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RESEARCH ARTICLE

Antimicrobial effect of dissolved curcuminoid in natural deep eutectic solvents (NADES) to *Escherichia coli* and *Staphylococcus aureus*: A promising candidate for antimicrobial photodynamic therapy (aPDT)

Orchidea Rachmaniah*, Galang Ramdhani Fitra Gama, Zandhika Alfi Pratama, Muhammad Rachimoellah

Department of Chemical Engineering, Faculty of Industrial Technology, Institut Teknologi Sepuluh Nopember, 60111 Sukolilo, Surabaya, Indonesia

* *Corresponding author: orchidea@its.ac.id*

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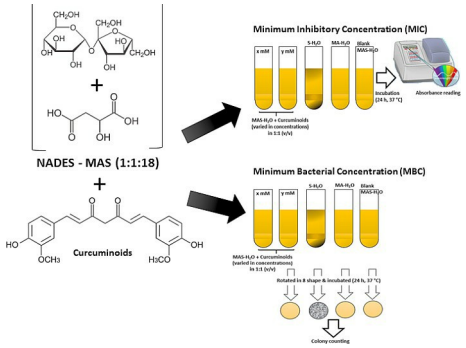
Graphical abstract



Abstract

An antimicrobial photodynamic therapy (aPDT) is a local antimicrobial treatment which utilizes a photosensitizer dye, visible light, and oxygen. It is considered as an alternative treatment for bacterial or fungal resistance. In this treatmetn, a pure, stable and non-toxic natural photosensitizer compound as a host cell which soluble in water and capable of producing reactive photoproducts is required. Curcumin as a natural yellow-orange photosensitizer dye with anti-inflammatory, anti-carcinogenic, anti-bacterial, and anti-infection activities is believed to be safe for human consumption. Combining curcuminoids as a photosensitizer dye with NADES as solvent instead of solving the low solubility drawback of curcuminoids in water, as well as becoming a potential candidate of aPDT. However, an antimicrobial effect of dissolved curcuminoids in NADES need to be studied first. Antimicrobial tests of curcuminoids to both of *Escherichia coli* and *Staphylococcus aureus* using minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) methods were conducted. Three NADES i.e. malic acid-sucrose-water (MAS-H_2O = 1:1:18); fructose-glucose-water (FG-H_2O = 1:1:1); and fructose-sucrose-water (FS-H_2O = 2:1:15) were tested by applying nine different concentrations of curcuminoids (2.00-4.00 mM). A blank of sample (no dissolved curcuminoids) as well as a pure solution of each constituent compounds of NADES such sucrose, malic acid, fructose, and glucose were also applied. Bacterial suspension approx. 10^8 cells/mL of 1 mL (24 h incubated at 37°C) was used for the test. MAS-H_2O (1:1:18) shown the most effective antimicrobial activity compared to both of FG-H_2O (1:1:1) and FS-H_2O (2:1:15). The toxicity of MAS-H_2O (1:1:18) to both *E. coli* and *S. aureus* may due to the low pH condition of NADES itself since malic acid has high acidity (pH <3). Meanwhile, both other NADES contains sugars, i.e. fructose, glucose, and sucrose, showing lower pH value (pH >5). Both on the concentration of the curcuminoids and bacteria effects the observed toxicity.

Keywords: Antibacterial, curcuma, deep eutectic solvent, ionic liquid

INTRODUCTION

Staphylococcus aureus strain has shown high resistance against each new class of licensed antibiotics. It is recently reported that a strain of *S. aureus* is resistant to vancomycin, a glycopeptide antibiotic considered as a last line generation of antibiotics. The problem is made more severe by social factors such as an inappropriate prescription of antibiotics, or an excessive prescription of antibiotics leading to either bacterial or fungal resistance. Moreover, more and more frequent transmissions of microorganisms occur due to global traveling, expansion of poverty among the population of the third world countries, and by the large variety of mechanisms adopted by microbial cells, thus increasing the resistance of the bacteria to external insults. This results in a thickening of their outer wall, encoding of new proteins which prevent the penetration of drugs, onset of mutants deficient in those porin channels allowing the influx of externally added chemicals, among others (Heger *et al*., 2014). Therefore, a comprehensive strategy is required to resolve this problem, such as an alternative solution to inactivate pathogens or bacteria.

A photochemical reaction among a photosensitizer (PS) or a dye, visible light, and oxygen produces reactive oxygen species (ROS) which is applied in antimicrobial photodynamic therapy (aPDT). The produced ROS, free radicals and/or other reactive photoproducts may cause damage to bacterial cell structures, which ultimately results in bacterial inactivation. An alternative way to combat bacteria resistances (Kumavat *et al*., 2013) is by overcoming the antibiotic resistances. A visible light at an appropriate wavelength is used to excite the PS molecule to produce superoxide radicals (type 1 reaction) or generate singlet oxygen (type 2 reaction) (Kumavat *et al*., 2013) which subsequently lead to the destruction of the bacterial cells by an oxidative burst (Cieplik *et al*., 2018) of biomolecules such proteins, lipids, and nucleic acids (Hamblin, 2016). It has been reported the effectiveness of aPDT against Gram-positive, such *Staphylococcus aureus* and *Bacillus subtilis,* and Gram-negative bacteria, such

Escherichia coli and *Salmonella typhimurium* (Cieplik *et al*., 2018) as well as yeasts and fungi (Sperandio *et al*., 2013).

An ideal compound for PS should pose a low toxicity to host cell, read human cell, especially when exposed to visible lights. Porphyrins, phthalocyanines, chlorins, phenothiazines, 5-aminolevulinic acid (5-ALA), xanthanes, fullerene, phenalenone, and triarylmethanes class of derivatives compounds as well as riboflavin derivatives are generally used as PS compound in aPDT studies. In case of new natural dyes of PS, curcumin is a promising candidate due to its capability in absorbing a blue light.

Curcuminoids, a hydrophobic polyphenol, which is presents in the rhizomes of *Curcuma longa* L, consist of curcumin, desmethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC). On top of its wide range of bioactivity including anti-inflammatory, anti-carcinogenic, anti-infection, and anti-flatulent, it can act as an antibacterial agent by inhibiting bacterial cell proliferation process. This is due to its ability to affect the function and modify the lipid bilayer of bacterial cell membranes that acts as a barrier to the autolytic enzymes (Prima and Handajani, 2013). Inhibition of proliferation process of bacterial suspected as crucial target of antibiotics. Curcumin is also known for its photo-toxicity against microorganism Tortik *et al*. (2014.

The application of curcuminoids is seem rather complicated by its low solubility in water at acidic (above pH 1) and physiological pH. It rapidly hydrolyzes under alkaline condition and susceptibility to photochemical degradation. The solubility of curcuminoids in water is approximately or less than 3×10^{-8} M (Tønnesen and Karlsen, 1985) while Patra and Sleem (2013) reported only 4 ppb (pH = 7.3). Therefore, it is difficult to interact with body tissue due to high water content in human body such that approximately 90% of water is contained in blood (Patra and Sleem, 2013). Subsequently, these issues have been addressed through Natural Deep Eutectic Solvent (NADES) as a solvent of curcuminoids. Moreover, Tortik *et al*. (2014) bounded curcumin to plyvinylpyrrolidone (PVP-C) and applied micellar formulation of curcumin as NovaSol®-curcumin; increasing its solubility in water and oral bioavailability. Additionally, curcuminoids with cationic substituents prepared by synthetic routes was applied by Spaeth *et al*. (2018). The phenolic OH group of the curcumin are blocked by attaching alkyl chains with cationic charged, enhancing the photo-dynamic effectivity against gram-negative bacteria (Hamblin, 2016). Cationic PS will effectively attach the microbial cell while short exposure to light. These findings are useful in overcoming the drawbacks of curcumin such weakness to adhere the gram-negative bacteria, low photo-stability, as well as its solubility limitation in water.

A higher yield of curcuminoids was obtained compared to ethanol or water when NADES was applied as solvents (Zullaikah *et al*., 2018). Curcuminoids was well-extracted in acidic and ionic type of NADES, e.g. citric acid-sucrose-water (CAS-H_2O), malic acid-sucrose-water (MAS-H_2O), choline chloride-citric acid-water (CCCA- H_2O), and choline chloride-malic acid-water (CCMA- H_2O). However, it is not stable within 96 h (Zullaikah *et al*., 2018). Moreover, higher water content of NADES ca. 60% of water, i.e. MAS-H_2O and CAS-H_2O (1:2:15), yielded more or less similar value of curcuminoids compared to 20-30% of water content (Rachmaniah *et al*., 2018). Therefore, MAS-H_2O, an acidic type of NADES was applied in this study along with neutral type of NADES, i.e. FG-H_2O and FS-H_2O. Differentiating in NADES constituents will alter both of physical and chemical properties of NADES.

The use of NADES as a solvent for PSs intended for aPDT has been reported recently (Wikene *et al*., 2017). The ability of highly polar NADES, i.e. glucose-sucrose (GS) and maleic acid-choline chloride (MC), to solubilize a hydrophobic PSs such curcumin may have been caused by intermolecular interactions with the NADES namely hydrogen bonds (Wikene *et al*., 2017). Both toxicity in the absence of light and photo-toxicity of the curcumin have been reported to increase when dissolved in NADES compared to conventional solutions of the PS prepared in phosphate buffered saline (PBS) (Wikene *et al*., 2017). NADES maleic acid-choline chloride (MC = 1:3) containing 1.25 μM curcumin photo-inactivated *E. coli* (OD_{600} = 0.03) (Wikene *et al*., 2015). This presumably due to the diketo conformer of curcumin in NADES. NADES seems to be able to lock the curcumin in its keto form.

TMPyP (5, 10, 15, 20-tetrakis(N-methylpyridinium-4-yl)porphyrin) and ZnTPPS4 (inc-5, 10, 15, 20-tetrakis(4-sulphonatophenyl)porphyrin were used as PS compounds in complex with hp-β-cyclodextrin. It is found that TMPyP (at 12.5-100 μmol/L range of concentration) is a promising PS for both aPDT and photodynamic therapy (PDT), while ZnTPPS4 is only suitable for PDT. A ZnTPPS4 needs higher concentration for suppressing *S. aureus* (Gram-positive bacteria) compared to TMPyP (Hanakova *et al*., 2014).

Other porphyrin compounds as PS was also studied in nano molar concentration, i.e. THPP (meso-tetra(*p*-hydroxyphenyl)porphine) and TCPP (meso-tetra(*p*-carboxyphenyl)porphine), in combination with NADES. Both THPP and TCPP that were solubilized in organic acid containing NADES combined with blue light were sufficient to induce >99.99% reduction in Gram-positive and Gram-negative bacteria (Wikene *et al*., 2017). The toxicity of dissolved THPP in citric acid-sucrose (CS) of NADES to *E. coli* either due the pH of the solution or chelation of outer membrane-bound cations. It might be concluded that the bacterial photo-toxicity of the porphyrins dissolved in NADES increase due to a low pH of the solution, protonation of the PSs, and an increase in permeability of the bacterial membrane, or other currently unknown factors (Wikene *et al*., 2017; Mbous *et al*., 2017).

The aim of the present study was to investigate the antimicrobial effect of dissolved curcuminoids in polar type of NADES: (MAS-H_2O (1:1:18), FG-H_2O (1:1:1), and FS-H_2O (2:1:15)) against both of *E. coli* and *S. aureus*. The obtained results can be used as a recommendation for NADES application as an aPDT.

EXPERIMENTAL

Materials

Curcuminoids standard were purchased form Merck (Darmstadt, Germany) as well as NADES constituent compounds such D (-)-Fructose (≥ 99,0 %), D (+)- Glucose, sucrose, and DL-Malic Acid (≥ 99.5%). All chemical materials for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) works were supplied from Laboratory of microbiology and biotechnology (Biology Department, Institut Teknologi Sepuluh Nopember, Surabaya, Indonesia), including both strains of *Staphylococcus aureus* and *Escherichia coli*.

Preparation of natural deep eutectic solvents (NADES)

NADES used in this study were prepared by a method from Dai *et al*. (2013) with slight modifications. A freeze dry was used instead of a vacuum evaporation especially for NADES with low molar ratio of water, i.e. malic acid (MA)-sucrose (S)-water (H_2O) (MAS-H_2O = 1:1:18); while NADES with high molar ratio of water, i.e. fructose (F)-glucose (G)-water (H_2O) (FG-H_2O = 1:1:1) and fructose (F)-sucrose (S)-water (H_2O) FS-H_2O = 2:1:15), a heating method was more appropriate. Initially, each compound of NADES, such malic acid, and sucrose for MAS-H_2O, were precisely weighted according to its molar ratio (1:1:18), mixed, and dissolved with water in sealed pot using a magnetic stirrer in a water bath (70 °C); obtaining a clear liquid mixture which is called as NADES.

Dissolving curcuminoids in natural deep eutectic solvents (NADES)

Curcuminoids should be previously dissolved in NADES, i.e. MAS-H_2O (1:1:18), FG-H_2O (1:1:1) and FS-H_2O (2:1:15), before used both in minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Nine different concentrations of curcuminoids: 2.00 mM, 2.25 mM, 2.50 mM, 2.75 mM, 3.00 mM, 3.25 mM, 3.50 mM, 3.75 mM, and 4.00 mM, were applied as well as a blank of sample (without any addition of curcuminoids). Standards powder of curcuminoids was precisely weighted following the determined concentrations and added to 5 mL of NADES. Sonication was applied, making the curcuminoids well dissolved in NADES.

Minimum inhibitory concentration (MIC)

The method was applied to find NADES with the best bacterial inhibition. A bacterial suspension was prepared by inoculating *E. coli* or *S. aureus* from nutrient broth slant agar to nutrient broth media; incubated for 24 h (37 °C) in rotary shaker at 120 rpm. Subsequently the nutrient broth media was sampled, and measured at λ_{max} = 600 nm till obtaining 0.4-0.6 optical density (OD); which is equivalent to the density of 10^8 cells/mL. A suspension of bacteria (density of 10^8 cells/mL) was mixed with NADES containing curcuminoids in specified concentration with ratio of 1:1 (v/v) by vortexing. The OD of the suspension sample was measured right after the vortexing step and 24 h incubation. The method was also applied for each variable of NADES.

Minimum bactericidal concentration (MBC)

The MBC method was applied to determine NADES with the best suppression bacteria effect. A counting method was used to count number of cells (CFU/mL). As explained previously, 1 mL of bacterial suspension was poured to Petri dish of test plate for further MBC analysis. The test plate was prepared previously by mixing MAS-H_2O containing in specified concentration of curcuminoids in a nutrient broth agar. The petri dish was rotated with 8 pattern number; therefore, agar and culture were well-mixed and incubated (24 h, 37°C). The method was also applied for each variable of NADES. A plate contains ≥250 colonies, cannot be counted, considering as too numerous to count (TNTC), while plate contains ≤ 25 colonies considering as too few to count (TFTC) (Joanne *et al*., 2008).

RESULTS AND DISCUSSION

Minimum inhibitory concentration (MIC)

The MIC or anti-bacterial activity test was conducted to know the minimum concentration of curcuminoids that inhibit the growth of bacteria. The MIC test was performed for each NADES, MAS-H_2O (1:1:18), FG-H_2O (1:1:7), and FS-H_2O (2:1:15). The MIC value was indicated by the absence of turbidity in the test tube after 24 hours of incubation at 37 ℃ (Owuama, 2017). If the turbidity of the test tube seems clear as the blank, means that the bacteria growth starts being inhibited. This indicates the presence of MIC qualitatively.

In case of MAS-H_2O (1:1:18), curcuminoids concentrations of 2.00 mM, 2.25 mM, 2.50 mM, 2.75 mM, 3.00 mM, 3.25 mM, 3.50 mM, 3.75 mM, and 4.00 mM were applied. No changes in turbidity were observed. All the test tubes were equally clear as a blank tube. The blank solution of MAS-H_2O (without any dissolved curcuminoids) showed inhibition of both *E. coli* and *S. aureus*.

Since qualitative visualization of the turbidity has many disadvantages, a spectrophotometry method based on absorbance was applied in the MIC test, instead of using the diffusion method due its quantitative result. A spectrophotometer was used to measure the absorbance of the samples, especially for FG-H_2O (1:1:7) and FS-H_2O (2:1:15). In case of absorbance of pre-incubation which is higher compared to absorbance of post-incubation, the bacterial growth was inhibited, and vice versa.

Similar to MAS-H_2O (1:1:18), nine different concentrations of curcuminoids were also applied to FG-H_2O (1:1:7) and FS-H_2O (2:1:15) (Fig. 1 and Fig. 2). A decrease in the ΔOD (difference between absorbances of pre-incubation and absorbances of post- incubation) was observed at curcuminoids dissolved at FG-H_2O (1:1:7) to *E. coli* (Fig. 1(a)) after 24 hours of incubation. Maximum inhibition appears to happen at 3.00 mM and decreased when curcuminoids concentration increased, ca. 3.25-4.00 mM. This probably happened due to bacteriostatic properties of *E. coli*. Initially, *E. coli* seems to be inhibited by the curcuminoids but after the microbes adapted with the new environment (read as curcuminoids dissolved in FG-H_2O (1:1:7)), the growth is starting to be affected. An impermeable outer cell membrane typically shown by gram-negative bacteria such *E. coli*; containing endotoxins and can block dye for guarding the inner membrane and cell wall (Sperandio *et al*., 2013).

However, the opposite trend was observed with *S. aureus* (Fig. 1(b)), means that curcuminoids dissolved at FG-H_2O (1:1:7) did not significantly inhibit the growth of *S. aureus*. No MIC value had been found for *S. aureus* at curcuminoids concentrations 2.00-4.00 mM. Moreover, 3.00 mM concentration of curcuminoids in FG-H_2O (1:1:7) was further used as the base concentration in determining the MBC. These bacteria have a fairly good resistance to some antimicrobials such curcuminoids (Wasitaningrum, 2009) and commonly to methicillin (Tortik *et al*., 2014) due to its polysaccharide layer which is responsible for the virulence of the bacteria. These presence of biofilm on bacteria causes slow penetration of antimicrobials compound to the cell wall and subsequently invulnerable.

Moreover, this phenomenon presumable due to the high concentration of bacteria, ca. 10^8 cells/mL (OD_{600} = 0.4-0.6). It is comparably relatively high to OD_{600} = 0.045 with CS (Citric acid-Sucrose = 1:1) and MFG (Malic acid-Fructose-Glucose = 1:1:1) of NADES at 0.20 nM (Wikene *et al*., 2017) and OD_{600} = 0.030 with GS (b-(+)-Glucose-Sucrose = 1:1) and MC (Maleic acid-Choline chlorine = 1:3) of NADES at 1.25-2.60 μM (Wikene *et al*., 2015).

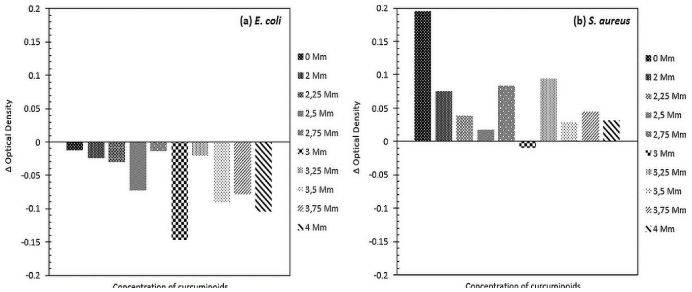


Fig. 1 Optical Density of Dissolved Curcuminoids in FG-H_2O (1:1:7) on *E. coli* (a) and *S. aureus* (b).

Curcuminoids concentrations of 2.00-4.00 mM in FG-H_2O (1:1:7) successfully inhibit the growth of *E. coli* (24 h), whereas in *S. aureus* (Fig. 2) did not. The lowest concentration of curcuminoids, 2.00 mM, dissolved in FG-H_2O (1:1:7) was conclusively the MIC for *E. coli*.

Furthermore, for FS-H_2O (2:1:15), curcuminoids concentrations of 2.00-4.00 mM gave good inhibition of both *E. coli* and *S. aureus* during 24 h (Fig. 2). Curcuminoids concentration of 2.00 mM in FS-H_2O (2:1:15) was conclusively the MIC of both *E. coli* and *S. aureus*.

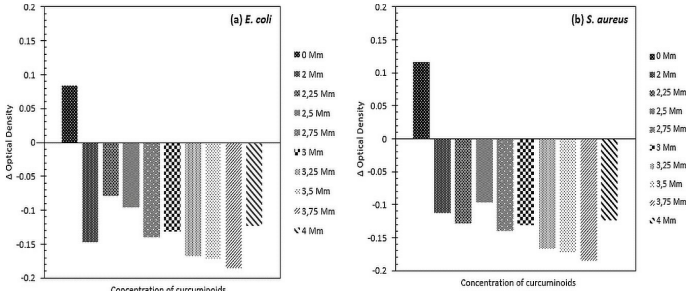


Fig. 2 Optical Density of Dissolved Curcuminoids in FS-H_2O (2:1:15) on E. coli (a) and S. aureus (b).

Minimum bactericidal concentration (MBC)

The MBC test was applied to determine the type of variables that could provide the best bacterial suppression effect, approximately >99.99% where no bacterial growth was seen on the agar plate (Bhatt and Mondal, 2015).

The MBC was also performed at MAS-H_2O (1:1:18); FG-H_2O (1:1:7); and FS-H_2O (2:1:15) as well as the MIC test. Due to the MBC test was applied further the MIC test, the concentrations of the dissolved curcuminoids in NADES for each of the studied NADES variables were based on the result of the MIC test. Following the result of the MIC test on MAS-H_2O (1:1:18), blank of MAS-H_2O (1:1:18) (without dissolved curcumin in NADES) shows the inhibition of both *E. coli* and *S. aureus*. Therefore, the MBC test for MAS-H_2O (1:1:18) was applied on the blank of MAS-H_2O (1:1:18), MA-H_2O (1:18) and S-H_2O (1:18) (Fig. 3).

The MBC test result on MAS-H_2O (1:1:18) (Fig. 3) showed that no bacterial grew on blank of MAS-H_2O (1:1:18) and MA-H_2O (1:18). However, bacterial growth was observed in S-H_2O (1:18).

Regarding the MBC test for FG-H_2O (1:1:7) and FS-H_2O (2:1:15), the following 3 variables of concentration from MIC were taken: (1) Blank solution of NADES, (2) The concentration of curcuminoids which had the greatest difference in OD value, and (3) The concentration of curcuminoids which had the smallest difference in OD value. Hence, for FG-H_2O (1:1:7), the 3 mM dissolved curcumin concentration was tested by MBC; while for FS-H_2O (2:1:15), the MBC test was performed at different dissolved curcuminoids concentrations for each of bacterial variables. The dissolved curcuminoids concentrations of 2.25 and 3.00 mM were tested by MBC in *S. aureus*, while the dissolved curcuminoids concentrations of 2.50 and 3.75 mM tested by MBC on *E. coli*.

It demonstrated that 3.00 mM concentration of curcuminoids in FG-H_2O (1:1:7), FG-H_2O blank (1:1:7), F-H_2O (1:7), and G-H_2O (1:7) showed bacterial growth. There was a total number of bacteria of 4.27 x 10^2 CFU/mL of *S. aureus* and 5.04 x 10^2 CFU/mL of *E. coli* (MBC test results not shown) for G-H_2O (1:7) (from 1.3 x 10^8 CFU/mL added bacteria from inoculum). Meanwhile, MBC test result of FS-H_2O (2:1:15) showed that the curcuminoids concentrations at 2.25, 2.50, 3.50, and 3.75 mM in FS-H_2O (2:1:15), and a blank of FS-H_2O (2:1:15) contained overgrowth of bacterial categorized as too numerous to count (TNTC). Thus, FS-H_2O (2:1:15) did not show any MBC on the studied curcuminoids concentrations, 2.00-4.00 mM. While MAS-H_2O (1:1:18) was proven by the effectively surprising bacterial growth up to >99.99% against either *E. coli* or *S. aureus*.

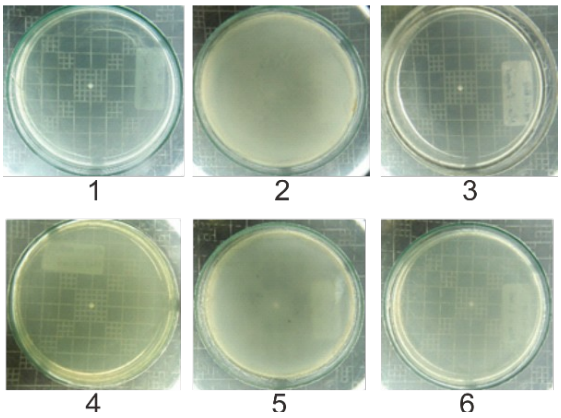


Fig. 3 Minimum Bactericidal Concentration (MBC) tested on *S. Aureus*: (1) MAS-H_2O (1:1:18), (2) S-H_2O (1:18), (3) MA-H_2O (1:18); MBC tested on *E. coli*: (4) MAS-H_2O (1:1:18), (5) S-H_2O (1:18), and (6) MA-H_2O (1:18).

NADES effect on the antimicrobial activity

Both MIC and MBC tests showed that MAS-H_2O (1:1:18) had the most effective anti-microbial activity compared with FG-H_2O (1:1:7) and FS-H_2O (2:1:15). NADES with organic acid compounds has higher anti-microbial activity than NADES containing alcohol, amino, and sugar. Similar results were also reported by Radosevic *et al*. (2017). The effect of an additional hydroxyl group for organic acids such malic acid as Hydrogen Bond Donor (HBD) increases anti-bacterial activity (Zhao and Xu, 2015). This fact also supported by Spaeth *et al*. (2018), hydrogen bonding of PS or dye is an important factor affecting the interaction between PS with the outer membrane cell of bacteria. MAS-H_2O (1:1:18) of NADES presumably creates more hydrogen bonding with curcuminoids as PS than FG-H_2O (1:1:7) and FS-H_2O (2:1:15). Hence, this creates more interactions with outer membrane cell of bacterial and finally disrupted the cell system.

The toxicity of NADES to a microorganism can also be affected by the pH of NADES itself. NADES containing malic acid has high acidity (pH <3), while NADES containing sugar has low acidity (pH >5). Thus, microbial tests on NADES containing malic acid showed high effectiveness in suppressing both of *S. aureus* and *E. coli* bacteria. Several reports indicated that NADES increases the permeability of lipid membranes of eukaryotic cells (Mbous *et al*., 2017). However, the factors affecting the bacterial membrane are still unknown. The solubility of the components in bacterial membrane; osmolality; or chelation may also be affected (Wikene *et al*., 2017). The effect of NADES on bacteria is possibly caused by: (1) the presence of antibacterial effects on NADES due to low pH, ca. pH <3. The acidic environment conditions (pH <3) are not being conducive to bacterial growth, weakening bacteria, and ultimately making their growth susceptible to other factors, (2) the presence of a peptidoglycan, a long chain of polysaccharides composed of N-acetylmuramic acid and an N-acetylglucosamine residue cross-linked by short peptide (Radosevic *et al*., 2017), and (3) NADES may be partially segregated in solution and the cations in NADES interacting with the polysaccharide chain through hydrogen bonds or electrostatic interaction causing cell wall interference (Wen *et al*., 2015). However, our study was not focused on the mechanism. This acidic environment conditions effect was observed from the results of MAS-H_2O (1:1:18) and MA-H_2O (1:18). Both of these variables indicated an inhibition and an eradication of bacterial growth up to >99.99% either by MIC and MBC tests. Maleic acid-choline chloride (MC3 = 1:3) of NADES shown highly photo-inactived *E. coli* at 1.25 μM of curcumin. Moreover, without PS and color-activated by exposed in visible light, MC3 possessed antibacterial activity to *E. coli* (Wikene *et al*., 2015). However, both low concentration of bacteria (OD_{600} = 0.030) and pH solution of MC3 (pH = 0.6) were applied. Strengthen the conclusory, low acidic solution not suitable for the bacteria.

NADES can weaken bacteria by extracting water-soluble and insoluble components from the bacterial membrane; since NADES dissolves some small molecules and macromolecules, including DNA (Dai *et al*., 2013). The disruption of the bacterial cell wall is due to the delocalized charge in NADES (Mbous *et al*., 2017; Wen *et al*., 2015) The presence of interference of the charge interaction in bacteria causing lysis due to NADES has acid properties (low pH).

The gram-negative bacteria, i.e. *E. coli*, having an extra lipopolysaccharide outer membrane on its cell wall. Therefore, *E. coli* was not susceptible to the disruption of NADES addition. Choline Cloride (ChCl)/malic acid and ChCl/malonic acid causing greater impact of lysis of gram-positive bacteria than that of gram-negative bacteria (Wen *et al*., 2015). This phenomenon was proven on the result of G-H_2O, when the growth of E. *coli* is more numerous than *S. aureus*. NADES composed of sugars, i.e. FG-H_2O and FS-H_2O, had no anti-microbial activity. This was proven by the growing number of bacteria in too numerous to count (TNTC). It is reasonable since carbohydrates (especially glucose and fructose) are the sources of carbon and energy for the growth of bacterial cells. Glucose, in particular, is metabolized through glycolysis; providing energy and metabolic intermediates for tricarboxylic acids. Glucose also provides a ribosome with *pentose phosphate*, which is needed for the synthesis of nucleic acids (Butler, 2004).

The result of this study indicated the role of NADES as a curcuminoids solvent in inhibiting the growth activity of bacteria especially NADES containing organic acid compounds such as MAS-H_2O (1:1:18) as well as those with high hydrogen bonding.

Curcuminoids effect on the antimicrobial activity

No bacterial growth was found at MAS-H_2O (1:1:18) both by MIC and MBC tests at various concentrations of curcuminoids (2.00-4.00 mM). This might be explained due to the low pH value of MAS-H_2O (1:1:18), ca. pH =1.1. *Escherichia coli* and *S. aureus* grows optimally at pH 7.0-7.5; tough *S. aureus* can be growth at pH range of 4.0-9.8 (Mailia *et al*., 2015). Moreover, curcuminoids has a broad spectrum of antibacterial activity against various types of gram-positive and gram-negative bacteria. In case of neutral type of NADES, FG-H_2O (1:1:7), the MIC test results shown an inhabitation of *E. Coli* only at 3 mM of curcuminoids concentration while not applied on *S. aureus*; due to bacteriostatic properties of *E. coli* (Fig. 1(a)). However, FS-H_2O (2:1:15) of NADES at curcuminoids concentrations of 2.00-4.00 mM gave good inhibition of both *E. coli* and *S. aureus* during 24 h (Fig. 2).

Antimicrobials mechanism of curcumin is similar to other phenolic compounds which have shown bactericidal properties. Thereof, phenolic often used as disinfectants. These bactericidal properties are effective active against vegetative bacterial cells but not against

bacterial spores. These bactericidal mechanisms can be achieved by: (1) damaging the cells wall, resulting cells lysis; (2) inhibiting formation of the cell wall components in growing cells; (3) changing the permeability of the cytoplasmic membrane causing leakage of nutrients from cells; (4) denaturing proteins of the cells; and (5) inhibiting the action of enzymes in the cells. However, the activity of phenol decreases with dilution and reaction with other organic compounds. Phenol compounds are very active at acidic pH (Abdullatif, 2016). Hence, a bactericidal activity was observed when curcuminoids dissolved at MAS-H_2O (1: 1: 18). No bacteria growth was detected after 24 hours after incubation. However, a bacteriostatic phenomenon is observed both in FG-H_2O (1: 1: 7) and (FS-H_2O = 2: 1: 15) of NADES. An inhibition was only effective for 24 h of incubation.

Moreover, curcuminoids is very unstable at pH >7 and optimally stabile at pH <7 (Tønnesen and Karlsen, 1985). Under acidic condition (pH <7), curcuminoids degradation proceeds much more slowly and less than 20% of curcuminoids decomposes at 1 hour (Kumavat *et al*., 2013). The stability of the curcuminoids is thought to be closely related to the pH conditions of NADES. MAS-H_2O has low pH due to the malic acid constituent of NADES while the neutrals type of NADES, containing sugar, has a pH>5.

Curcuminoids stability is also due to the presence of hydroxyl or carboxyl groups in malic acid. The presence of hydroxyl groups (-OH) or carboxyl (-C=O-) allows more hydrogen bonds to be formed (Dai *et al*., 2013), therefore curcuminoids are more bonded and preserved through hydrogen bonds, consequently more stable. Acidic type of NADES, which is consisted of malic acid-sucrose-water (MAS-H_2O = 1:1:18) has more hydroxyl groups (-OH) than neutral type of NADES, i.e. FG-H_2O (1: 1: 7) and (FS-H_2O = 2: 1: 15). The more hydroxyl and/or carboxyl groups in the NADES constituent components, the more curcuminoids are bound and preserve through the hydrogen bonds.

CONCLUSION

Acid type of NADES, MAS-H_2O, has the most effective anti-microbial activity compared to the neutral types (FG-H_2O and FS-H_2O). The toxicity of MAS-H_2O was affected by the pH of NADES since malic acid was a strong acid (pH<3), while neutrals NADES (sugar contains) had a pH>5. In addition, the active compound of curcuminoids had broad spectrum antibacterial activity that was antibacterial active against various types of gram-positive and gram-negative bacteria. The observed toxicity depends both on the concentration of the curcuminoids and bacteria.

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