IDJPCR
Indonesian Journal of Pharmaceutical and Clinical Research

p-ISSN 2615-6199
e-ISSN 2620-3731

Indonesian Journal of Pharmaceutical and Clinical Research

Vol. 01, No. 01, April 2018

Journal Homepage: https://talenta.usu.ac.id/index.php/idjpcr

Editorial Team

Articles

Indonesian Journal of Pharmaceutical and Clinical Research

Antimicrobial Activities and Mechanism of Action of *Petiveria alliacea* Stem Extract

Yani Mulyani[1*], Ika Kurnia Sukmawati[1] , Jajang Jafar Sodik[1]
[1]Sekolah Tinggi Farmasi Bandung, Bandung, Indonesia

Abstract. This research aimed to determine the antimicrobial activity of ethanol extract of *Petiveria alliacea* stem (EEPS) against *Staphylococcus aureus, Pseudomonas aeruginosa*, *Escherichia coli*, and *Candida albicans*. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by perforation and broth micro dilution methods. Study on the mechanism of action of EEPS was conducted by molecular docking and Scanning Electron Microscopy (SEM) techniques. The results showed that EEPS had an inhibitory activity against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Candida albicans* with MIC values of 256, 128, 256, 512 µg/ml, consecutively. These values are included in to the medium category. Through the process of molecular docking, the best interaction was observed between S-benzyl-L-cysteine sulfoxide with penicillin-binding protein receptor of *Pseudomonas aeruginosa* characterized by free energy change (ΔG) of 4.32 kcal/mol, and the Ki value of 682.16 µM. Four folds of MIC of the EEPS caused changes in the morphology of *Pseudomonas aeruginosa*. EEPS possessed antimicrobial activities against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, and Candida albicans*.

Keywords: Antimicrobial Activity, Molecular Docking, *Petiveria alliacea*, SEM.

Abstrak. *Penelitian ini bertujuan untuk mengetahui aktivitas antiinfeksi ekstrak etanol batang Petiveria alliacea singawalang (EEBS) terhadap mikroba Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, dan Candida albicans. Konsentrasi hambat minimum (KHM) dan konsentrasi bakterisida ektrak minimum diuji menggunakan metode perforasi dan broth micro dilution. Mekasisme kerja EEBS dianalisis dengan metode molecular docking dan Scanning Electron Microscope. Penelitian ini menunjukkan bahwa EEBS mempunyai aktivitas penghambatan terhadap Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, dan Candida albicans dengan nilai KHM berturut turut 256, 128, 256, 512 ppm dan mempunyai diameter hambat kategori sedang. Melalui proses docking molekular, didapatkan interaksi terbaik antara S-benzyl-l-cysteine sulfoxide dengan reseptor Penicillin-binding protein bakteri Pseudomonas aeruginosa ditandai dengan diperoleh energi ikatan (ΔG) sebesar 4.32 kcal/mol, dan nilai Ki 682.16 µM, Konsentrasi pada 4 x KHM EEBS menyebabkan perubahan morfologi sel bakteri Pseudomonas aeruginosa berupa tonjolan, pengkerutan dan permukaannya kasar. Ekstrak etanol batang singawalang memiliki aktivitas antiinfeksi terhadap Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, dan Candida albicans.*

Kata kunci*: Antiinfeksi, Molecular docking, Petiveria alliaceae, SEM.*

Received 02 June 2017 | Revised 17 October 2017 | Accepted 12 March 2018

*Corresponding author at: Sekolat Tinggi Farmasi Bandung, Bandung, Indonesia

E-mail address: yani.mulyani@stfb.ac.id

1. Introduction

According to the World Health Organization (WHO), infectious diseases and parasites represent the leading cause of mortality worldwide. Bacterial infections contributed to about 2 million of mortality globally [1,2]. *Staphylococcus aureus*, *Escherichia Coli*, and *Pseudomonas aeruginosa* are phatogenic bacteria that commonly cause infection in humans [3]. *Staphylococcus aureus* causes diarrhea, inflammation, necrosis, pneumonia, endocarditis and septicemia [4]. *Escherichia coli* causes infection in the urinary tract, diarrhea, sepsis and meningitis [3]. *Pseudomonas aeruginosa* was reported as the most frequent cause of nosocomial infections, especially in the Intensive Care Unit (ICU) [3]. In 2011, 1.5 million cases of fungal infections were caused by Candida species [5]. To cure infectious disease, the treatment using antibiotic is still the most effective approach. The microbial resistances to antibiotics keep increasing from year to year. Thus, new sources of antimicrobial agents need to be discovered as alternative drugs which are more effective, cheaper and safer.

Indonesia is known as one of the countries in the world which has the biological diversity.Based on The Agenda of National Research 2015-2019 [6], the research on herbal medicines using the biological diversity of Indonesia is encouraged, so the studies to discover new sources of antimicrobial agents are very relevant. *Petiveria alliacea* belongs to Phytolaccaceae family, and has different names in many countries [7]. This plant contains many compounds, including alkaloids, flavonoids, triterpenoids, steroids and sulfurs. In Brazil, *Petiveria alliacea* is used as antispasmodic, diuretic and stimulant [8]. In Indonesia, *Petiveria alliacea* has been reported to have analgesic and anti-inflammatory effects. This plant is also used to treat hemoptysis [9].

The presence of thiosulfinates and trisulfides are estimated as the most active compounds as antibiotic and antifungal agents [10]. Kubec et al (2001) isolated the compound of S-benzyl-l-cysteine sulfoxide in various macerates, extracts and other preparations of *Petiveria alliacea*, this compound is estimated has antimicrobial effect. Therefore, the present study aimed to determine the antimicrobial activity, and to review the mechanism action of *Petiveria alliacea* stem against microbial pathogens, including *Staphylococcus aureus, Escherichia coli*, *Pseudomonas aeruginosa,*and *Candida albicans*, applying in vitro perforation and micro dilution methods. Insilico method was also used by adding a receptor to a ligand, and observing the morphological changes using a Scanning Electron Microscope (SEM).

2. Materials and Methods

2.1. Preparation of Extract

Fresh stems of *Petiveria alliacea* were obtained from Balitro, Bogor. The sample was determined at the Laboratory of Plant Taxonomy, UNPAD. The plant material was collected, shaded, dried and powdered. As much as 1000 g of powdered stems were macerated in 10 l of a mixture of ethanol and water (4:1) for 24 h. It was filtered and concentrated to a small volume

to remove the entire ethanol using a rotary evaporator. The extract was concentrated again in a water bath at 50-60 ^{o}C until totally dry.

2.2. Characterization and Phytochemical Screening of Extract

The extract was characterized for its water level, ash level, ethanol soluble concentrates level, water soluble concentrate level and density. The ethanol extract of *Peveria alliacea* stems (EEPS) was screened for the presence of alkaloid, flavonoid, saponin, quinine, tannin, steroid/triterpenoid and sulfur compounds.

2.3. In Vitro Studies

This in vitro study was done using micro dilution, perforation, and Scanning Electron Microscope (SEM) method based on the previous method done by Yani M (2012) and Valgas (2007) [9;11]. Briefly, 100 µl of Mueller-Hinton Agar (MHA) media were used for the growth of bacteria. Mueller-Hinton Broth (MHB) media were used for the growth of fungi. Each media were poured into microplates in the first column (negative control) using micro dilution method. As much as 5 µl of bacterial or fungal suspension were poured into 10 ml of MHA/MHB and homogenized using the vortex. An amount of 100 µl of the mixture was poured into microplate in the second until the twelfth column. In the twelfth column, 100 µl of antibiotic solution/extract was added with some concentration, then homogenized. One hundred µl solution obtained from the twelfth column was placed into the eleventh column. The third column would be the lowest concentration. The plates were incubated at 25 ^{o}C for 72 h for fungi and at 37 ^{o}C for 24 h for bacteria. The area with no microbial growth (clear zone) was then observed.

The lowest concentration of an antimicrobial that will inhibit the visible growth of microorganisms after overweight incubation called Minimum Inhibitory Concentration (MIC). About 5 µl of aliquot from each of clear zone was placed into the sabouroud dextrose agar in a Petri dish. It was incubated at 25 ^{o}C for 72 hours for fungi and at 37 ^{o}C for 24 hours for bacteria and then was observed. The lowest concentration that inhibits the visible growth of microorganism was defined as Minimum Fungicidal Concentration (MFC) and Minimum Bactericidal Concentration (MBC) [12].

For perforation method, the bacterial or fungal inoculums was uniformly spread using a sterile cotton swab in a sterile petri dish of Muehler Hilton Agar. Six serial dilutions with concentration of 20, 15, 10, 7.5, 5, and 2.5µg/ml for extract. As much as 50 ml of each dilution of EEPS was added to each of 6 wells (Holes were bored on the agar gel with perforator of 7 mm diameter and 20 mm apart from each other). The plates were incubated at 25 ^{o}C for 72 h (fungi) and at 37 ^{o}C for 24 h (bacteria). After incubation, the bacterial and fungal growth was observed. Inhibition of the bacterial growth was measured in mm using Vernier caliper [11].

2.4. In Silico Studies

The study of mechanism of action of EEPS as antibiotic and antifungal agents was carried out using in silico docking and SEM method. The docking result was determined and bacterial assay was chosen for the study of mechanism of action.

In the docking method, ligand compound used was sulfur group, S-benzyl-1-cysteine sulfoxide which was commonly found in *Petiveria alliacea* stem, assumed to have antibiotic and antifungal activities. S-benzyl-1-cysteine sulfoxide was modeled in the 2D and 3D form using an application known as ChemDraw Ultra 8.0. The examined compound/ligand that would be used was geometrically optimized to gain the stability of energy of molecule compound assay and to know the most stable of molecule compound position using DFT (Density Functional Theory) method with B3LYP function basis by setting 3-21G with Gaussian09 application.

D-alanine carboxypeptidepenicillin binding protein receptor can be found in *Escherichia coli* (PDB ID: 2EX8), *Pseudomonas aeruginosa* (PDB ID: 4WEL), *Staphylococcus aureus* (PDB ID: 3VSL) and *Sterol 14alpha-demethylases (CYP51)* receptor which can be found in *Candida albicans* (PDB IDP: 1X8V), they were obtained from The Data Bank of Protein (Rcsb.org). The next step was the validation of docking method using the calculation of Root Mean Square Deviation (RMSD) value (RMSD $\leq$ 2 A^{o}). The docking was processed on each of the receptor in bacteria and fungi with the same ligand and then observed for free energy, constant inhibitor, and the interaction between ligand and receptor.

The morphological changes from bacteria and fungi after exposing the EEPS was studied using SEM and observed from the result of in silico studies using the docking method with the best binding energy between ligand and protein. If the binding energy of the conformation getting smaller, the result of docking is better.

2.5. Observations of Cell Morphology

The bacterial suspension (24 h) was poured into the ethanol extract with a concentration of 4x minimum inhibitory concentration (MIC). The solution was incubated at 37^{o}C for 24 h, it was then centrifuged at 3500 rpm for 20 min. The supernatant was separated from the pellet. The pellet was soaked in glutaraldehyde 2% for 24 h, then it was soaked in cocodylate buffer for 20 min. After that, the pellet was soaked in osmium tetraoxide 1 % for 1 h, it was then dried using alcohol of 70%, 80%, 95% and absolute for 20 min. The pellet was suspended with butanol and put down on the dried cover slip. The cover slip was coated with the gold using a vacuum process for 20 min and it was observed using a SEM [9].

3. Results and Discussion

3.1. Phytochemical Screening

The study of anti-infective activity of EEPS against bacteria and fungi was started by collecting fresh *Petiveria alliacea* from Balitro, Bogor. Authenticated voucher samples were deposited at the Laboratory of Plant Taxonomy, Biology department, UNPAD. The extraction result showed the *Petiveria alliacea* stem has yield of 8.24% extract.

Phytochemical screening of extract revealed the presence of alkaloid, tannin, flavonoid, triterpenoid and sulfur compounds as shown in Table 1.

Table 1. Results of Phytochemical Constituents Screening

Test	Dried powder of *Petiveria alliacea* stem	Extract of *Petiveria alliacea* stem	Literature
Alkaloid	+	+	+
Tannin	+	+	+
Flavonoid	+	+	+
Steroid	-	-	-
Triterpenoid	+	+	+
Quinine	-	-	-
Saponin	-	-	-
Sulfur	+	+	*

+: Presence of constituent -: Absence of constituent

The characterization of extract and dried powder aimed to know and ensure the quality of dried powder in accordance with the requirements (Table 2).

Table 2. Characterization of Dried powder and Extract of *Petiveria alliacea* stem

Test	Dried powder (%)	Extract (%)
Water level	9.0	20.0
Water soluble concentrates level	5.7	11.0
Ethanol soluble concentrates level	7.5	13.1
Ash level	8.03	5.1

3.2. In Vitro Studies

Based on the measuring of the diameter of minimum inhibitory concentration of EEPS against S*taphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli,* and *Candida albicans,*Table 3 showed that EEPS exhibited antimicrobial activity against S*taphylococcus aureus, Escherichia coli* with a inhibition zone diameter of 6.0 mm and 6.6 mm and considered as medium category with concentration of 5 µg/ml, whereas with concentration of 2.5 µg/ml the EEPS has inhibitory effect against *Pseudomonas aeruginosa* and *Candida albicans* with diameter of inhibition zones of 6.9 mm and 6.0 mm and considered as medium category.

The results showed in Table 3 indicated that EEPS is effective as an antibiotic against S*taphylococcus aureus* (Gram +), *Pseudomonas aeruginosa*, *Escherichia coli* (Gram -) and also effective as an antifungal agent against *Candida albicans.*

Table 3. Diameter of Inhibition Zones

Type of bacteria	20 µg/ml	15 µg/ml	10 µg/ml	7.5 µg/ml	5 µg/ml	2.5 µg/ml	0.5 A µg/ml	0.5 T µg/ml	0.5 K µg/ml
Staphylococcus aureus	10.7±0.45	6.9±0.15	6.6±0.30	6.5±0.26	6.0±0.1	5.6±0.21	11.3±0.15	12.1±0.25	-
Pseudomonas aeruginosa	10.8±0.87	9.9±0.30	9.7±0.47	8.6±0.46	8.6±0.15	6.9±0.32	8.6±0.15	10.6±0.1	-
Escherichia coli	9.4±0.45	8.0±.65	7.7±0.58	7.5±0.23	6.6±0.47	5.6±0.64	10.0±0.20	11.46±0.15	-
Candida albicans	8.6±0.8	8.9±0.37	8.6±0.65	7.8±0.62	6.6±0.12	6.0±0.37	-	-	10.9±0.55

The results showed that EEPS had activity as anti-infective towards fungi and bacteria, which were S*taphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli*,and *Candida albicans* with the Minimum Inhibitory Concentration (MIC) of 256 µg/ml, 128 µg/ml,256 µg/ml, and 512 µg/ml, respectively. The Minimum Bactericidal Concentration (MBC) of those bacteria were >512 µg/ml, 512 µg/ml,>512 µg/ml, and>1024 µg/ml, respectively, as shown in the Table 4.

Table 4. Determination of MIC, MFC and MBC Values

	EEPS (µg/ml)		K (µg/ml)		A (µg/ml)		T (µg/ml)	
Microbe	**MIC**	**MFC/MBC**	**MIC**	**MFC**	**MIC**	**MBC**	**MIC**	**MFC**
Staphylococcus Aureus	256	>512	-	-	1	128	1	256
Pseudomonas Aeruginosa	128	512	-	-	1	512	1	256
Escherichia Coli	256	>512	-	-	1	128	1	128
Candida Albicans	512	>1024	2	512	-	-	-	-

MIC = Minimum Inhibitory Concentration; MFC= Minimum Fungicidal Concentration;MBC = Minimum Bactericidal Concentration; K= Ketoconazole; A= Amoxicillin; T= Tetracycline; EEPS = Ethanol Extract of *Petiveria alliacea* Stem; (-) = not tested

3.3. In Silico Studies

Binding of S-benzyl-L-cysteine sulfoxide which is contained in the EEPS with active receptors of bacteria and fungi in the creation of peptidoglycan and ergosterol is performed by docking in silico method. To support the in vitro data, the selection of bacteria and fungi to be continued with SEM.

Table 5. Docking Compound Test Result Using 4 Receptor

Ligand	**PBP** ***Escherichia coli***		**PBP** ***Pseudomonas aeruginosa***		**PBP** ***Staphylococcus aureus***		***sterol 14alpha-demethylase (CYP51)***	
	(ΔG)	**Inhibitory Constant**	**(ΔG)**	**Inhibitory Constant**	**(ΔG)**	**Inhibitory Constant**	**(ΔG)**	**Inhibitory Constant**
S-benzyl-L-cysteine sulfoxide	-2.64	11.52 mM	-4.32	682.16 uM	-3.73	1.83 uM	-4.00	1.18 mM
Amoxicillin	-3.14	4.98 mM	-3.46	2.91 mM	-4.8	304.85 uM	-	-
Tetracycline	-2.24	22.66 mM	-4.47	526.18 uM	-5.57	82.49 uM	-	-
Ketoconazole	-	-	-	-	-	-	-8.03	1.30 uM

The docking results of S-benzyl-L-cysteine sulfoxide compound with Penicillin-binding protein receptor 3 *Pseudomonas Aeruginosa, Penicillin-binding protein 4 (dacB) Escherichia coli,* Penicillin-binding protein 3 *Staphylococcus Aureus,* and *Estriol-bound and ligand-free structures of sterol 14 alpha-demethylase (CYP51)* that natural ligands produced Gibbs Free Energy (ΔG) were -4.32 kcal/mol, -2.64 kcal/mol, -3.73 kcal/mol, and- 4,00 kcal/mol, respectively, as shown in Table 5. The low value of ΔG showed that conformation was stable. The lower the ΔG value, then more stable the interaction between ligand and receptor. Besides Gibbs Free Energy (ΔG), the natural ligands also yielded 682.16 uM , 11.52 mM 1.83 uM, and 1.18 mM of Inhibition Constant (k_i). The lower the value of k_i, the better the affinity between ligand-receptor [13]. From the data above, it can be concluded that the best interaction happened between S-benzyl-L-cysteine sulfoxide with *Penicillin-binding protein* receptor of *Pseudomonas aeruginosa*showed with the value of Gibbs Free Energy (ΔG) and Inhibition Constant (k_i). The binding of S-benzyl-1-cysteine acetate with PBP3 *Pseudomonas aeruginosa*can be seen in Figure 1.

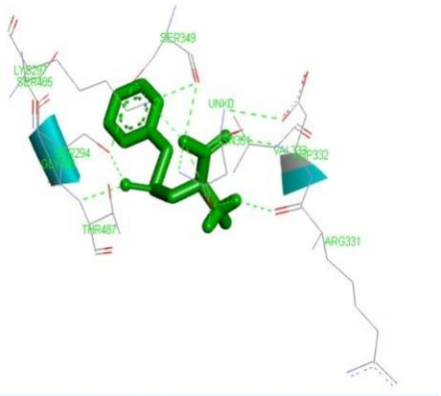

Figure 1. The binding of docking of S-benzyl-l-cysteine acetate with *Penicillin-binding protein 3 Pseudomonas Aeruginosa*

The result of docking of S-benzyl-1-cysteine acetate with PBP 3 *Pseudomonas aeruginosa* in Table 6 has ten hydrogen bonds with the residue of THR, serine (SER), asparagine (ASN), lysine (LYS), valine (VAL), and arginine (ARG). The lower distance between donor atom with acceptor atom showed the better interaction between ligand-receptor [14]. The substance that has better activity can bind to more amino acid. Moreover, with the mutual amino acid bonds of substance and natural ligand towards receptor proved that the five best substances were the candidates of *Penicillin-binding protein 3 Pseudomonas aeruginosa*[15].

Table 6. Binding between S-benzyl-L-cysteine sulfoxide and Penicillin-binding protein of *Pseudomonas aeruginosa*

Ligand	Residue of The Amino Acids	Atomic Binding		Space
		Atomic Ligand	Atomic Receptor	
S-benzyl-L-cysteine sulfoxide	THR 487	O	N	3.2092
	SER 294	O	OG	2.7342
	ASN 351	OD1	H	2.1487
	ASN 351	OD1	H	2.4035
	ASN 351	O	O	3.1941
	ASN 351	O	ND2	2.5542
	LYS 297	O	NZ	2.8324
	SER 294	O	O	2.4655
	VAL 333	O	N	2.9689
	ARG 331	O	H	2.3693

Table 6 showed that S-benzyl-L-cysteine sulfoxide substance that was present in EEPS was the best substance to inhibit the formation of the cell wall of bacterium *Pseudomonas aeruginosa*. This results confirmed the findings in the in vitro test that EEPS was the most effective on *Pseudomonas aeruginosa* bacterium because it had the smallest MIC of 128 µg/ml and the smallest MBC of 512 µg/ml using the broth micro dilution method and the MIC of 5 µg/ml with a inhibition zone diameter of 8.6±0.15 mm using perforation method.

3.4. Observations of Cell Morphology

The observation results using SEM (Figure 2) showed that *Pseudomonas aeruginosa* cell which was treated with EEPS of 4x MIC (128µg/ml) for 24 hours at 37^0C had the abnormal cell wall which is shown by the presence of porous, bumps and shrinking on the cell wall. The formation of pores on the cell surface and the shrinking cell wall was expected because cell wall was not synthesized perfectly. As for the formation of small bumps on the bacteria cell was caused by the inability of the damaged peptidoglycan cell of antibiotic substance to hold the high intracellular tension, so that the cytoplasm forced out from the bump. This is usually shown in the area which was weakened by the antibiotic substance.

The antibiotic mechanism of action of EEPS towards *Pseudomonas aeruginosa* was determined by comparing cell morphology between *Pseudomonas aeruginosa* which was treated with antibiotic and extract. Antibiotics used as controls were tetracycline HCl and amoxicillin. SEM

microgram on Image D and E showed the effect of amoxicillin on *Pseudomonas aeruginosa.* Normally, cell wall was formed at the cell splitting phase. When *Pseudomonas aeruginosa* was treated with amoxicillin, cell wall wasn't formed, causing malicious substances to enter the cell leading to the cell death, which was evident from visible wrinkles, bumps, and pores. According to Gale (1981), amoxicillin impedes bacteria growth by slowing several stages in cell wall synthesis. Amoxicillin is a structural analog D-Ala-D-Alanine antibiotic, which contains covalent bonds as provided by penicillin binding protein (PBP) at active sites. When penicillin G is connected to PBP, peptidation reactions are stopped, disallowing alanine to be removed to form bonds with nearby peptides, which halts peptidoglycan synthesis causing cell death. Microgram obtained from SEM of the cell *Pseudomonas aeruginosa* that were treated with HCl tetracycline as shown in Figures 2 (F and G) which showed formations of only wrinkles on cell walls. As stated by Gale (1981), tetracycline enters microorganisms through passive diffusion and an active process which relies on energy. Once inside the cell, the tetracycline will reversibly bind itself to the 30S subunit of the ribosome of bacteria to impede the formation of tRNA-Aminoacyl bonds to the receptor sites in mRNA ribosome. This behavior prevents amino acid from reacting with incomplete peptides. This process proved tetracycline's role in impeding protein creation, and *Staphylococcus aureus* cell surface will develop abnormal shapes after being treated with tetracycline, as observed with SEM. Based on the morphological destruction pattern ratio of the *Pseudomonas aeruginosa* cell as observed through SEM microgram, we can conclude that the role of the antimicrobial of EEPS is similar to that of amoxicillin, which is to impede cell wall growth.

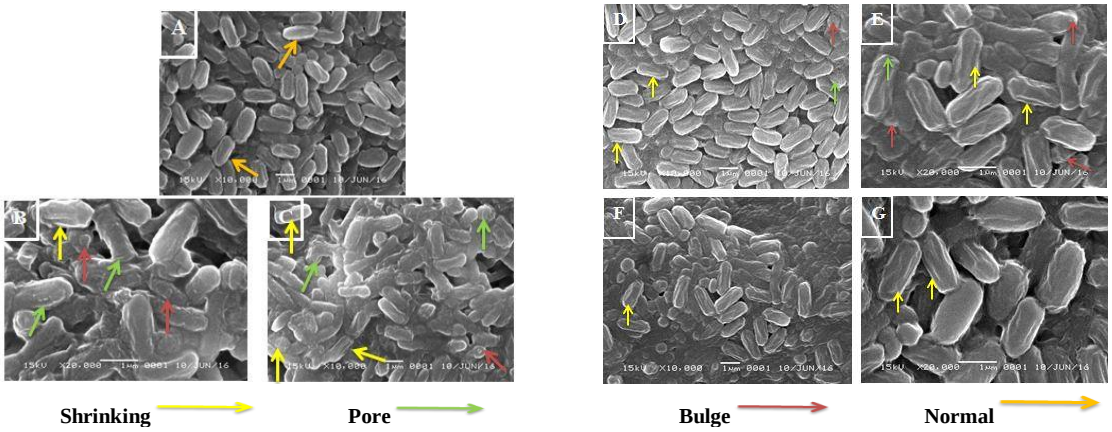

Figure 2 . The observation of *Pseudomonas aeruginosa* morphology after exposing with extract, Amoxicillin and Tetracycline

A = normal cell of *Pseudomonas aeruginosa* (magnification 10000x), B = *P. aeruginosa cell wall* (magnification 10000x),C =*Pseudomonas aeruginosa* cell wall after exposing with the EPPS 4x (128 µg/ml) (magnification 10000x), D= *Pseudomona*. *aeruginosa*cell wall after exposing with Amoxicillin 4x MIC (1µg/ml) (magnification 10000x), E = *Pseudomonas aeruginos a*after exposing with Amoxicillin 4x MIC (1µg/ml) (magnification 20000x), F = *Pseudomonas aeruginosa* cell wall after exposing with Tetracycline 4x MIC (1µg/ml)

(magnification 10000x), G= *Pseudomonas aeruginosa* cell wall after exposing with Tetracycline 4x MIC (1µg/ml) (magnification 20000x).

4. Conclusion

Ethanol extract of *Petiveria alliacea* had antimicrobial effects on *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, and Candida albicans* with minimum inhibition concentrations of 256 µg/ml, 128 µg/ml, 256 µg/ml, and 512 µg/ml, respectively using the broth micro dilution method, and had medium inhibition diameter category when using the perforation method. The molecular docking method produced the best interaction between S-benzyl-L-cysteine sulfoxide with the *Penicillin-binding protein* receptor of *Pseudomonas aeruginosa*. The obtained 4.32 kcal/mol value of bond energy (ΔG), 682.16 µM of Ki coefficient, as well as ten hydrogen bonds with THR amino acid, serine, asparagine, lysine, valine, and arginineas residues. Based on the morphologic destruction pattern ratio of the cell *Pseudomonas aeruginosa* as observed using the microgram obtained from SEM, we could conclude that the antimicrobial activity of *Petiveria alliacea* extract was similar to that of amoxicillin, which was to impede cell wall synthesis.

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