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ORIGINAL ARTICLE

Antibacterial activity of endosymbiotic bacterial compound from *Pheretima* sp. earthworms inhibit the growth of *Salmonella* Typhi and *Staphylococcus aureus*: *in vitro* and *in silico* approach

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ABSTRACT

Background and Objectives: Earthworms coexist with various pathogenic microorganisms; thus, their immunity mechanisms have developed through a long process of adaptation, including through endogenous bacterial symbionts. This study aims to identify earthworm endosymbiont bacteria compounds and their antibacterial activity through an *in vitro* approach supported by an *in silico* approach.

Materials and Methods: This research was conducted using the *in vitro* inhibition test through agar diffusion and the *in silico* test using molecular docking applications, namely, PyRx and Way2Drugs Prediction of Activity Spectra for Substances (PASS).

Results: The *in vitro* results showed a potent inhibition activity with a clear zone diameter of 21.75 and 15.5 mm for *Staphylococcus aureus* and *Salmonella* Typhi, respectively. These results are supported by chromatography and *in silico* tests, which showed that several compounds in endosymbiotic bacteria, cyclo (phenylalanyl-prolyl) and sedanolide, have high binding affinity values with several antibiotic-related target proteins in both pathogenic bacteria. Cyclo (phenylalanyl-prolyl) has the highest binding affinity of -6.0 to dihydropteroate synthase, -8.2 to topoisomerase, and -8.2 to the outer membrane, whereas sedanolide has the highest binding affinity to DNA gyrase with approximately -7.3. This antibiotic activity was also clarified through the Way2Drugs PASS application.

Conclusion: Ten active compounds of endosymbiont bacteria, Cyclo (phenylalanyl-prolyl) and sedanolide were potential candidates for antibacterial compounds based on the inhibition test of the agar diffusion method and the results of reverse docking and Way2Drugs PASS.

Keywords: Antibiotics; Endosimbiotic bacteria; Endosymbiotic bacterial compound; *Pheretima* sp.

INTRODUCTION

Antibiotic resistance has become an interesting and challenging study for researchers (1). Antibiotic resistance is described as a condition when pathogenic microorganisms are resistant to antibiotics (2). The development of any new antibiotic drug has been accompanied by its resistance. However, resistant strains can spread if there is no compliance with their infection control measures. The lack of new findings to replace antimicrobials that are no longer effective is a problem amid the need to protect the effectiveness of existing antimicrobials (3).

Currently, experts are starting to develop new antibiotic discoveries from various sources, such as plant

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extracts (4, 5). Parts of plants that are used also vary, such as tubers, seeds, leaves, and fruit (6, 7). New sources of antibiotics from earthworm extracts have also begun to be developed, such as in *Pheretima* and Lumbricus (8, 9), however, the research about the antibiotic potential of earthworm endosymbionts was limited, so the effectiveness of these antibiotics still requires further study.

Earthworms such as *Pheretima* sp., Lumbricus, and Eudrilus live in rich organic compound conditions. However, these soil conditions are also loaded with pathogenic organisms that can threaten the life of the earthworms (10). Various studies related to antibiotic testing of earthworms mostly lead to the use of their extracts, but Byzov et al. (11) stated that earthworms have the potential endosymbiont bacteria to inhibit pathogenic bacteria. Therefore, further research is needed to determine the antibiotic potential of earthworm endosymbionts. These potentials can be known through inhibition tests and compound analyses that were validated through the *in silico* using the molecular docking method and Way2Drugs Prediction of Activity Spectra for Substances (PASS) (12, 13).

MATERIALS AND METHODS

Endosymbiotic bacterial culture from *Pheretima* sp. *Pheretima* sp. was obtained from the lake area of Hasanuddin University (5°08.254′S, 119°29′E). The area has a high organic content. Endosymbiotic bacteria from *Pheretima* sp. was isolated and coded as R4. The R4 isolates were then grown on tryptic soy broth media and incubated for 24 h at 28°C (14).

Purification of endosymbiotic bacteria. R4 isolates (*Pheretima* sp.) washed with sterile water and dissected. After that, a serial dilution was made at concentration of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}. 1 mL solution from the inoculated dilution in the TSA me- dium (the pour plate method), then incubated at 37°C for 24-48 hours. This step was conducted until a sin- gle colony was obtained (14).

***In vitro* antibiotic activity test.** Antibiotic testing was conducted using the modified agar well diffusion method (15, 16), with ciprofloxacin (30 µg) as the control solution. The turbidity of bacterial suspension (24 h incubation) was determined with the McFarland 0.5 standard using a spectrophotometer in the wavelength of 580 nm, in which the transmittance value of 25% was equivalent to 10^8 CFU/mL bacteria. After centrifugation, both supernatant and sonicated pellets were used in the antibiotic test, using the agar diffusion method and incubated at 37°C for 24 h, and the resistance zone was measured with a caliper. The incubation was then continued for 48 h to see the properties of the active compound.

***In silico* antibiotic activity test.** The supernatant of R4 isolates was analyzed using high-performance liquid chromatography (HPLC) and a high-resolution mass spectrometer. The HPLC instrument used a Thermo Scientific Dionex Ultimate 3000 RSLC-nano equipped with a micro flow meter with 0.1% formic acid in water and 0.1% formic acid in acetonitrile as solvents. The Hypersil GOLD aQ 50 × 1 mm × 1.9 um particle size was used as the analytical column of the instrument. The analytical flow rate was set at 40 µL/min with a period of 30 min and the column temperature at 30°C. The high-resolution mass spectrometer instrument used the Thermo Scientific Q Exactive, with a full scanner at 70,000 resolution and data-dependent MS2 at 17,500 resolution with a span of 30 min. The three-dimensional structure of the R4 supernatant compound was downloaded from the PubChem online page (https://pubchem.ncbi.nlm.nih.gov/). The structure was then saved in SDF format and converted to PDB format through the Avogadro application. Furthermore, the target protein contained in the bacteria was selected. Information regarding the function and activity of the target protein was obtained from the Uniport database (https://www.uniprot.org); then, the three-dimensional structure was downloaded via the Protein Data Bank (https://www.rcsb.org/). The three-dimensional structure of the target protein was visualized in the PyMOL v1.7.4.5 application. The reverse docking process was performed using the Vina Wizard feature integrated into PyRx 0.8, which predicts the potential bonds between compounds produced by bacteria and target protein (12, 16). The interactions between ligands, target proteins, and control compounds were visualized and analyzed using PyMol. The activity test used the Way2Drugs PASS online application that can be accessed through http://www.pharmaexpert.ru/passonline/ex.php (13, 17) by entering the canonical SMILE data and analyzing the PA value and biological activity of the compound.

RESULTS

The antibiotic activity test of R4 isolates was performed *in vitro* using the agar diffusion method. The clear zone that forms around the well is an indication of the ability of antibiotics against pathogenic microbes (18).

The inhibition test used cell-free supernatant and bacterial pellets. After 24 h, a large inhibition zone was formed with a diameter of 21.75 mm in *Staphylococcus aureus* and 15.5 mm in *Salmonella* Typhi, and ciprofloxacin (control) showed clear zones of 34.5 and 33.5 mm in *Staphylococcus aureus* and *Salmonella* Typhi, respectively (Fig. 1). The clear zones were reduced as the increase of incubation time (48 h). For the treatment group, the inhibition zone was 20.75 and 14.75 mm in *Staphylococcus aureus* and *Salmonella* Typhi, respectively (Fig. 2), whereas that for ciprofloxacin was 33.5 and 32.75 mm in *Staphylococcus aureus* and *Salmonella* Typhi, respectively.

The molecular docking of the endosymbiotic bacterial compound to the DHPS target showed that the cyclo (phenylalanyl-prolyl) had the lowest binding affinity value of -6 (Table 1). Cyclo (phenylalanyl-prolyl) has the lowest binding affinity for three receptors targets (DHPS, topoisomerase, dan outer membrane) among 10 endosymbiont bacterial compounds. This indicates the potential for these compounds to react with targets to achieve the activation required in the antibiotic action process to inhibit bacterial growth.

The antibiotic activity of the cyclo compound (phenylalanyl-prolyl) found in the endosymbiotic bacterial compound is almost the same as that of quinolone in inhibiting topoisomerase, which is an enzyme responsible for nucleic acid synthesis (Table 2).

The results of molecular docking support the results of *in vitro* tests, that the compounds of R4 isolate have potential as an antibiotic in inhibiting the growth of pathogenic bacteria *Salmonella* Typhi and *Staphylococcus aureus*. The energy required for binding is in line with the binding affinity value. It also affects the position of the attachment, namely, the surface. The easier the compound binds to the target protein, the deeper it will be attached and the stronger the interactions that may occur (Fig. 3).

Cyclo (phenylalanyl-prolyl) has acted as a glycopeptide-like antibiotic with a value of 0.673, whereas sedanolide has a PA value of 0.32 (Table 3). Several important activities were identified, such as the activity of cyclo (phenylalanyl-prolyl) as an antagonist to membrane permeability inhibiting DNA synthesis. Sedanolide also has antagonistic activity against membrane permeability and is an inhibitor of protein synthesis through interaction with DNA ligase and DNA polymerase.

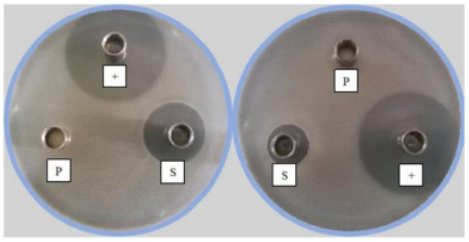

Fig. 1. The inhibition test of R4 isolates after 24 h. Supernatant (S), pellet (P), and control (+). *Salmonella* Typhi (left), *Staphylococcus aureus* (right)

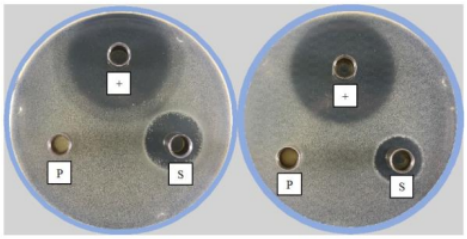

Fig. 2. The inhibition test of R4 isolates after 48 h. Supernatant (S), pellet (P), and control (+). *Salmonella* Typhi (left), *Staphylococcus aureus* (right)

DISCUSSION

Salmonella Typhi is a common pathogenic bacteria that cause typhoid fever (19, 20) and enteric fever (21). Approximately 200,000 deaths per year are caused by *Salmonella* Typhi, with >20 million new cases per year. Based on the latest news, approximately 11-21 million cases and 128,000-161,000 typhoid-related deaths occur annually worldwide (21, 22). Another common main cause of human infection is *Staphylococcus aureus* (23). All strains of *Staphylococcus aureus* can produce compounds that may attack innate and adaptive immunity (24). *Staphylococcus aureus* is well known for its ability to acquire resistance to antibiotics (25, 26). Therefore, it is necessary to find new antibiotics to control the diseases caused by these bacteria.

Table 1. Molecular docking of bacterial R4 isolates metabolites

No	Compound	DHPS	Topoisomerase	Gyrase	Outer Membrane
1	Oleamide	−4.8	−5.4	−6.5	−5.2
2	Dibutyl phthalate	−4.9	−6.9	−5.5	−7.2
3	Hexadecanamide	−4.6	−5.1	−5.2	−5.9
4	4-methoxycinnamic acid	−5.2	−6.3	−6.5	−7.2
5	Cyclo (phenylalanyl-prolyl)	−6	−8.2	−6.4	−8.2
6	Caprolactam	−4.6	−5.3	−4.9	−5.1
7	Linolenic acid ethyl ester	−4.8	−5.7	−5.6	−6.3
8	Bis(2-ethylhexyl) phthalate	−4.7	−6	−5.7	−6.7
9	Eicosapentaenoic acid	−4.6	−6.5	−5.5	−7.3
10	Sedanolide	−5.3	−6.4	−7.3	−8.1
11	Sulfamethoxazole (Control)	−6	-	-	-
12	Quinolone (Control)	-	−9	-	-
13	Ciprofloxacin (Control)	-	-	−8.3	-
14	Penicillin (Control)	-	-	-	−8.2

Table 2. Way 2 Drugs test results for cyclo (phenylalanyl-prolyl) compounds

PA Value	Activity
0.702	Membrane integrity antagonist
0.673	Glycopeptide-like antibiotic
0.581	Antieczematic
0.468	Diuretic inhibitor
0.399	Membrane permeability inhibitor
0.377	Antiprotozoal *(Leishmania)*
0.348	Antiviral (Picornavirus)
0.348	Antifungal
0.301	DNA synthesis inhibitor

Inhibition test: *in vitro*. Ciprofloxacin is a broad-spectrum synthetic antibiotic used against both Gram-positive and Gram-negative bacteria (27, 28). Although the inhibition zone of ciprofloxacin is larger, based on the classification Oldak et al. (29), the endosymbiotic bacterial compound R4 was considered as very high in inhibiting the growth of pathogenic bacteria as it has an inhibition zone of >12 mm. The results in both observation times show that the antibiotic compound in R4 isolate is bactericidal because it does not show a significant narrowing of the inhibition zone; the bactericidal agent kills bacteria (30-32). The antibiotic activity of the supernatant showed differences in Gram-positive and Gram-negative bacteria, of which the supernatant was more effective against Gram-positive than Gram-negative bacteria. It is due to differences in the

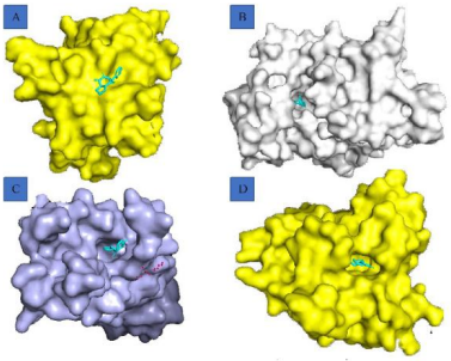

Fig. 3. Visualization of Ligand (control compound) – Macromolecule (receptor) interactions, (A) Cyclo(phenylalanyl-prolyl) (blue) and DHPS (yellow); (B) Cyclo(phenylalanyl-prolyl) (blue) and topoisomerase (green); (C) Cyclo(phenylalanyl-prolyl) (blue) and outer membranes (blue); and (D) Sedanolide (blue) and DNA gyrase (yellow)

Table 3. Way2Drugs test results for sedanolide compounds

PA Value	Activity
0.717	Anti-inflammatory
0.506	Membrane integrity antagonist
0.502	Antifungal
0.5	Antiviral (Rhinovirus)
0.442	Antiprotozoal (Leishmania)
0.43	Antiviral (Influenza A)
0.425	DNA ligase (ATP) inhibitor
0.322	DNA polymerase I inhibitor
0.32	Antibacterial

cell wall structure of the two bacteria (33). Moreover, the test results showed that the endosymbiont bacteria secreted their compounds out of the cell (extracellular). It indicated by the formation of an inhibition zone in a supernatant. Our result is in accordance with previous results, showing the effectiveness of symbiont bacteria from sponges as antibacterial (34).

Gram-positive bacteria have a peptidoglycan polymer that is very close to the cell surface allowing the antibiotic to easily penetrate the cell. Gram-negative bacteria contain an outer membrane consisting of lipopolysaccharides that act as a barrier to hydrophobic and hydrophilic compounds that have a certain molecular weight. The outer membrane serves as an impenetrable barrier for some antibiotics. Tiny hydrophilic antibiotics diffuse through water channels in the outer membrane formed by proteins called porins (33, 35).

Inhibition test: *in silico*. The in-silico test aims to determine the interaction between the desired compound and the target/receptor molecule. The interaction can be visualized using informatics-based methods. Antimicrobial activity testing began with the determination of bioactive compounds (ligands) through the LCMS test for the supernatant. Ten bioactive compounds, which are secondary metabolites of the earthworm endosymbionts *Pheretima* sp., were obtained through the LCMS. These compounds include oleamide, dibutyl phthalate, hexadecanamide, 4-methoxycinnamic acid, cyclo (phenylalanyl-prolyl), caprolactam, linolenic acid ethyl ester, bis(2-ethylhexyl) phthalate, eicosapentaenoic acid, and sedanolide. The target compounds (macromolecules) used in this study were important proteins contained in the bacteria, including DNA gyrase, dihydropteroate synthase (DHPS), topoisomerase, and outer membrane proteins. DNA gyrase plays a crucial role in the DNA replication process (36). Topoisomerase participates in nucleic acid synthesis (37), DHPS takes part in the synthesis of folic acid, and the outer membrane protein is important in bacterial cell membranes (38). The bioactive compounds of the endosymbiotic bacteria *Pheretima* sp. and the receptors (macromolecules) were then analyzed through docking applications. We used sulfamethoxazole, quinolone, penicillin as a control because its antibiotic synthetic that proven its activity. Moreover, these antibiotic have different mechanism action and useful to predict interaction and mechanism action of endosimbiont compound with receptor or macromolecule.

Molecular docking predicts bonds between compounds based on the structure of these compounds to form a conformation in a bond (binding mode) (39). Cyclo (phenylalanyl-prolyl) has a compound structure that is able to bind the three compounds with low affinity (39). The same result was found in the sulfamethoxazole antibiotic. Sedanoline also has a low binding affinity of -5.3 (Table 1). Sulfamethoxazole is a class of antibiotics that works by inhibiting bacterial synthesis from tetrahydrofolic acid, a physiologically active form of folic acid and a cofactor needed in the synthesis of thymidine, purines, and bacterial DNA. Sulfamethoxazole is a structural analog of para-aminobenzoic acid and inhibits dihydrofolic acid synthesis through inhibition of DHPS (40). DHPS receptors enact an essential role in folate biosynthesis (38). DHPS catalyzes the reaction that produces 7,8-dihydropteroate. The next steps in folate synthesis involve the conversion of 7,8-dihydropteroate to finally produce folate compounds (41). The molecular docking of R4 isolates against the topoisomerase receptor resulted in the lowest binding affinity of −8.2 by the cyclo (phenylalanyl-prolyl), whereas quinolone (positive control) had a binding affinity of −9. Quinolone works by inhibiting the separation of double-strand DNA (42).

In present study, molecular docking aims to determine the interaction between the active compound (ligan) and receptor (macromolecule). This similarity indicates the binding affinity quinolone and Cyclo (phenylalanyl-prolyl) to bind with topoisomerase almost same. We showed that the lower the binding affinity value of the compound, the less energy is required to perform the binding. Therefore, the formation of bonds between the two compounds will be easier than another endosymbiont compound.

The docking of the DNA gyrase receptor resulted in the lowest binding affinity of −7,3 by sedanolide, slightly higher than the binding affinity of ciprofloxacin (-8,3). Ciprofloxacin works by inhibiting the gyrase enzyme. The gyrase enzyme allows the relaxation of supercoiled DNA by breaking the two strands of the DNA chain, crossing them, and finally resealing (28). The outer membrane has the lowest binding affinity against cyclo(phenylalanyl-prolyl) with a value of −8.2 and sedanolide with a value -8.1. Penicillin (positive control), which has a mechanism of action of disrupting cell wall synthesis (33, 43) a

binding affinity of −8.2.

The biological activity of cyclo (phenylalanyl-prolyl) and sedanolide compounds was tested using Way2Drugs PASS. PASS is a software designed for evaluating the general biological potential of molecules, such as organic compounds. PASS provides a simultaneous prediction of many types of biological activity based on the compound structure (Way2Drug.com). In a present study, sedanolide and cyclo (phenylalany-prolyl) are the most potential compound from molecular docking result. Molecular docking shows sedanolide and cyclo (phenylalanyl-prolyl) have the lowest binding affinity compared to other endosymbiont compounds.

The antibiotic potential of R4 isolates compounds was clarified through molecular docking, visualization of interactions, and the Way2Drugs PASS test. This study suggests that endosymbiotic bacteria can be explored further for their very high potential as a new source of antibiotics.

CONCLUSION

The inhibition zone (with 21.75 and 15.5 mm in *Staphylococcus aureus* and *Salmonella* Typhi, respectively) of the endosymbiotic bacterial metabolite of *Pheretima* sp. earthworm appeared to be very strong. The *in silico* test supported these results, which showed the antibacterial potential of the compound based on its binding affinity value of −6 on DHPS, −8.2 on topoisomerase and the outer membrane, and −7.3 on DNA gyrase. The antibiotic activity was also clarified through Way2Drugs PASS application that shows the antibiotic activity of cyclo (phenylalanyl-prolyl) and sedanolide compounds. Based on these results, the endosymbiotic bacterial compound of *Pheretima* sp. has the potential as an antibiotic agent.

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