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RESEARCH ARTICLE

In Vitro Effect of Alfa Mangostin on Multiresistant Uropathogenic Escherichia Coli

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ABSTRAK

Pendahuluan: Di Indonesia, resistensi terbesar pada uropatogen E. coli adalah resistensi terhadap ampisilin (91,9%), siprofloksasin (83,7%) dan sefiksim (67,6%). α–mangostin merupakan salah satu senyawa kimia yang dikembangkan sebagai antibiotik baru dari isolat herbal Garcinia mangostana L, namun belum terbukti efektifitasnya terhadap E. coli multiresisten. **Tujuan:** Penelitian ini menguji aktivitas α- mangostin secara in vitro pada pertumbuhan bakteri uropatogen E. coli multiresisten.

Metode: Perlakuan terhadap bakteri uropatogen E. coli dilakukan secara in vitro, menggunakan α–mangostin menggunakan kadar 14; 28,13; 56,25; 112,5; 225; dan 450 µg/mL dengan replikasi 4 kali pada masing-masing konsentrasi. Aktivitas antibakteri dari α–mangostin diukur dengan melihat pertumbuhan atau kematian bakteri pada setiap konsentrasi menggunakan metode tidak langsung, yaitu metode pembacaan absorbansi sampel. Sampel atau uropatogen E. coli yang telah diberi perlakuan berbagai dosis α–mangostin diinkubasi selama 18 – 20 jam, kemudian dibaca absorbansinya menggunakan spektrofotometer UV-Vis λ 625 nm.

Hasil: Nilai Kadar Hambat Minimal (KHM) pada penelitian ini adalah 450 µg/mL. Berdasarkan uji regresi linear (STATA 13.1) hubungan konsentrasi α–mangostin dan aktivitas hambatan pertumbuhan bakteri, didapatkan nilai uji F 0,0001 < 0,05 menyatakan bahwa semua konsentrasi α–mangostin secara simultan mempunyai pengaruh signifikan terhadap pertumbuhan uropatogen E. coli.

Kesimpulan: α–mangostin tidak efektif untuk menghambat pertumbuhan bakteri uropatogen E. coli multiresisten karena nilai KHM 450 µg/mL relatif besar. Aktivitas relevan berpotensi di klinik akan terjadi apabila nilai KHM suatu zat secara in vitro < 100 µg/mL. Bahkan industri farmasi lebih menyukai pengembangan antibiotik yang mempunyai nilai KHM in vitro ≤ 2 µg/mL.

Kata kunci : MDR, antibiotik, Garcinia mangostana L.

ABSTRACT

Introduction: *In Indonesia, the most commom uropatogen E. coli resistance has been to ampicillin (91.9%), ciprofloxacin (83.7%) and cefixime (67.6%). α-mangostin, a chemical compound, has been developed as a new antibiotics isolaated from herbal Garcinia mangostana L, but its effectiveness against multiresistant uropathogenic E. Coli has not been established.* ***Objective:*** *This study examined the effect of α-mangostin on growth of multiresistant E. coli*

Methods: *α-mangostin Treatment of E. coli uropatogen bacteria was administered in vitro, using 14 levels of concentration 14; 28,13; 56.25; 112.5; 225; And 450 µg/mL with 4 times replication at each concentration. The antibacterial activity of α-mangostin was determined by evaluating bacterial growth at each concentration using the indirect method by sample absorbance reading. The Samples of uropatogen of E. coli treated with various doses of α-mangostin were incubated for 18-20 hours and then subjected to the absorbance reading using a UV-Vis spectrophotometer λ 625 nm.*

Results: *Minimum inhibitory concentration (MIC) in this study was 450 mg/mL. Based on linear regression (STATA 13.1) relationship between α-mangostin concentrations and bacterial growth inhibition activity showed 0.0001 <0.05 showing that all concentrations of α-mangostin simultaneously had a significant effect on the growth of uropathogenic E. coli.*

Conclusion: *α-mangostin has not been effective to inhibit the growth of multiresistent uropathogentic E. coli due to a relatively high MIC (450 mcg/mL). a Potentially relevant activity in the clinical setting will occur if the value of the MIC of a substance in vitro <100 µg /mL. Even the pharmaceutical industry prefers the development of antibiotics with in vitro MIC value of ≤ 2 µg/mL.*

Keywords: *MDR, antibiotics, Garcinia mangostana L.*

INTRODUCTION

The most common extraintestinal infections caused by the bacteria of Escherichia coli (E. coli) has been urinary tract infection (UTI). This disease occurs at least 25-35% adul women during their life (Sukandar, 2009). Meanwhile, the incidence of multi drug resistance of E. coli has become a global problem (Alhashash *et al.,* 2013).

The incidence of multi-drug-resistant uropathogenic Escherichia coli has been reported in Nepal (Baral *et al.,* 2012) represeting 38.2% total isolates of patient with UTI. Moreover, 55.2% uropathogenic E. coli was detected toproduce Extended Spectrum Beta Lactamase (ESBL). Another study stated that the most

common E. coli resistece was to co-trimoxazole (86%), amoxicillin (76%), class of cephalosporins (53.75% - 70%), and followed by the fluoroquinolone group (52.5% - 57.5%) (Ihsan *et al.,* 2014). Meanwhile, broad-spectrum antibiotics such as amoxicillin clavulanate, flurokuinolon, and cephalosporin have been avoided due to the risk of resistance. In Indonesia, the most commom resistance in uropatogen E. coli is resistance to ampicillin (91.9%), followed by ciprofloxacin (83.7%) and cefixime (67.6%). The resistance is caused by the irrational use of antibiotics (Khalilullah *et al.,* 2011).

The multiresistant uropathogenic E. Coli incidence requires us to look for alternative treatment. Despite efforts to optimize the parameters of the available pharmacokinetics/pharmacodynamics of each has been done, but optimizing the parameters PK/PD is not completely reduce the incidence of antibiotic resistance (Gloede *et al.,* 2010). Other efforts that can be done is infection therapy using herbal medicine or chemical isolates from herbal ingredients. Mangosteen (Garcinia mangostana L.) Are found in Indonesia, some active isolates were tested possess antibacterial activity. Mangosteen extracts are reported to have pharmacological activity as anti-inflammatory, cytotoxic agents, antitumor, allergy, antioxidant, antiviral, and antibacterial. Primary isolates α–mangostin showed a fairly high cytotoxic activity against bacteria (Al Massarani *et al.,* 2013). The antibacterial activity of α–mangostin is bactericidal with the mechanism of action undermines the integrity of bacterial cytoplasmic membrane so that the components of intracellular bacteria out (Koh *et al.,* 2013).

Several studies have demonstrated the effectiveness of alpha-mangostin against Gram + especially Staphylococcus aureus, Leptospira and Mycobacterium tuberculosis (Seesom *et al.,* 2013). Another study of Bhat and Daihan (2013) showed a moderate susceptibility of bacteria to xanthones in the extract of Garcinia mangostana L. is Staphylococcus aureus, Streptococcus pyogenes, Bacilus subtilis and Escherichia coli. But among the four strains tested, Streptococcus pyogenes was the most susceptible to the extract of Garcinia mangostana L. The antibacterial activity of α–mangostin against multidrug resistat uropathogenic E. coli has not been proven. Therefore, this study aimed to determine antibacterial activity of α–mangostin against multiresistant drug uropathogenic E. coli in vitro.

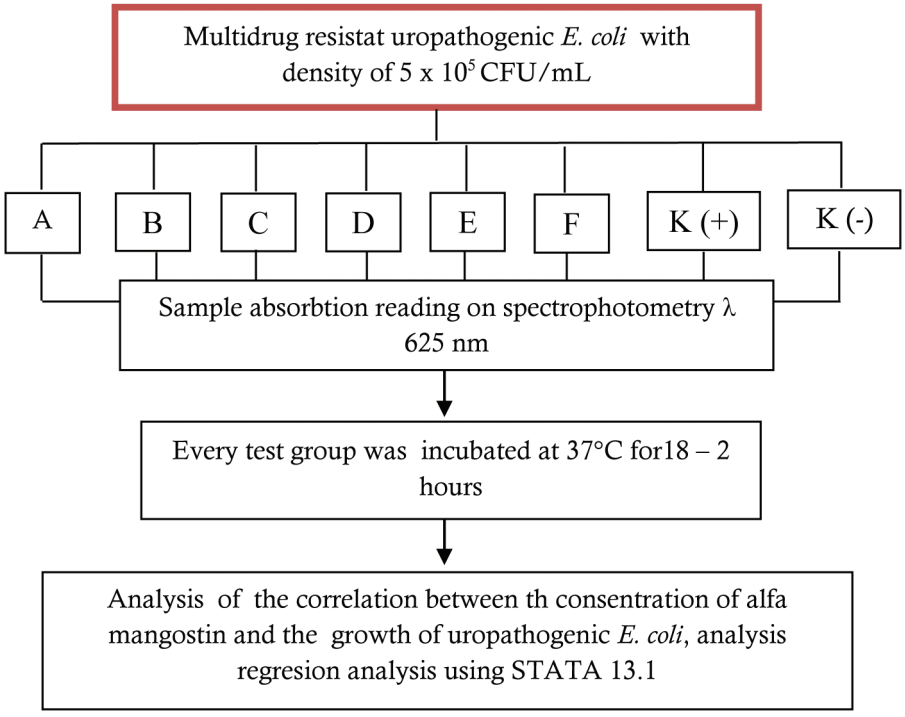
METHODS

A. Population and sample research

The study population was uropathogenic E. coli isolated from patients with UTIs in Muhammadiyah Roemani hospital of Semarang. The multidrug resistent uropathogenic E. coli samples were confirmed with a series of biochemical tests

B. Uropathogenic biochemical test of multidrug Resistent uropathogenic E. coli

The isolates obtained from UTI patients were grown in MacConkey media containing Eosin Methylene Blue (EMB), before gram staining. A series



of biochemical tests were performed to idetify the isolate. The series of biochemical test materials included IMViC (Indol test tube, Methyl red, Voges proskaur, and Citrate). Having idetified uropathogenic E. coli, the pattern of resistance were determined using media Mueller Hinton Agar (MHA, Merck). The Antibiotics used to assess uropathogenic resistance patterns of E. coli were amoxicillin, erythromycin, cotrimoxazole and ciprofloxacin. The multidrug Uropathogenic resistet E. coli were then used to test α–mangostin active substance (purity ≥ 98%, Sigma-Aldrich) that was dissolved in methanol solution(Merck).

C. Test the effectiveness of α–mangostin against E. coli MDR uropathogenic E. coli

The density of the bacterial suspension for determinig the effectiveness of alpha mangostin refers to the standard as described by Matuschek *et al*. (2013) suggesting a minimum density of the bacterial suspension used in the test of minimum inhibitory concentration (5 x 10^5 CFU/mL).

The effectiveness of alpha-mangostin against E. coli bacteria uropatogen was evaluated with broth dilution method using a series of specific levels of alpha-mangostin in broth or liquid media (MHB) (Figure 1). The level range was as described by Al Massarani *et al*. (2013), who reported that alpha activity mangostin dimetilsulfoxide diluted with dimethylsulfoxide (DMSO) for E. coli in the form of IC50 values of v> 200 μM (equivalent to 82.09 mg/mL). In addition to the use of a serial level, one tube without alpha mangostin (positive control) was used as a control of bacterial growth and a negative control tube (sterile bacterial media and alfa mangostin without bacteria) as a sterility control.

Figure 1. Schematic flow of treatment A, B, C, D, E, and F in each group of E. coli bacteria using different concentrations of alpha mangostin. (A) 450.0 μg/mL, (B) 225.0 μg/mL, (C) 112.5 μg/mL, (D) 56.2 μg/mL, (E) 23. 1 μg/mL, dan (E) 11. 5 μg/mL, for control K (+) containing media uropathogen MHB and E. coli bacteria, as well as K (-) alpha mangostin containing 6.75 μg/and MHB media were used. Each treatment was tested with 4 repetitions.

The antibacterial activity of alpha mangostin was measured by observing the growth or death of bacteria at each concentration. The growth or death of bacteria were tested using the indirect method, absorbance readings using UV-Vis λ 625 nm (wavelength readings McFarland standard). Samples or uropathogen of E. coli treated with various doses of alpha-mangostin were incubated for 18-20 hours.

D. Statistical analysis

Absorbance in each treatment group was compared with that of positive and negative control group. To analyze the relationship between the concentration of alpha mangostin and the growth of uropathogenic E. coli, regression analysis test was performed using STATA 13.1.

RESULTS

a. Biochemistry test of Multidrug resistant Uropathogenic of E. coli

Uropatogen E. coli is a Gram-negative growing in the agar media of Mac Conkey. E. coli is a strong lactose fermenter, so that the production of acid produced is enough to precipitate bile salts. Colonies produced by E. coli on MacConkey agar medium was pink with a halo effect around the colony. For bacteria that can not perform lactose fermentation, the Gram bacteria colony - growing in MacConkey media will be transparent or yellowish (Allen, 2013).

The second identification test using EMB agar medium, colonies of purple E. coli and have a metallic green color luminescence characteristics. Agar media of EMB was used to distinguish E. coli lactose fermentant from Enterobacter aerogenes (Oxoid, 2010). biochemical tests, ie indol test, methyl red, proskaur voges, and citrate were done for confirmation. The results of biochemical tests on samples of the uropathogenic E. coli isolates of patients with UTI is shown in table 1:

Table 1. Results of biochemical test on the samples of uropathogenic E. Coli

No	Type of test	result
1	Uji *Indol*	+
2	Uji *Methyl red*	+
3	Uji *Voges Proskaur*	-
4	Uji *Citrate*	-

To get Multidrug resistant uropathogenic E. coli, the bacteria confirmed by chemical were subjected to sensitivity test to assess the patterns of resistance. Multiresistant bacteria are bacteria resistant to three or more antibiotics. Samples of uropathogenic E. coli used uropatogen included antibiotics multiresistant bacteria. The uropatogenic samples of E. coli were resistant to amoxicillin, erythromycin and cotrimoxazole (Satari and Mieke, 2012). The Interpretation of sensitivity test results is presented in table 2 below:

b. Minimum Inhibitory Assay Results of α -Mangostin against Uropathogenic E. coli

Minimal inhibitory concentration assessment

Table 2. Results of uropathogenic E. coli sensitivity test along with a reference standards (EUCAST 2015 and Cockerill *et al.,* 2012)

No	Antibiotics	Diameter results (mm)	Interprestasion	Reference Standard		
				Sensitive (mm)	Intermediate (mm)	Resistent (mm)
1	amoxicillin 25 µg	8	Resistent	≥17	14-16	≤13
2	erythromycin 15 µg	9	Resistent	≥23	14-22	≤13
3	cotrimoxazole 25 µg	10	Resistent	≥16	11-15	≤10
4	Siprofloxacin 5 µg	35	Sensitive	≥21	16-20	≤15

was performed by broth dilution method using a Mueller Hinton Broth (MHB) media -mangostin α concentration range 14-450 µg/mL. the absorbtion after the treatment on uropathogenic E.coli is shown as follows;

Table 3. Mean ± SD difference (decrease/increase) in absorbance after treatment α - mangostin on uropathogen E. coli in MHB media

No	consentration (µg/mL)	repetition	Absorbansion*	Mean Absorbansion ± SD
1	450	1	(0.293)	(0.288) ± 0.022
		2	(0.311)	
		3	(0.291)	
		4	(0.258)	
2	225	1	0.242	0.196 ± 0.039
		2	0.163	
		3	0.164	
		4	0.215	
3	112.5	1	0.172	0.117 ± 0.068
		2	0.049	
		3	0.068	
		4	0,180	
4	56.25	1	0.196	0.179 ± 0.017
		2	0.157	
		3	0,175	
		4	0.189	
5	28.13	1	0.120	0,139 ± 0,024
		2	0,127	
		3	0.137	
		4	0.173	
6	14	1	0,093	0.084 ± 0.038
		2	0.054	
		3	0.053	
		4	0.134	
7	control (-)	1	(0.001)	(0.001)
8	control (+)	1	0.155	0.155

* The absorbance was determined from the absorbance value of the treatment after incubation minus the absorbance value of the treatment prior to incubation. The brackets () shows a negative value, meaning that there was a decrease in treatment absorbance (decrease in the number of bacteria), whereas increased absorbance means there is an increase in the number of bacteria after incubation.

The results in Table 3 showed that the concentration of α-mangostin 450 µg /ml showed a decrease in the number of bacteria sindicated from from a decrease in absorbance of the solution. For -mangostin α concentrations of 14-225 mg/mL there was an increased absorbance or an increase in the number of bacteria after incubation. It can be concluded that the minimal inhibitory concentration of α-mangostin against uropathogenic - E. coli was 450 µg /mL. Furthermore, α-mangostin at concentrations of 14-225 µg/mL showed no bacterial growth inhibition against uropathogenic E. coli.

The control group was used as control of the media and control the growth of uropathogenic E. coli. the control positive group showed bacterial growth, whereas negative control showed bacterial growth. Results of negative controls showed that α-mangostin and media used was sterile. Positive control results indicated that uropathogenic E. coli can grow on the medium.

c. Statistic analysis

The statistical analysis of linear regression was applied to determie the effectiveness of a dose of α-mangostin agaist E. coli. the tests were carried out by finding the relationship between the value of the dose of α-mangostin and the decrease/increase in absorbance after incubation. The analysis was performed using STATA 13.1 and the results is shown in Figure 2:

The lowest absorbance value was found at concentrations of 450 µg/ml. The coefficient of determination ((R2) was 0.6115 = 61.15%. This indicates that the sample absorbance variable variation can be explained by variable concentrations of α-

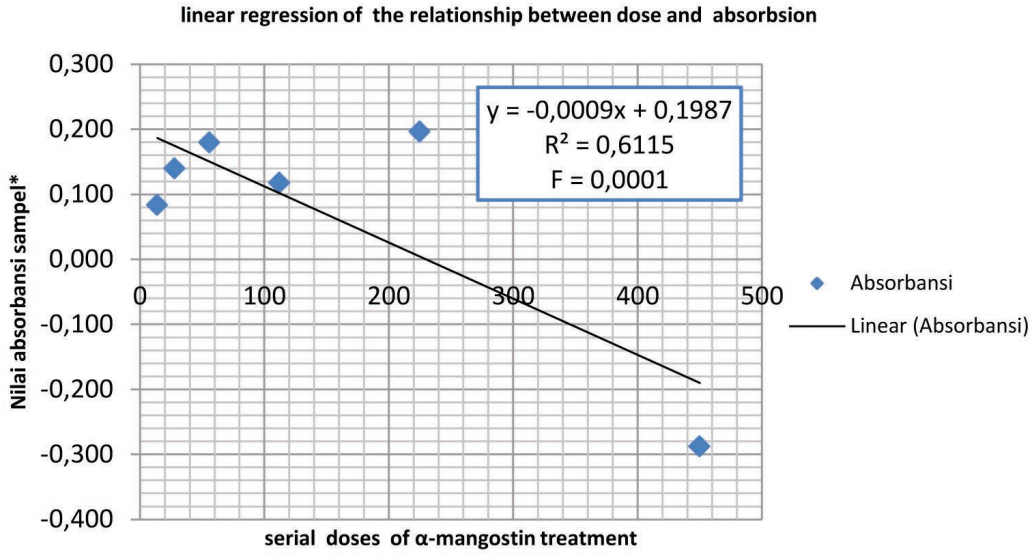


Figure 2. linear regression relationship between the dose of α-mangostin and absorbance values *
* The absorbance is determined by evaluating the absorbance value of the treatment after incubation minus the absorbance value of the treatment prior to incubation.

mangostin was 61.15%. The equation obtained by the regression line was y = - 0.0009x + 0.1987. The value of y is predicted log of the number of bacterial colonies of uropathogenic E. coli (UPEC), while the value of x is the concentration of α-mangostin. F test value 0.0001 <0.05 stated that all concentrations of α-mangostin simultaneously have a significant effect on the absorbance, in other words they have an effect on the growth of uropathogenic E. coli.

DISCUSSION

the treatment with a differet levels of concentration of α-mangostin, showed that the minimum inhibitory concentration of α-mangostin by evaluating the turbidity in the test tube after incubation for 18-20 hours and at 370 C could not be done. This is due to the fact that turbidity can also be caused by α-mangostin, not only due to the presence of bacteria. The minimum inhibitory concetration were determined by calculating the difference in absorbance before and after 18-20 hours incubatio at 37 ^{0} C.

The Minimum inhibitory cocentration againts uropathogenic E. coli was established at 450 µ g/ml. This showed a negative result or decreased absorbance of the treated solution compared to absorbance prior to incubation. In α- mangostin with a concentration 14 to 225 µg/mL showed an increase i absorbance after incubation incicating no bacterial growth ihibition againts uropathogeic E. coli.

Alfa xanthon mangostin is derivataives compounds found in the peel of the mangosteen fruit (Garcinia mangostana L) and has the IUPAC name 1,3,6-trihydroxy-7-methoxy-2,8-bis (methyl-2-butenyl) -9H-xanten- 9-on (MY Ibrahim *et al.,* 2014). Xanthon is the main compound of Garcinia mangostana L., the widely distributed plant in Indonesia. This plant belongs to the family Clusiaceae. The optimum temperature for Garcinia mangostana L. is 25-35^0 C (Brunner and Payan, 2011). Some of the pharmacological activities associated with α- mangostin include antioxidants, anti-inflammatory, anticancer, hypo-allergenic, antiviral, antibacterial, antifungal, and antimalarial activity. Other Xanthones such as gamma mangostin and xanthon derivatives has bee show to have no antibacterial activity (Jindarat, 2014).

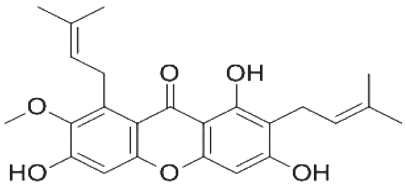


Figure 1. Chemical structure of α- mangostin (MY Ibrahim *et al.,* 2014)

The minimum inhibitory levels were obtained by the study conducted by Zou et al. (2013) which showig that the α- mangostin less effective in killing gram-negative bacteria with the minimum inhibitory concentration >200 µg/mL. Uropathogenic Escherichia coli belongs to the gram-negative bacteria with outer cell membrane composed of lipopolysaccharide. This lipopolysaccharide serves as a non permeable barrier that is not permeable to hydrophobic molecules. Test

substances in this treatment, α- mangostin are oe of the xanthon derivatives composed of hydrophobic molecules. This causes reduction in the antibacterial activity of α- mangostin against bacteria. Alpha mangostin was reported to be more effective against gram-positive bacteria than gram-negative (Koh et al., 2013). Gram-positive bacteria susceptible to alpha mangostin include Streptococcus mutans, the cause of dental caries (Nguyen and Marquis, 2011).

Other studies related to α- mangostin as antibacterial stated that α- mangostin potently inhibit the growth of methicillin-resistant Staphylococcus aureus (MRSA). Koh *et al*. (2013) states that the α-mangostin bactericidal activity against in vitro can quickly reduce the bacterial colony to 103 5 minutes. Alpha mangostin damage the integrity of the cytoplasmic membrane of bacteria rapidly, causing damage in intracellular component bacteria and cause the bacteria to die. A study conducted by Seesom *et al*. (2013) stated that α- mangostin has the potential to inhibit the growth of Mycobacterium tuberculosis. Other studies on antibacterial activity of mangostin was coducted by Zou *et al*. (2013) stating that the test on the effect of mangostin against Pseudomonas aeruginosa and Klebsiella pneumoniae resulted in MIC of 40-50 μg/mL. The studies on effect of α- mangostin against several bacteria including Escherichia coli have also been carried out by Al Massarani *et al*. in 2013 resulting in E. coli IC 50 >200 μg/mL.

Previous studies showed that α- mangostin has effective antimicrobial activity shown by a low the minimum inhibitory concentration, the rapid bactericidal activity, and the ability to minimize the occurrence of bacterial resistance. However, α- mangostin did not show a sufficient selectivity between of bacterial and mammalian cell membranes. Xanthon hydrophobic properties contained in the α- mangostin provides a strong driving force for fat solution to penetrate the of eukaryotic and prokaryotic membrane. However, some studies have reported that this can be overcome by modifying smaller molecules to be specific to bacterial cell membranes (Zou *et al.,* 2013).

The high value of the MIC in this study showed lack of effectiveness of alpha-mangostin against E. Multidrug uropathogenic E. coli. The high value of α-mangostin MIC resulted in consumption in the form of mangosteen peel extract or pure compound in very large quantities. This will affect the toxicity of the alpha mangostin in the human body. A study showed that the oral administration did not obtain a complete concentration profile due to the low bioavailability of α- angostin. Chitchumroonchokchai *et al*. (2012) stated that 60 ml of mangosteen juice provides ± 3.07 mmol/L α- mangostin. α- mangostin maximum levels in the blood is reached at 3.7 ± 2.4 hours with maximum concentration on one subject of 450 nmol/L, equivalent to the levels of 0.18 μ g/mL. This suggests that to get the levels of 225 μg/mL of blood requires mangosteen juice as much as 7500 ml.

According to Ramaiya *et al*. (2012) α- mangostin when administered in cotton seed oil in rats with an equivalent dose of 615 mg in a 90 kg adult human, reaches a maximum plasma concentration of 1.300 nmol/L and are detected up to 24 hours. In -mangostin α administration in rats through the veins is stated in the study of Li *et al*. (2011) that the disposition of α-mangostin in rat plasma is biphasic, divided into fast distribution and slow elimination phase. The half-life of the distribution phase is 3 minutes and the terminal elimination phase is 3.5 hours. It indicates a high bond between α- mangostin with tissue.

In this study, the determination of the activity of α-mangostin was conducted to merely evaluate the value of the minimum inhibitory concentration. Determination of minimal bactericidal acticity was not conducted. Some literature state that potentially relevant activity in the clinical setting will occur if the value of MCI <100 μg/mL. Even the pharmaceutical industry prefers the development of antibiotics with ≤ 2 μg/mL. Minimum bactericidal activity is greater than MIC value, because the KHM value obtained in this study was 450 μg/mL (or greater than 100 μg/mL), then the MIC was not determined (Sanchez and Kouznetsov, 2010; Levison and Levison, 2009).

The antibacterial activity of alpha mangostin, especially against Gram negative bacteria requires further studied. The study of synergism with some antibiotics also did not show effective results. The combination of alpha mangostin with ampicillin, penicillin G, tetracycline and streptomycin did not showed effectiveness against Gram - (E. coli) and Gram + (S. aureus) (NHS Ibrahim *et al.,* 2014). The crude extract activity of either the ethanol or water fraction of Garcinia mangostana Linn was also tested on several pathogenic bacteria. The results of these studies suggested that the required concentration of MIC and MBC is quite large (4000 mg/mL to extract water and 2000 mg/mL ethanol extract), to be able to inhibit and kill bacteria E. coli (RV Geetha *et al.,* 2011).

CONCLUSION

α- mangostin is not effective to inhibit the growth multi drug resistat uropahogenic E. coli multiresistant

uropatogen due to a relatively high MIC (450 mcg/mLz). The antibacterial activity of alpha mangostin, especially its activity against Gram negative requires futher studied. The study of synergism with some antibiotics also did not show effective results.

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CONFLICTS INTERES

There is no conflict of interest with any party, including the pharmaceutical industry.

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