethyl acetate, v/v) of 1.0 mL were injected by an autosampler in the split mode (1/153). The oven temperature was programmed from 70C to 230C at a rate of $5^oC\ ml^{-1}$. Helium was used as the carrier gas. Identification of compounds was based on comparisons of their mass spectra with those recorded in the MS library database NIST (National Institute of Standards and Technology) 12, NIST62 and WILEY229. Quantitative analysis of each essential oil component (expressed as area percentage) was carried out by peak area normalization measurement. For dead-time and Kovats Retention Index determination were conducted under isothermal condition (70^oC).

NMR analysis

In order to obtain information about the structure of the isolated active compound, ^{1}H-NMR (Nuclear Magnetic Resonance) spectroscopy analysis was performed. NMR spectra was recorded using Bruker DRX 500 (^{1}H: 500 MHz, ^{13}C: 125.77 MHz). The isolated fraction (20 mg/1.0mL) was dissolved in $CDCl_3$ into a 5mm NMR tube, and the solvent signal was used for the spectra calibration (^{1}H at 7.26 ppm).

Effect of essential oil isolated compound on bacterial biofilm formation and fungal preformed biofilms

Biofilms of *C. albicans* were formed on polystyrene flat bottom 96-well microtiter plates (Sarstedt Inc., Newton, NC, USA). Briefly, 100 µL of a standardized cell suspension (10^7 CFU/mL) grown 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 h at 37^oC to yield a intermediate biofilm phase. RPMI 1640 medium was used because in this medium *C. albicans* is

capable to induce hyphal formation in *C. albicans*. Following incubation, the cell suspension was aspirated and each wells was washed twice with 150 µL of PBS to remove loosely adhered cells. A total of 100 µL concentrated compound ranging from 100 to 6.25 µg/mL in methanol (MeOH) were added to the washed wells. For vehicle control we used ethanol at concentration 1% v/v, and for the negative control the biofilm was not exposed to anti-fungal agent. The plates were then incubated at 37^{o}C for 24 h. Quantification of the biofilm formed was done using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay describes below with a microtiter plates reader (Bio-Rad 680 XR) at 550 nm. Testing was performed in triplicate. Fluconazole concentration ranging from 1,024 – 128 µg/mL was used as a positive control in this study. When a compound was found to reduce at least 50% of biofilm formation (Minimum biofilm inhibition concentration, $MBIC_{50}$), it was considered as biofilm preventive (Ramage *et al.,* 2002).

To test for the inhibition of active compounds on *P. aeruginosa* PAO1 and *S. aureus* Cowan I biofilm formation inhibition, a PVC (polyvinyl chloride) flexible U bottom 96 wells plates was used (Falcon 3911, Becton Dickinson, Franklin Lakes, NY). Compound concentrations ranging from 100-6.25 µg/mL in MeOH were used. Negative control (cells + media : TSB for *S. aureus* Cowan I and M63 supplemented with 20% casamino acid, 20% glucose and 1mM MgSO4 for *P. aeruginosa* PAO1), positive control (cells + media + streptomycin), vehicle controls (cells + media +methanol concentration 1% v/v), and media controls were included. For the positive controls of the streptomycin concentration of 1000-100 µg/mL were used. Blanks undergo the same treatment as test samples, but without incubation. All tests were performed in triplicate. The MTT assay was used to estimate and validate the effects of plant essential oils on the biofilms formation. Inhibition of biofilm formation was calculated as % of inhibition on the basis of color absorbance by using the formula mentioned below to determine the final $MBIC_{50}$ values:

[(ODgrowth control – Odsample) / ODgrowth control] x 100

ODt= optical density (595 nm) of the test well; ODmc24: optical density (595 nm) of the media control well; ODvc: optical density (595 nm) of the vehicle control well (Sandasi *et al*., 2009). The concentration at which the extract depleted the bacterial biofilm by at least 50% was labeled as the $MBIC_{50}$.

Essential oils isolated compound in combination with anti-microbial against microbial biofilm

The anti-microbial activity of isolated plant essential oils isolated in combination with fluconazole or streptomycin was assessed by the chequerboard method in triplicate (Karpanen *et al.,* 2008).

Microtiter plates containing *C. albicans* biofilms were washed three times with 200 µL sterile PBS per well to remove any unbound cells. The isolated compounds used at concentration that inhibit 50% planktonic cells and fluconazole at various concentration were prepared in RPMI 1640 medium. Next, 50 µL of the compound was added to the wells of the microtiter plates in diminishing concentrations, and 50 µL of fluconazole was added to the columns. The isolated compound alone at various concentration, fluconazole alone and saline were used as controls. The microtiter plates were incubated at 37^{o}C for 24 h (intermediate phase *Candida* biofilm).

The compound isolated from essential oil at concentration that inhibited 50% of planktonic cells and streptomycin were prepared in LB medium. Next, 50 µL of the essential oil's isolated compound solution was added to the rows of 96 well microtiter plates in diminishing concentrations, and 50 µL of streptomycin was added to the columns in diminishing concentrations. The compounds isolated from essential oil alone at various concentrations, streptomycin alone and saline were used as controls. The microtiter plates were incubated at 37^{o}C for 24 h (*P. aeruginosa* biofilm) and 48 h (*S. aureus* biofilm). Following the incubation time, the effects of plant essential oil's isolated compound on the fungal and bacterial biofilms were estimated using the MTT assay as describes below. The test compound found to reduce at least 50% of the biofilm formation was considered as biofilm preventive (Ramage *et al.,* 2002).

The analysis of the combination of massoia lactone and streptomycin or fluconazole was obtained by calculating the Fractional Inhibitory Concentration Index (FICI) as follows: FICI = (MIC of the combination of massoia lactone and streptomycin or fluconazole /MICa alone) + (MIC of the combination of massoia lactone and streptomycin or fluconazole /MICb alone), where MICa is the Minimal Inhibitory Concentration of massoia lactone from *M. aromatica* oil, and MICb is the Minimal Inhibitory Concentration of streptomycin or fluconazole. The FICI was interpreted as follows: (1) a synergistic effect when FICI ≤ 0.5; (2) an additive or indifferent effect when FICI >0.5 and <1 and (3) an antagonistic effect when FICI >1 (konate *et al*., 2012).

MTT assay

The activity of the active compound in inhibit fungal and bacterial biofilm was quantified by determining biofilm cell viability using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. MTT (Sigma) solution (5 mg/mL in PBS) was prepared, filter-sterilized using a 0.22-µm pore size filter. The preformed biofilms were first washed three times with 200 µL PBS, and then 100 µL of PBS buffer and 20 µL of the MTT solution were added to each of the prewashed wells. The microtiter plate was then incubated for 2 h at 37^{o}C. Following incubation, the PBS-MTT solutions

were removed from the wells and replaced with 100 µL of DMSO in each well to dissolve the water soluble purple formazan crystal. The color intensity of the soluble formazan was determined using microplate reader (Bio-Rad 680 XR) at 550 nm. Percentage of cell survival is expressed as the formula below:

$$\textit{Percentage cell survive} = 100\% \text{ x } \frac{\textbf{absorbance of treated cells}}{\textbf{absorbance of control cells}}$$

Qualitative analysis on *C. albicans* biofilm

A Carl Zeiss LSM 5 Exciter Laser Scanning Confocal Microscope (Leica Microsystems, Germany) was used to observe the architecture of the *C. albicans* biofilms in the presence of massoia lactone. Candida biofilms were formed by dispensing standardized cell suspension (500 µL of a suspension containing 1.0 x 10^7 cells/mL in LB medium) onto 13 mm diameter pre-sterilized plastic coverslips (Thermanox; Nulge Nunc International) placed in the wells of pre-sterilized flat bottomed twelve-well plates (Iwaki). The plate was incubated at 37^0C for 90 minutes (adhesion phase). Following incubation period, the supernatant was removed and a total of 500 µL RPMI medium containing different concentrations of massoia lactone (100 – 6.25 µg/mL) were added to the washed wells. The plate then incubated for 8 h (adherence phase), 24 h (intermediate phase) and 48 h (maturation phase) at 37 ^{0}C.

The coverslips then were washed twice with PBS and stained using the LIVE/DEAD fluorescent stains (10 µL of 3.34 µM SYTO9 and 10 µL of 20 µM Propidium Iodide (PI) both in DMSO) (Molecular Probes, USA) before examined under CLSM. Serial sections in the xy plane were obtained at 1 µm intervals along the z axis. A 20× and 40x oil immersion objective were used with 488 nm Ar laser excitation and 500–640 nm band pass emission setting. The image were subsequently analysed using the freely available image processing software imageJ version 1.46 (Rasband, National Institutes of Health (NIH), Bethesda, Maryland, USA : http://rsb.info.nih.gov/ij/) including the LSM reader plugin to open LSM5 formatted image stack created by the microscope software. The images' scale bar used to calibrate the ImageJ area measurement algorithm. The observations were made in triplicates and representative images are presented here (Ramage *et al.,* 2002; Jin *et al.,* 2005; Dusane *et al.,* 2012).

Measurement of Hemolytic Activity

The hemolytic activity was investigated by incubating 100 µL of serially diluted massoia lactone (100-6.25 µg/mL) in phosphate-buffered saline (PBS) with 100µL of a suspension of 1% red blood cells in 96-wells V-bottom plate (Greiner). Fresh human red blood cells (RBCs, 30 mL) were suspended in 10 mL of PBS and washed 3 times by centrifugation (5 minutes at 1500 rpm) until the supernatant was clear and resuspended in PBS. Erythrocyte suspensions (5×10^6 cells/mL) were incubated with different concentrations of massoia lactone (100 – 6.25 µg/mL) at 37°C for 1 hour and then centrifuged at 2000 rpm for 10 minutes to precipitate intact erythrocytes and debris. The supernatant in each well was transferred to a new plate and were assayed for absorbance at 450 nm to determine the percentage of hemoglobin released from the lysed erythrocytes. 100% hemolysis data was obtained by adding 10 mL of TRITON-X solution (5 % v/v in DMSO), a strong surfactant, to the above-prepared RBC suspension. The hemolytic activity of the massoia lactone was expressed as the percentage of absorbance compared with that observed after 100% lysis induced by Triton X-100. The supernatant of an untreated erythrocyte suspension in PBS was used as a spectrophotometric blank.

Statistical methods

Statistical analysis was conducted with one way ANOVA followed by Dunnett's tests. Differences were considered significant with P-values of 0.05 or less.

RESULT AND DISCUSSION

TLC profile and Bioautography assay of *M. aromatica* oil

The essential oil obtained had a yield of 0.5 % (w/w).The TLC profile of the *M. aromatica* essential oil separation using Toluene:ethylacetate 93:7, and anysaldehyde sulphuric acid as stain solution showed three spots (Table 1). Using GC-MS analysis, the majority of the oil constituents were identified using the data sources available (Table 2). Among the identified compounds, Massoia lactone (77.06 %) shown to be the main component in *M. aromatica* oil.

Table 1: TLC profiling of *M. aromatica* Essential Oil

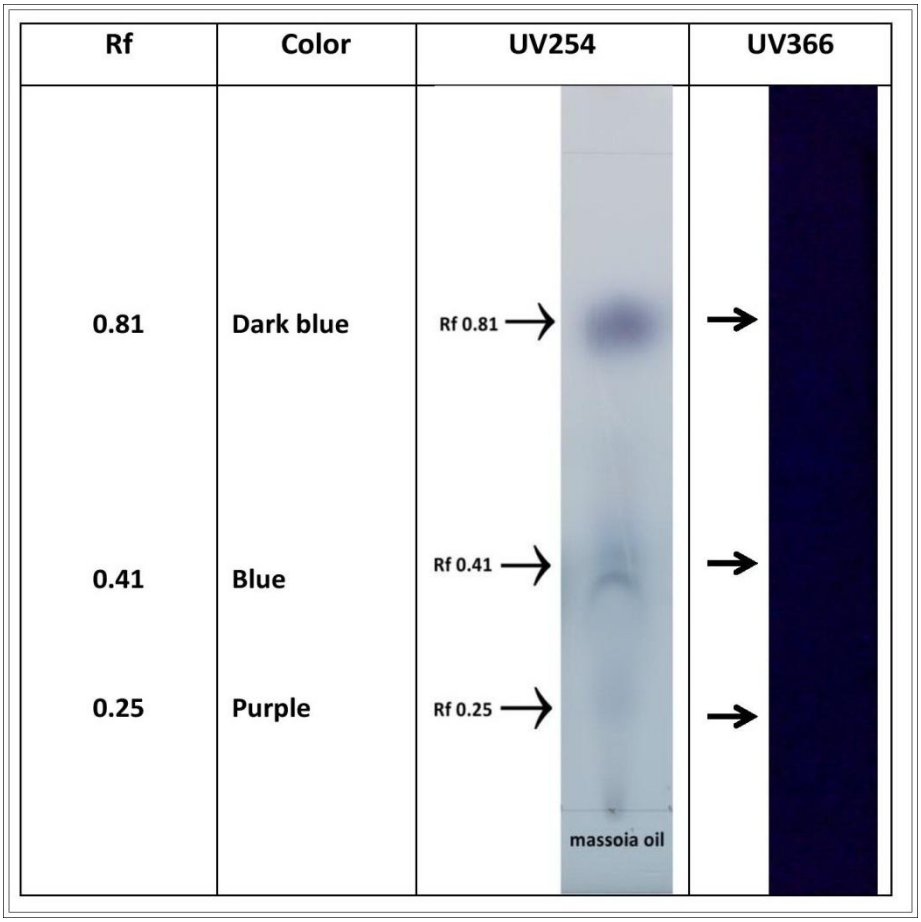

Table 2: Chemical composition of the essential oil from the bark of *M. aromatica*

No	Compound	Retention Time (min)	Peak Area (%)
1	L-Linalool $$	10.491	1.95
2	Alpha Copaene	15.243	2.94
3	Beta Elemene $$	15.467	1.24
4	Alloaromadendrene $$	16.411	3.17
5	5-Hydroxy-2-Decenoic Acid Lactone $$ 2H-Pyrane-2-one, 5,6-dihydro-6-penthyl-, (R)-(CAS) (-)-Massoia lactone	17.242	77.06
6	Cyclopentane, 1,1'-ethylidinebis-$$ Ethane, 1,1-dicyclopenthyl-$$ 1,1-Dicyclopenthylethane	19.591	8.54
7	Benzyl Benzoate	20.443	5.11

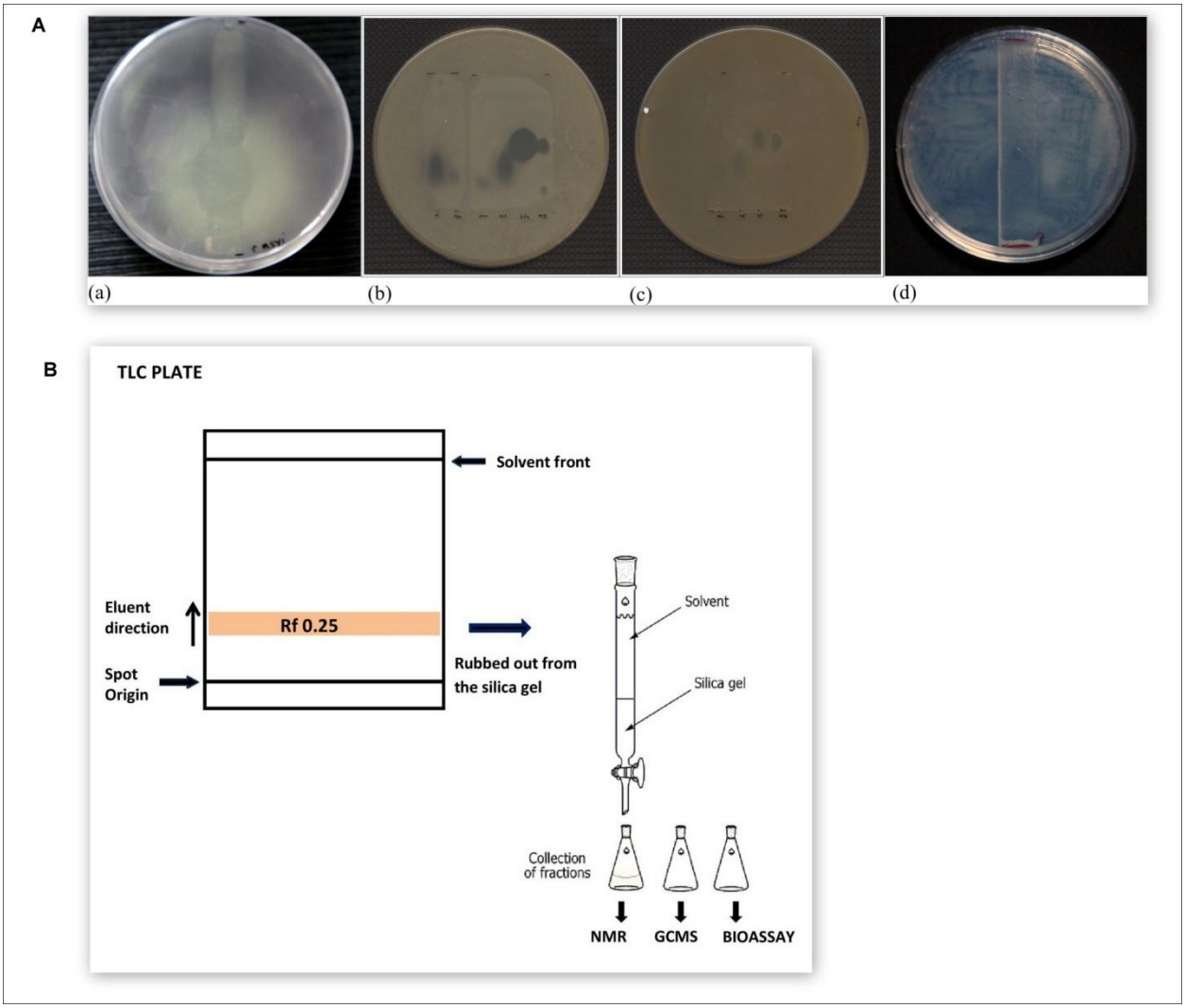

Fig. 1: Isolation of *M. aromatica* oil's active compound. (A) Bioautography analysis of (a) *M. aromatic*a oil on *C. violaceum* WT plate, (b) *M. aromatica* oil, isolated *M. aromatica* oil active compound (Mlact), cynamaldehyde and eugenol on *C .albicans* 10231 plate, on *P. aeruginosa* PAO1 plate, and (d) on *S. aureus* Cowan I plate. (B) Scheme of isolation procedure for *M. aromatica* active compound using preparative TLC.

Figure 1 shows a bioautography profile applied for *M. aromatica* essential oil using *P. aeruginosa* PAO1. The method resembles a disk assay, and combined with TLC separation provide a valuable screening method that quickly detection of components of an essential oil with anti-microbial activity. In this experiment we used contact bioautography, where the microbials diffuse from a TLC plate to an inoculated agar plate. The bioautography against *P. aeruginosa* showed that the component at Rf value 0.25 appear to be the main one responsible for the biological properties of the oil. This compound was then subjected to the TLC preparative analysis to isolate the active.

Isolation of *M. aromatica* oil active compound, GC-MS and NMR analysis

A preparative thin layer chromatography method was used to isolate the active compound of the *M. aromatica* essential oil. The result gave 3 isolated compound (24, 22, and 32 mg/mL). TLC profiling of the isolated compound showed one single dark blue spots at Rf value of 0.25.

In order to obtain information about the chemical compounds contain in the *M. aromatica* essential oil, GC-MS analysis was performed. Table 2 and Figure 2 show the chemical composition of *M. aromatica* essential oil and the GC-MS spectra. Massoia lactone was shown to be the major compound (77%).

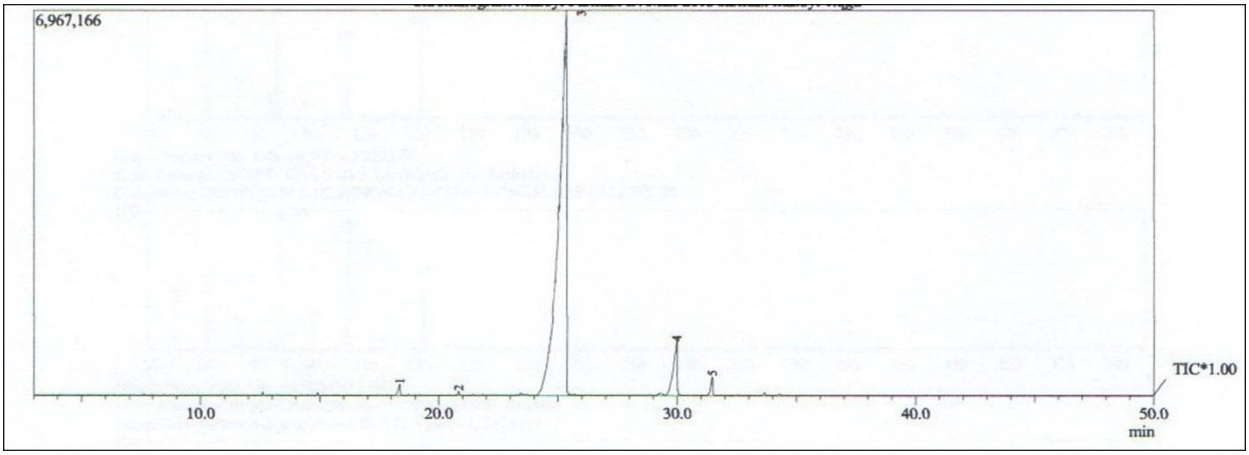

Fig. 2: GC-MS profile of *M. aromatica* essential oil. The indicated compounds in the chromatograms are shown in Table 3.

GC-MS analysis was also performed to check the chemical composition of the isolate compound. Table 3 and figure 3 showed that the isolated compound from *M. aromatica* oil obtained by preparative chromatography method followed by GC-MS analysis is 5-Hydroxy-2-Decenoic Acid Lactone $$ 2H-Pyrane-2-one, 5,6-dihydro-6-penthyl-, (R)-(CAS) (-)-Massoia lactone $C_{10}H_{16}O_2$ (94%). In our previous study, we determined the amount of massoia lactone in massoia oil using TLC-densitometry at 211 nm maximum wavelength. The content of massoia lactone in 1% v/v massoia oil was 0.55 % w/v respectively (Hertiani *et al*., manuscript in preparation).

1D ^{1}H-NMR elucidation was performed to ensure the structure of the massoia lactone obtained. H-NMR profile obtained (Figure 4) convinced the proposed structure recommended by the GC-MS i.e. $C_{10}H_{16}O_2$ which was supported further by comparison to the data reported by Siminiatt (2007) and Harbindu and Kumar (2011) reccorded on $CDCl_3$ as well (Table 4). The present of an aliphatic chain was confirmed by resonance peaks at the upfield region integrated as 11H which support a propyl

substituent. A shift to more down field area was observed at δ 4.40-4.46 (1H, m) which represent an H-containing chiral carbon next to the lactone moiety (H-6). A cis-vicinal H-H coupling of an alkene was observed between resonances at δ 6.87-6.92 and δ 6.03-6.06 having a coupling constant of 9.75 Hz. These finding support the present of an α,β unsaturated lactone. Further splitting of the resonance peak is resulted from the present of an adjacent methylene (H-5). Considering that the only the R isomer is available in nature, the isolate is elucidated as Figure 4.

We also assessed the anti-biofilm activity of massoia lactone in combination with antibiotic and anti-fungal drug. The result showed that the median MBIC of individual fluconazole for *C. albicans* biofilm was 512 μg/mL, and the median MBIC of individual streptomycin for *P. aeruginosa* PAO1 and *S. aureus* Cowan I biofilm was 500 μg/mL. The MBIC of fluconazole in combination with massoia lactone decreased four fold for *C. albicans* biofilm. The MBIC of streptomycin, in combination with massoia lactone decreased from 500 μg/mL to 125 μg/mL (Table 5, Figure 7). Therefore, the combination of streptomycin/massoia lactone yielded synergism activity against the biofilm of *P. aeruginosa* and *S. aureus* (FICI 0.5), whereas the combination of fluconazole/massoia lactone also gave a synergistic effect where the effect of the combination is greater than the sum of the individual effects, with FICI value of 0.375. The use of anti-microbial combinations may improve the management of microbial biofilm-associated infection, disrupt the biofilm and prevent the emergence of microbial resistance. Although the result showed that there is a synergistic activity of massioa lactone with streptomycin and fluconazole, the exact mechanism are still unknown and has to be elucidated.

Table 3: Major chemical constituents of *M. aromatica* essential oil as identified by GC-MS

Peak	Retention time (Rt)	Kovats Retention Index (RI)	Area	Area (%)	Similarity index (SI)	Chemical Component
3	25.346	1168.629	156590945	92.05	94	5-Hydroxy-2-Decenoic acid lactone (Massoia lactone)
4	29.961	1195.847	10522375	6.19	86	5-Hydroxy-2-Decenoic acid lactone (Massoia lactone) *

*The compound obtained was predicted to be a 5-Hydroxy-2-Decenoic acid lactone (Massoia lactone) derivate with higher boiling point and have the same fragmentation as Massoia lactone.

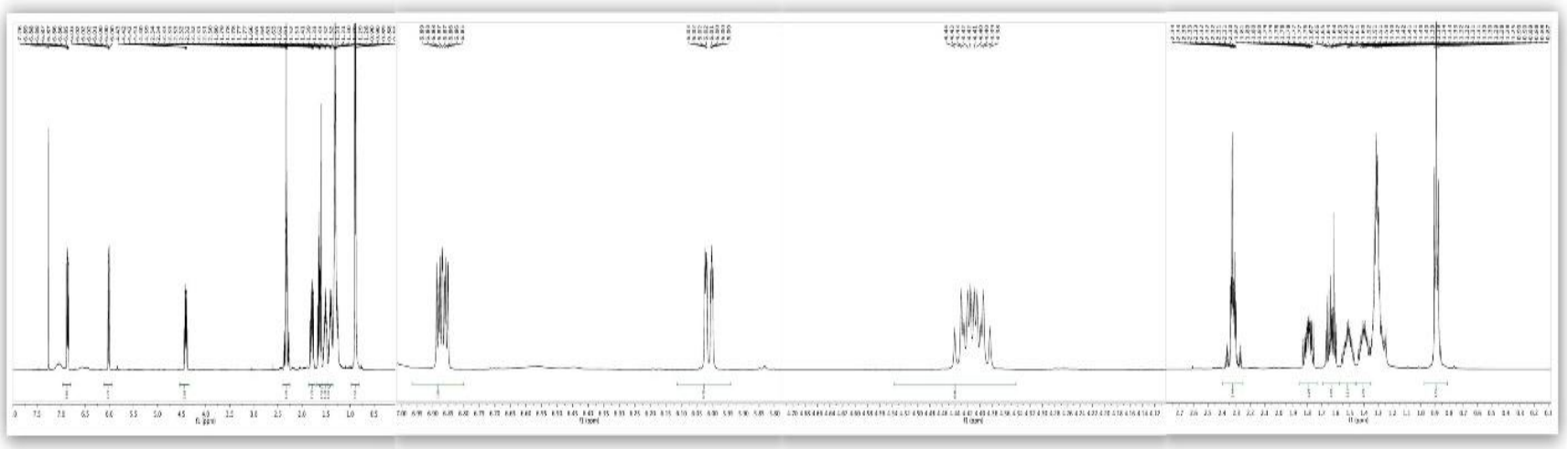

Fig. 3: ^{1}H-NMR profile of massoia lactone

Table 4: NMR data of isolate in comparison to (-)-Massoia lactone ($C_{10}H_{16}O_2$)

Atom No.	**Isolate** [a]		(-)-Massoia lactone ($C_{10}H_{16}O_2$)[b]		(-)-Massoia lactone ($C_{10}H_{16}O_2$)[c]	
	^{1}H		^{1}H		^{1}H	
	δ	Integration, multiplicity, *J* in Hz	δ	Integration, multiplicity, *J* in Hz	δ	Integration, multiplicity, *J* in Hz
H-4	6.87-6.92	1H, ddd, *J* = 9.75, 5.4, 4.4	6.78-6.83	1H, m	6.87-6.90	1H, m
H-3	6.03-6.06	1H, ddd, *J* = 9.75, 1.25, 1.4	5.93-5.97	1H, m	6.04	1H, d, *J* = 10
H-6	4.40-4.46	1H, m	4.32-440	1H, m	4.41-4.45	1H, m
H-5a	2.33-2.36	1H, dd, *J* = 1.40, 4.4	2.23-2.29	2H, m	2.32-2.38	2H, m
H-5b	2.34-2.37	1H, dd, *J* = 1.25, 5.4				
H-7	1.63-1.86	3H, m			1.64-1.82	3H, m
H-8	1.63-1.86	5H, m				
H-9	1.50-1.57					
H-10	1.39-1.47					
	1.31-1.36		1.24-1.93	8H, m	1.26-1.34	5H, m
H-11	0.92	1H, t, 7.1 Hz	0.82	3H, brs	0.90	3H, t, *J* =6.9 Hz

[a]Data were recorded in $CDCl_3$, at 500 MHz, multiplicities and coupling constant are given in Hz; [b] $CDCl_3$, at 400 MHz Siminionatto *et al.*, (2007); $CDCl_3$, at 200 MHz Harbindu and Kumar (2011)

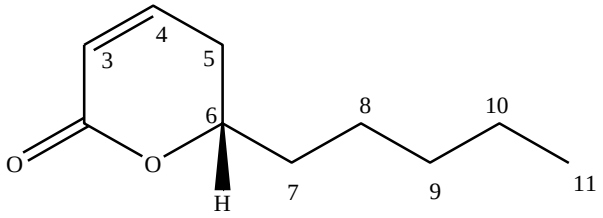

Fig. 4: Massoia lactone chemical structure

Effect of massoia lactone on microbial biofilms

Biofilm infections are a major medical problem since they are difficult to eradicate and the source of many chronic infections. The discovery of anti-infective agents, which are active not only against cells but also against microbial biofilms, represents an important goal (Schillaci *et al.,* 2008). Essential oils are mixtures of numerous organic chemicals, and some individual components are known to exhibit anti-microbial properties.

The MTT assay was used to measure the efficacy of massoia lactone against microbial biofilm. In this assay, microorganisms with active electron transport system metabolically reduce the yellow tetrazolium salt (MTT) to water soluble purple formazan product. The color intensity of the soluble formazan was determined using a microplate reader at A_{550} (Mossmann, 1983). The colorimetric assay using MTT is quicker, more sensitive compare to the traditional anti-bacterial assay that use colony counting of bacteria on nutrient agar plates, and less expensive compare to XTT (Stevens and Olsen, 1993; Roehm *et al*., 1991). The disadvantage of MTT is that the formazan forms a crystalline presipitate needs to be solubilized first prior to absorbance measurements.

In our study, we found the anti-biofilm potential of the massoia lactone. As the main compound isolated from *M. aromatica* bark essential oil, massoia lactone showed anti-biofilm activity against all the test organisms. A reduced level of biofilm formation was clearly observed against control (biofilm without the presence of the test compound). At concentration of 100 µg/mL, showed capability in inhibit the formation of *C. albicans* intermediate biofilm as much as 82.25 ± 0.54% (***P<0.001), whereas at lower concentration (25 µg/mL) massoia lactone capable to inhibit *C. albicans* intermediate biofilm formation as much as 49.98 ± 0.32% (**P<0.01). The median biofilm inhibition concentration ($MBIC_{50}$) for massoia lactone was at concentration of 50 µg/mL for *P. aeruginosa* biofilm (50.14 ± 0.08) (**P<0.01), and 100 µg/mL for *S. aureus* biofilm (67.28 ± 0.28) (**P<0.01) (Figure 5).

Efficacy massoia lactone in inhibit microbial biofilm tested in microtiter plate using MTT method was confirmed by CLSM analysis. Intermediate phase (24h) of *C. albicans* biofilms were grown on the surface of coverslips and stained with SYTO9 and PI for the monitoring of live/dead cells. We observed a preventive activity on biofilm formation, when we inoculated *C. albicans* with different concentration of massoia lactone. Growth suppression of *C. albicans* biofilm by massoia oil was clearly seen by CLSM with the Live/Dead stain, compared with the dense biofilm growth of the control (Figure 6).

Pretreatment of *C. albicans* biofilm with various concentrations of massoia lactone showed that there was a dose-dependent effect of massoia lactone on *C. albicans* biofilm formation. CLSM study showed that in the presence of massoia lactone (concentration of 100 µg/mL), a significant decrease of *C. albicans* biofilm biomass compare to negative control was evident (figure 6). When the concentration was decreased, we observed the increase amount of blastopores, pseudohyphae and mycelia in *C. albicans* biofilm biomass. The anti-biofilm activity thus may be due to the anti-fungal activity of the massoia lactone.

The effect of massoia lactone to microbial biofilms has yet been scrutinized. Massoia lactone possess a characteristic α,β-unsaturated δ-lactone, which is also a common feature in a number of biologically active natural products such as (+)-geniothalamin, (-)-callystatin A, canolide A and the kava lactones, either different in the substituents at the C6 position or have a molecular structure architecture that maintains the α,β-unsaturated δ-lactone skeleton (Rali *et al*., 2007). The mechanism of action of lactone against microorganims is not fully understood but it is speculated to be involved in membrane disruption, resulting in alterations in membrane permeability and leakage of intracellular materials. This compound could be able to penetrate through the polysaccharide matrix of a biofilm and dissolve lipids in the biofilm matrix (Cowan, 1999; Trombetta *et al*., 2005). In the presence of lactone, *Yarrowia lipolytica* lossed its cultivability and membrane integrity and addition of lactone in the medium provoked a decrease in the concentration of ergosterol (Ngoc Ta *et al*., 2010). However, a study by Kishimoto *et al.* (2005) suggested that the anti-fungal activity of massoia lactone is possibly due to its capability in inhibit respiratory system of *C. albicans,* because it arrests oxygen consumption by *C. albicans*. Since the exact mechanism of anti-biofilm activity of massoia lactone remains unclear, study on a gene expression of *C. albicans* cells in response to massoia lactone will be required.

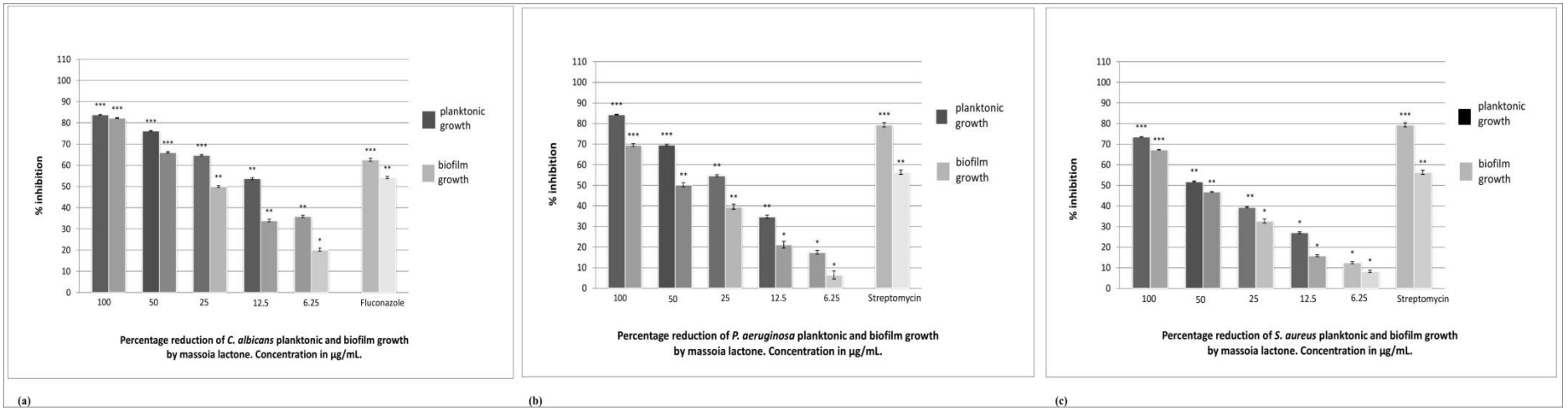

Fig. 5: Activity of massoia lactone against *C. albicans* ATCC 10231 (a), *Pseudomonas aeruginosa* PAO1 (b), and (c) *Staphylococcus aureus* Cowan I planktonic growth and biofilm formation. Concentration in µg/mL. Streptomycin concentration of 500 µg/mL and and Fluconazole concentration of 512 µg/mL were used as positive control. The bars indicated the standard deviations of the means. Asterisks indicate a significant difference between treatment and negative control.

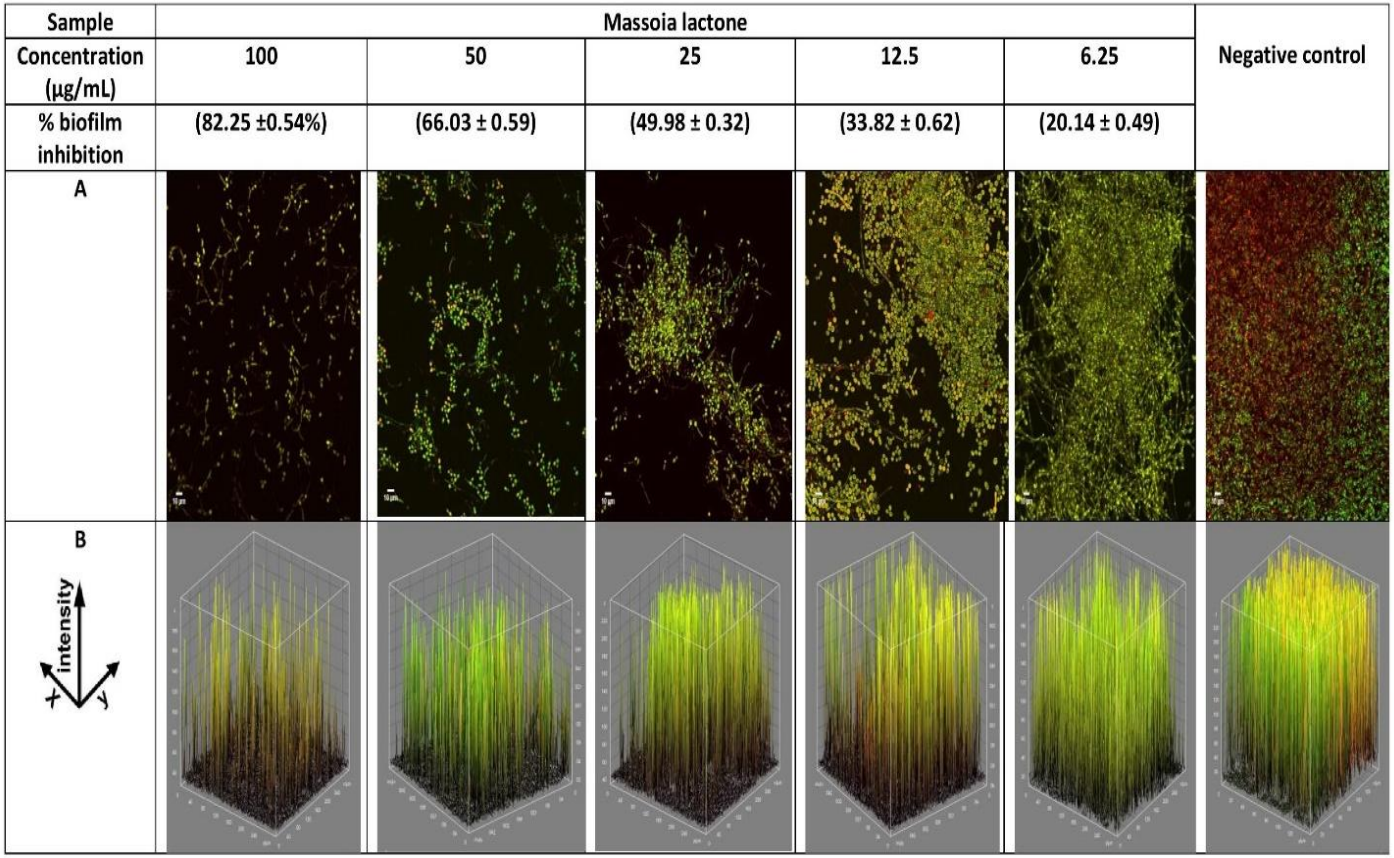

Fig. 6: Representative confocal scanning micrograph images of biofilm inhibition activity of massoia lactone at concentration of 100 – 6.25 µg/mL against intermediate phase of *Candida albicans* biofilm. A: projected upper view of the biofilm, B: estimated three-dimensional view of the biofilm. CLSM images were taken at 20x magnification.

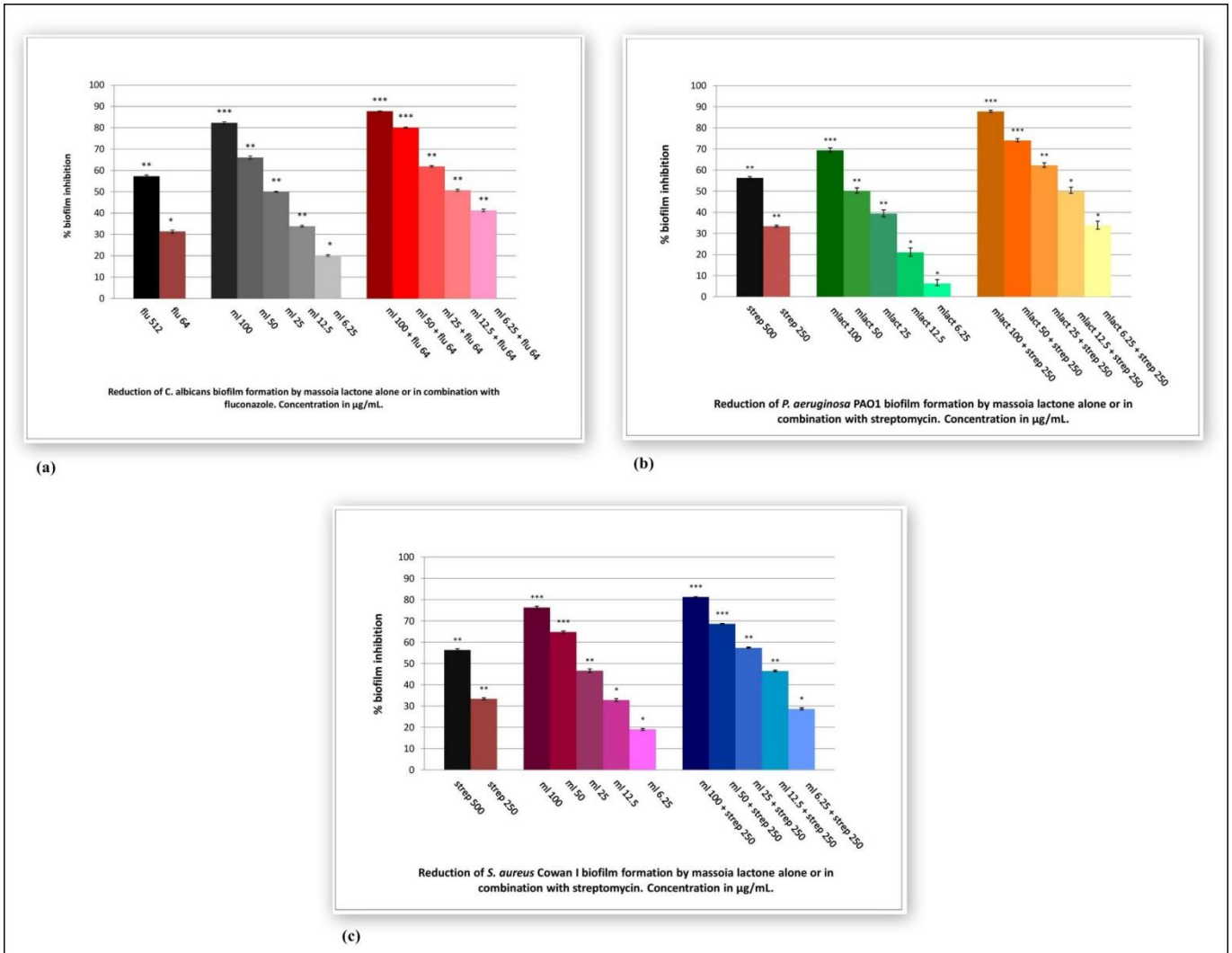

Fig. 7: Efficacy of massoia lactone (ml) alone and in combination with: (a) fluconazole (flu) against *C. albicans* 10231, (b) streptomycin (strep) against *Pseudomonas aeruginosa* PAO1, and (c) streptomycin (strep) against *Staphylococcus aureus* Cowan I. The bars indicated the standard deviations of the means. Asterisks indicate a significant difference between treatment and negative control.

Table 5: MBICs of Massoia lactone and antibiotics alone and in combination, and fractional inhibitory concentration index (FICI) values against *P. aeruginosa, S. aureus* and *C. albicans* biofilm formation

Microorganism	Compound Test (µg/ml)	$MBIC_{50}$-o (µg/mL)	$MBIC_{50}$-c (µg/mL)	FIC_{50}	$FICI_{50}$	Result (Interpretation)
P. aeruginosa PAO1	massoia lactone	50	12.5	0.25	0.5	synergy
	streptomycin	500	125	0.25		
S. aureus Cowan I	massoia lactone	100	25	0.25	0.5	synergy
	streptomycin	500	125	0.25		
C.albicans ATCC 10231	massoia lactone	50	12.5	0.25	0.375	synergy
	fluconazole	512	64	0.125		

Massoia lactone has been synthesized from various methods (Ramachandran *et al.,* 2000, Sabitha *et al*., 2007; Sato *et al.,* 2001), however no investigation about its anti-biofilm activity has ever been conducted. In future research, the effect on *P. aeruginosa* and *S. aureus* biofilm architecture in the presence of massoia lactone could be examined.

Hemolysis assay

In order to measure the cytotoxicity of the massoia lactone, hemolytic activity test was performed using human red blood cells. Result demonstrated that massoia lactone showed a dose dependent increase in destroying red blood cell and release hemoglobin (Figure 8). At to the highest concentration tested (100 µg/mL), exerted as much as 12.4 ± 0.9 % human red blood cells hemolysis compare to Triton X-100 which gave 100% hemolysis. This result indicates that at the range concentration tested, massoia lactone is less toxic to the human erythrocytes.

In nature massoia lactone is a rare essential oil component that was first characterized by Abe in 1937 (Abe, 1937). It is also present in *Hierochloe odorata* and *H. australis* that are commonly used in vodka production (Bernreuther *et al*., 1990), and has been isolated from mandibular glands formicine ants of the genus *Camponotus* as an alarm pheromone (Cavill *et al.,* 1968). The pleasant and sweet light coconut odor of massoia lactone make this compound very useful as a flavor for milk and butter, in the manufacturing of alcoholic drinks and as aromatizer in perfumes (Mineeva, 2012). IFRA (International Fragrance Association) prohibited the use of *Massoia aromatica* oil and massoia lactone because it was shown to be skin irritant and cause sensitization when it is applied in aromatheraphy. According to the study by Benoni and Hardebeck (1964), the oral toxicity (LD_{50}) for

massoia lactone is 1890 mg/kg, however the recommended safety dosage for massoia lactone to be use as a fragrance ingredient is not yet available (Anonim, 2009).

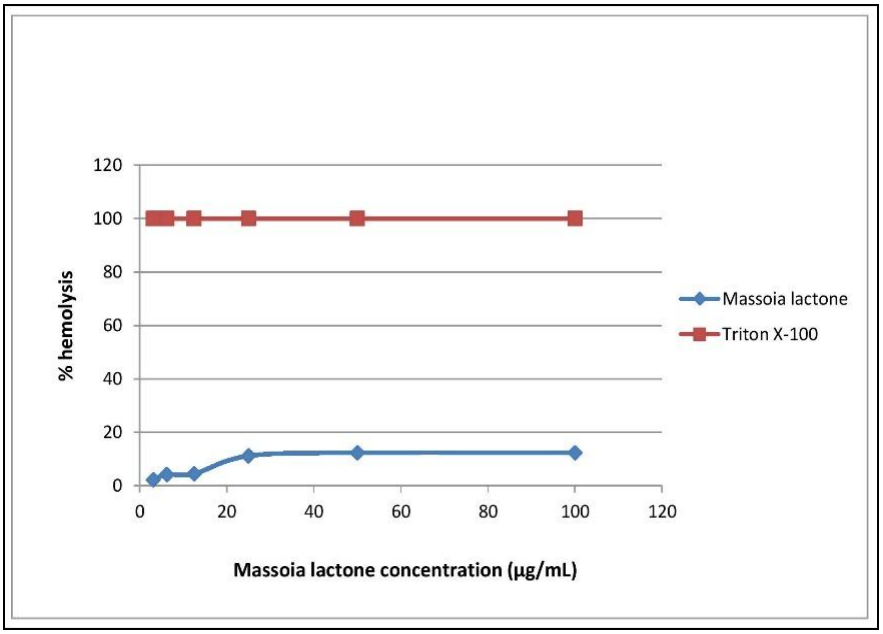

Fig. 8: Hemolytic activity of massoia lactone. Triton X-100 used as positive control.

In the future, massoia lactone could be used for therapeutical formulations to treat diseases caused by microorganisms. However, to be used as a drug for human and animal illness in pharmaceutical industry, safety tests in animal models have to determine the dose that causes no adverse effect.

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