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In vitro Antibacterial Activity of Cell Free Fermentation Supernatant of *Passiflora edulis forma flavicarpa* Sims. Fruit Fermented by de Man, Rogosa and Sharp Media

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Abstract

Antibacterial activities of cell free fermentation supernatant (CFFS) of passion fruit (*Passiflora edulis forma flavicarpa* Sims.) fermented in de Man-Rogosaand Sharpe (MRS) broth media against *Staphylococcus spp*., *Methicillin-Resistant Staphylococcus aureus* (MRSA), and Extended Strain Methicillin-Resistant (ESBL) *Escherichia coli* have been investigated. The fermentation broth was derived from 24 and 48 hours cultures collection after rotary shaking incubation at 37^{o}C. A bioassay was performed using well diffusion agar method on nutrient agar media, incubated at 37^{o}C for 24 hours. Minimum inhibitory concentration and potency of the CFFS were determined using kanamycin, streptomycin, vancomycin, erythromycin, and amoxicillin as standards. It was found that the fermentation broth containing 32 x 10^4 CFU/g exhibited inhibitory activity against *S. Aureus* ATCC 25923 and *S. epidermidis* FN-6 after 24 hours similar to 48 hours fermentation. The anti-bacterial activities of 24 hours fermentation supernatant against all the test bacteria were almost similar. The characteristic of the CFFS indicated the acid property with pH of 3$\pm$ 0.1. Lactic acid bacteria were detected by biochemical identification based on catalase, Gram staining, motility, H_2S, indol, Simon citrate, and Voges Proscower tests. Thin layer chromatography-contact bioautography was developed by KH_2PO_4 solution as eluent and *E.coli* as a test bacterium showed two spots by which two clear inhibition zones were obtained. The prospective of CFFS passion fruit as a potential antibacterial substance source is recommended for future development.

Keywords: Antibacterial activity, passion fruit, fermentation supernatant, *Staphylococcus spp*, ESBL, MRSA.

1. Introduction

Nowadays, the use of natural ingredients as raw materials in drug development is beginning to be in demand among the pharmaceutical industry communities. According to World Health Organization (WHO) data, about 80% of the world population are using products based on medicinal herbs. Plants, especially those with ethnopharmacological uses, have been the primary sources of medicine for early drug discovery (Sofija, 2017). During the last 10 years, the discovery of new antibiotic drugs is not considered comparable with the prevalence of antibacterial resistance, so research for the discovery of antibiotic raw materials began to be directed at natural sources (Asirvathamdoss, *et al.*, 2008).

Passion fruit (*Passiflora edulis*), a member of the Passifloraceae family, is also well-known as markisa fruit. It has more than 500 species (Paull and Duarte, 2012; Reis *et al.*, 2018). The plant originated from Brazil and has scattered to other countries in Asia, Australia, Africa, India, South America, and the Caribbean. It has other variants that can be identified by the color of their fruits such as yellow which is *P. edulis forma* flavicarpa, purple which is *P. edulis forma* edulis, and orange which is *P. edulis* var. caerulea (Reis *et al.*, 2018). The plant parts (flower, leaves, stem, fruits, and roots) have several medicinal effects and have been used traditionally. The flower is the part that is used mostly for its calming effect, anticonvulsant and antihypertensive properties, which are useful for patients with anxiety and insomnia. However, it is not as potent as *Passiflorain carnata* flower (Ramaiya *et al.*, 2014). Meanwhile, the fruits are almost round or oval in shape; about 4cm to 8cm in diameter, and mainly used as food since they have a juicy orange pulp. The pulp is sweet-sour in flavicarpa variant and sweeter in both edulis and caerulea variant. The skin is tough, smooth, and waxy but wrinkles when it is ripe. The seed is numerous, hard, small, and pitted inside the fruit. It has many common names according to the country they are grown in such as

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'*markisa*' in Indonesia and Malaysia, '*limangkan*' in Laos, '*maracuya peroba*' in Portugal, '*maracuja-do-campo*' in Brazil (Paull and Duarte, 2012; Aziz, 2016).

The fruits contain good amount of nutrients which are good for dietary consumption and have numerous phytochemicals such as glycosides including flavonoids (Ingale and Hivrale, 2010), e.g. luteolin-6-C-chinovoside, luteolin-6-C-fucoside, cyanogenic glycosides, e.g. passibiflorin, epipassibiflorin, passicapsin, passicoriacin, epipassicoriacin, cyanogenic-b-rutinoside, epitetraphyllin B, amygdalin, prunasin, triterpenoid glycosides, e.g.passiflorine), and salicylate glycosides. Other chemicals such as b-carboline alkaloids harman, harmine, harmaline and harmalol, phenols, carotenes, and g-lactones are also found in the fruit (Bernes *et al.*, 2007). Passion fruit is a fruit that has a high nutritional value where there are many multimineral contents and various vitamins, as well as high carbohydrates and water (Zibadi and Watson, 2004). These compounds may become the prospect of antimicrobial, antioxidant, anticancer,and anticarcinogenic (Ramaiya *et al.*, 2014; Oliveira *et al.*, 2016). Reis *et al* (2018) mention that the compound is also associated with antiplatelet, antiviral, antiallergic, and anti-inflammatory activities. Furthermore, the plant parts of the passion fruit including the peel extract and the pulp are positively tested for antibacterial and antifungal activity on certain microbial tested (4). It has been reported that antibacterial and antifungal compound has been isolated by Birner and Nicolls as cited by Ramaiya *et al* (2014).

In recent years, there have been many studies on the antimicrobial activity of the *Passiflora edulis* plant by which is strong to moderate inhibition both in Gram-positive and Gram-negative has been exhibited. Extracts from the leaves and flowers of the plant *Passiflora edulis* are able to inhibit the growth of pathogenic bacteria such as *V. Cholerae*, *Pseudomonas aeroginosa*, *Escherichia coli*, *Bacilus subtilis* (Ingale and Hivrale, 2010), *Salmonella typhi*, *Staphylococcus aureus*, *Streptococcus pyrogens*, and *Bacebuspenilis* (Asirvathamdoss, *et al*., 2008).

There have been numerous studies of passion fruit for its phytochemical properties; however, there are no reports concerning the antibacterial activities of the pulp of fruit fermented by de Man Rogose and Sharp (MRS) media against pathogenic bacteria. Zahroh (2014) has reported lactic acid bacteria (LAB) isolated from the passion fruit *Passiflora edulis* var. Sims. Some LAB have been known as sources of bioactive substances included antimicrobial. This study has performed antibacterial activities of cell-free fermentation supernatant (CFFS) of *Passiflora edulis forma* flavicarpa fruit against ESBL *Escherichia coli*, MRSA, and some species of *Staphylococcus* and non-ESBL *Escherichia coli*. Screening active substances by Thin Layer Chromatography-contact bioautography have also been reported.

2. Materials and Method

2.1. Plant source and determination

The yellow passion fruits (Figure 1) were collected freshly from a local farm in Sidoarjo, harvested in April 2019. The passion fruit plant was identified and determined based on the taxonomy character of leaf, flower, fruit, steam plant, and recommended by Herbarium Malangensis, Department of Biology, Faculty of Math and Science, Universitas Negeri Malang as *Passiflora edulis forma flavicarpa*, Sims.

2.2. Sample preparation, fermentation, and characterization

The passion fruits were washed and dried before they were divided into two parts and the 5 gram of fruit pulps were weighed and put into 50 mL of MRS broth media to be fermented with rotary shaker at 150 rpm and at 37^{o}C for 48 hours. The fermentation broth was taken after 24 hours of fermentation, centrifuged, and the supernatant was collected for characterization and bioassay.

2.3. Determination of Total Plate Count (TPC)

The supernatant was then made into a serial dilution of 1:10 until 10^7 using sterile normal saline solution. Each of the serial dilutions was inoculated on the MRS (Oxoid, UK) agar and incubated at 37ºC for 24 hours. Cell growth was observed and the plating colonies were counted using bacteria colony counter.

2.4. Inoculum preparation.

The selected bacteria strain was transferred aseptically to sterile saline water, vortex, and then the turbidity was measured using spectrophotometer against the sterile saline water to obtain 25% Transmittance (about 10^9 CFU/ml of bacteria) turbidity or optical density at 580nm (Isnaeni *et al.*, 2019).

2.5. Bacterial inhibitory activity.

Screening inhibitory activity of the passion fruit was performed after 24 and 48 hours fermentation. The bioassay was done by well agar diffusion method using NA media and *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 8739, ESBL and MRSA bacteria obtained from RSUD Dr. Soetomo as test bacteria. The test media were prepared by pour plate method. 10-12 mL of melted NA (45-50^{o}C) were poured into the empty sterile petri disk, used as a base layer. The seed layer was prepared by adding 3-5 µL of 25 % transmittance of the test bacteria inoculum into 8 mL of the melted NA, mixed well with vortex, poured on the surface of the base layer, and allowed it to solidify. The well was made by bored with 7 mm in diameter. Each well reservoir was filled with 50 µL of the solution test. Incubation was performed at 37ºC for 24 hours. Growth inhibitory zone diameter was measured by digital caliper (Isnaeni, 2019).

2.6. Thin Layer Chromatography-contact bioautography

The developing chamber used was CAMAG Chamber (10x10) with lid and was prepared aseptically. Hence, the size of the TLC plate used was 6cm x 10cm and it was dried at 100'C for 20 minutes before developing. The solvent used was 7.5% of KH_2PO_4 solution. The samples used were 20µL for each spot and "overspot" loading was used to concentrate the samples on one spot over again after the previous spot has dried. The samples were CFFS of passion fruit of 24-hour fermentation, streptomycin (100ul/ml), and kanamycin (100uL/ml) used as standard.

The Contact Bioautography was performed after the developed TLC plate had completely dried from the residual solvent and contacted, silica gel side down, onto the *Escherichia coli* seed layer as the test bacterium in the petri dish. The agar plate and the contacted plate were stored in the refrigerator for 1 hour to allow the diffusion

of the active substances in the chromatogram into the seed layer. Furthermore, the plate was removed from the agar plate,then incubated for 24 hours at 37'C. The growth of the *E. coli* and the inhibition zone were observed.

2.7. Minimum Inhibitory Concentration.

Determination of the minimum inhibitory concentration (MIC) of the passion fruit CFFS was done using the agar dilution method, where a number desired volume of the supernatant (1ml, 2ml, and 3ml) were mixed with the NA media. Then it was inoculated with six test bacteria by streaking using Öse needle about 1cm on the surface of the NA medium. Multiple replicates were performed on the same agar plate. The agar plates were then incubated at 37'C for 20-24hours. An agar plate without the passion fruit CFFS was performed as the negative control. Serial dilution of CFFS had been applied form 100%, 75%, 50%, 25%, 12.5%, and 6.25% of concentration on the paper disk with volume capacity of 10µL, placed on the surface of NA agar inoculated by *E.coli* as a test bacterium. Zone of growth inhibitory activity was observed after 20-24 hours of incubation.

3. Results

3.1. Characteristic of Free Cell Fermentation Supernatant

Performance of the fermentation broth of passion fruit in MRS media (Table 1) was brownies in color, pH value 6±0.1 before and decreased to 3±0.1 after 24 and 48 hours fermentation. Total plate count in MRS and NA media was 133 10^3 and 32 x 10^4 CFU / mL respectively. Several colonies suspected Lactobacilli and Streptococci were found, based on the identification of the isolates with biochemical reactions (Gram staining, catalase, and motility test, conformed to vitek-2).

Table 1. Characteristic of CFFS passion fruit fermented in MRS media

Parameters	Characteristics
Organoleptic	Liquid, browns colour
Odor	Specific smell of passion fruit
pH	6 ± 0.1 (0 hours), 3±0,1 (24 hours)
Total plate count in MRS and NAMedia	133 x 10^3 and 32x10^4CFU/ml
Inhibitory activity at 0 hour incubation	- (*S. aureus* and *E.coli*)
Inhibitory activity at 24 hours incubation	+ (*S. aureus* and *E.coli*)
Inhibitory activity at 48 hours incubation	+ (*S. aureus* and *E.coli*)
Lactic acid bacteria screening	+ based on Gram staining, morphology, catalase and motility test, conformed to VITEK-2

3.2. Antibacterial activity

The CFFS of passion fruit screening growth inhibitory activity against *S. aureus* ATCC 25923 showed that the potency of the CFFS after 24 hours and 48 hours fermentation was almost similar (Table 2 and Figure 1), as well as the activity against *S. epidermidis* FN-6 (Figure 2), ESBL, and non-ESBL *E. coli* after 24 hours of fermentation (Figure 3). Subsequent tests were carried out for fermented broth after incubation for 24 hours on several *Staphylococcus spp*. and *E. coli* (Table 2). The agar well diffusion test performed to 5 different strains of *E. coli* showed that the CFFS of passion fruit presented inhibitory activity against five different strains of *E. coli* with the zone of inhibition diameter from 13.80 mm to 22.05 mm (Figure 4).

Table 2. Inhibitory activities of CFFSpassion fruit after 24 hours fermetation againts various test bacteria

Bacteria	Zone of growth Inhibition (mm)					
	CFFS	Van 8ppm	Cefat 8 ppm	Strep 8 ppm	Am 16 ppm	Ery 8 ppm
Exteded Spectrum Beta Lactamase	17,78 ± 0,60					
Escherichia coli ATCC 8739	17,47 ± 0.57					
ESBL *E.coli*6110	15,10 ± 1,17					
ESBL *E.coli*6024	16,33 ± 0,31					
ESBL *E.coli*5949	20,65 ± 0,69					
Staphylococcus aureus ATCC 25923	17,76 ± 1,12	16.63±0.05	20.00±0.00	22.98±3.15	19.88±0.65	21.35±0.00
MRSA	15.13 ± 1.03					
Staphylococcus epidermidis FNG-1	15,68 ± 0,86					
Staphylococcus epidermidis FNG- 2	16,83 ± 0,47					
Staphylococcus epidermidis FNG- 3	15,80 ± 0,68					
Staphylococcus epidermidis FNG-4	16,48 ± 0,41					
Staphylococcus epidermidis FNG-5	16,99 ± 0,39					
Staphylococcus epidermidis FNG-6	15,43 ± 0,44					
Staphylococcus epidermidis FNG-7	16,16 ± 0,59					
Staphylococcus epidermidis FNG-8	17,03 ± 0,24					
Staphylococcus epidermidis FNG-9	17,06 ± 0,24					
Staphylococcus epidermidis FNG-10	17,80 ± 1,12					

Diameter of agar hole =7 mm.Van= Vancomycin, Cefad= Cefadorxil, Strep= Streptomycin, Am = Amoxycilin , Ery = Erythromycin

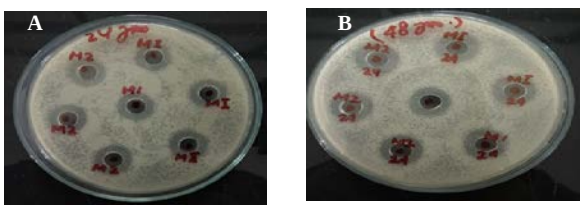

Figure.1. Growth inhibitory activity of passion fruit CFFS in MRS media against *S. aureus* ATCC 25923 after incubation at 37°C for 24 hours (A) and 48 hours (B).

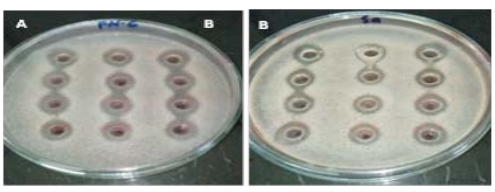

Figure.2. Growth inhibitory activity of passion fruit CFFS in MRS media against *S. epidermidis FN-6* (A) and *S. aureus* ATCC 25923 (B) after incubation at 37°C for 24 hours.

Figure.3. Growth inhibitory activity of passion fruit CFFS in MRS media against ESBL 6110 (A), non-ESBL *E.coli* (B), and ESBL 6024 (C) after incubation at 37°C for 24 hours.

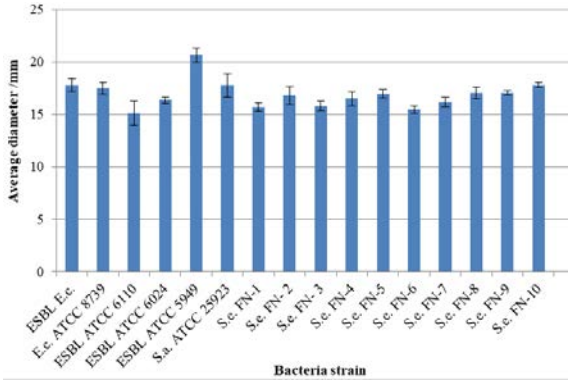

Figure 4.Average diameter (mm) of zone inhibition of CFFS againts test bacteria

3.3. Minimum inhibitory concentration

The MIC of CFFS passion fruit was performed after antibacterial sensitivity was done. Figure 5 depicted the sensitivity of CFFS against six test bacteria. The agar dilution test of the CFFS had shown that the negative control agar plate (without CFFS addition) was covered with full growth of all test bacteria, while the agar plate with 1mL of the CFFS had shown inhibition to most of the inoculated area with few insignificant bacterial growths. Furthermore, the agar plate with 2mL and 3 ml of the CFFS had completely inhibited all microbial growth (Table 3). Table 3 showed the susceptibility test of the agar dilution test against the test bacteria. In addition, determination of MIC using serial dilution of 10 µL CFFS 100%, 75%, 50%, 25%, 12.5%, and 6.25 % applied on the sterile paper disk indicated that 75% of exhibited zone of growth inhibitory activity were less than 13 mm, meanwhile the 50% CFFS solution did not exhibit inhibitory activity.

3.4. Statistical analysis

Statistical analysis using Kruskal Wallis test was performed to evaluate the significant difference of inhibitory activity of the CFFS against bacterial tests. From the analysis results, it was obtained Asymp sig value that was 0,000. CFFS provided the greatest inhibitory activity on ESBL *E. coli* 5949 and the smallest one in *Staphylococcus epidermidis* FNG-6.

Table 3. Sensivitity test of six test microbial against passion fruit CFFS after 24 hours using streak method

Bacteria	Volume of CFFS added to 10 ml nutrient agar media											
	Positive Control (Without CFFS)			1mL			2mL			3 mL		
	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3
ESBL	+	+	+	+	-	-	-	-	-	-	-	-
E ESBL6110	+	+	+	-	-	-	-	-	-	-	-	-
E ESBL6024	+	+	+	-	-	-	-	-	-	-	-	-
E ESBL5949	+	+	+	-	-	-	-	-	-	-	-	-
E ESBL8739	+	+	+	-	-	+	-	-	-	-	-	-
SA ATCC 25923	+	+	+	-	-	+	-	-	-	-	-	-

R = Replicate

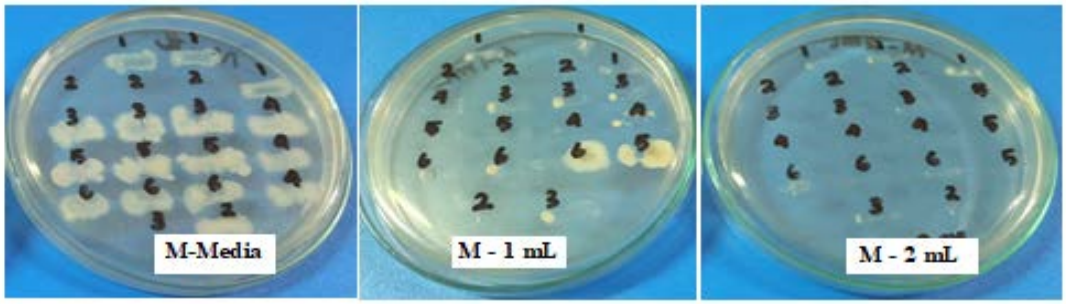

Figure 5. Sensitivity of six test bacteria against CFFS on Nutrient Agar mediaESBL *E. coli*, 2. NA-broth, 3. ESBL 6024, 4. ESBL 5949, non-ESBL *E.coli* ATCC 8739, *S.aureus* ATCC 25923, M-Media: media without CFFS, M-1 mL: NA Media + 1 mL of CFFS, M-2 mL: NA Media + 2 mL of CFFS.

4. Discussion

Passion fruit, as well as other fruits, is a suitable habitat for the growth of lactic acid bacteria (Askari *et al.*, 2012) and even probiotics, due to their adequate nutritional content. White and Sharareh (2018) have reported the results of their research on the fermentation process of *Lactobacillus rhamnosus* GR-1 using apple, orange, and grape juice. The main fermentation products that can be directly detected and dominant are lactic acids and other organic acids, thereby reducing the pH value in the fermentation process. In this study, pH value of the CFFS decreased at 24 hours of evaluation from 6 to 3. It was reported that passion fruit by-product stimulates the growth and folate production by starters and probiotic cultures in fermented soymilk (Mac *et al.*, 2017). The fermentation process using inulin apparently accelerates growth by up to 10 times compared to being stored in a refrigerator. The potential of the CFFS passion fruit as lactic acid bacteria by which many useful substances produced by fermentation processes might prospectively develop.

In this study, it has also been proven that before the fermentation process, the viability of the cell colonies in MRS media was less than 30 and increased to 32 x 10^4 CFU / mL after 24 hours of incubation. The MRS is a selective medium for the growth of lactic acid bacteria (Askari *et al.*, 2012). Before the fermentation process, CFFS passion fruit also did not show inhibitory activity against all test bacteria (Table 1). Antimicrobial activity of dried fruit has been widely reported (Aziz *et al.*, 2006). This phenomenon proves the importance of the presence of active compounds in fruit (Ingale and Hivrale, 2010; Reis *et al.*, 2018) as antimicrobial. Asirvathamdos *et al.* (2008) have reported in-vitro antimicrobial activity of passion fruit extract, but the effect of the fermentation process in selective media MRS for probiotic growth and antibacterial activities has not yet been reported. Fermentation with MRS media leads to increase the population of lactic acid bacteria that are able to produce lactic acid and other organic acids, as well as other active ingredients that can act as anti-bacterial. Therefore, the CFFS passion fruit was not only effective as a source of active compounds, but also a source of lactic acid bacteria that have various activities. The accumulation of inhibitory activity against pathogenic microbes is very interesting to be further studied in order to detect the dominant component contributing as an antibacterial.

Pathogenic bacteria used as test bacteria ESBL, MRSA, and non-ESBL *E.coli* and *Staphylococcus spp.* are usually resistant bacteria group against methicillin and beta lactam antibiotics, in which in this research was represented by vancomycin, meropenem, cefadroxil, amoxicillin, streptomycin, kanamycin, and erythromycin. The antibiotics standard solutions used at the CFFS passion fruit showed intermediate until sensitive against some test bacteria at 100%/50µL with MIC of 10% (Table 2, Figures 5 and 6). Antibacterial activity data from CFFS compared with various bacteria test were analyzed using the IBM SPSS ver 24 program. From the results of normality testing requiring one group of samples that had a sig value <0.05, we could obtain abnormal data distribution results.

Statistical analysis was using the non parametric Kruskall Wallis method with a confidence degree of 0.95 (α = 0.05). From the analysis results, it was obtained Asymp sig value of 0,000, so that the p value was<0.05. It can be said that there were significant differences between the antibacterial activity of CFFS with the type of bacteria test. CFFS provides the greatest inhibitory activity on ESBL *E. coli* 5949 and the smallest one on *Staphylococcus epidermidis* FNG-6. However, this phenomenon cannot prove the inhibitory potential of bacteria based on their Gram bacteria. The CFFS has a broad spectrum activities, but its inhibitory potential will depend on these individual bacterial strains. Therefore, further research is needed for this matter. In the future, it is interesting to examine the passion fruit CFFS activities on *Mycobacterium tuberculosis* (MTB) and Multi Drugs Resistant (MDR)-TB and other MDRs. On the other hand, the CFFS has potential opportunity to improve as an antimicrobial instant preparation.

The results of active substances identification by TLC-contact bioautography showed that CFFS contained two active compounds and the TLC chromatograms could be detected by UV lamps (Figure 7), but the Rf value of active compound on the TLC-bioautograms was still needed to be further investigated, whether it was derived from the same compounds as detected with UV lights. Based on the TLC chromatogram developed by a single eluent of KH_2PO_4 solution with a pH of 6.5, which was able to eluate the active compound with Rf 0.4-0.5 (tailing), the active compound was predicted to be polar. The TLC-bioautography method was very suitable for screening active compounds for both identification and separation through eluent system optimization. A very simple method can separate the active compound components from natural ingredients, two or more compounds from the same class, for example, the antibiotic streptomycin aminoglycosides and kanamycin (Febri *et al*, 2019) that have been validated (Isnaeni *et al.*, 2019).

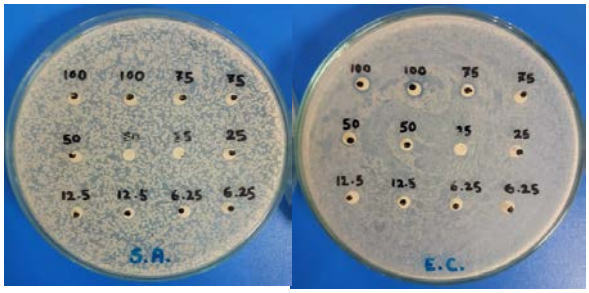

Figure 6. Determination of MIC of 10 µL CFFS on the paper disk against *S. aureus* (A) and *E. coli* (B) on NA mediaat 100%, 75%, 50%, 25%, 12.5%, and 6.25% concentration.

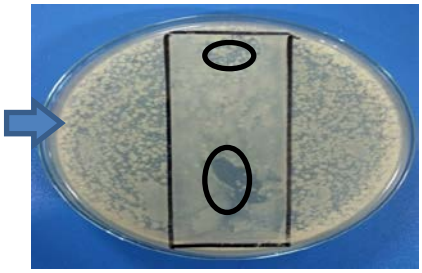

Figure 7. TLC-Chromatogram of CFFS on Silica Gel GF_{254} using 7.5% KH_2PO_4 solution as eluent under UV lamp detection (A) and TLC-contact bioautogram using *E.coli* as a test bacterium (B).

5. Conclusion

Cell-free fermentation supernatant of passion fruit (*Passiflora edulis forma*.flavicarpa Sims.) is recommended to be developed as a source of active substances for antibacterial against pathogenic bacteria event for multi drugs resistant. The active substance might be a polar compound. Furthermore, isolation, separation, and purification to obtain the active isolates or novel substances are very interesting to be studied in the future.

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