RESEARCH ARTICLE | JUNE 14 2016

Metabolic profiling of endophytic bacteria from Purwoceng (*Pimpinella pruatjan* Molkend) root and antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* FREE

Yekti Asih Purwestri; Nur'aini Kartikasari; Sartika Gunawan Putri; Wildiani Wilson; Langkah Sembiring

AIP Conf. Proc. 1744, 020063 (2016)
https://doi.org/10.1063/1.4953537

Metabolic Profiling of Endophytic Bacteria from Purwoceng (*Pimpinella pruatjan* Molkend) Root and Antibacterial Activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Yekti Asih Purwestri[1, a)], Nur'aini Kartikasari[1], Sartika Gunawan Putri[1], Wildiani Wilson[2] and Langkah Sembiring[1]

[1]*Faculty of Biology, Universitas Gadjah Mada, Jl. Teknika Selatan, Sekip Utara, Yogyakarta 55281, Indonesia*
[2]*Faculty of Nursing and Health Science, University Muhammadiyah of Semarang, Jl. Kedungmundu Raya No.18, Semarang, Central Java, Indonesia*

[a)]Corresponding author: yekti@ugm.ac.id

Abstract. Recent study on antibiotic produced by endophytic bacteria become emerging to fight against the increasing of drug-resistant bacteria. Twenty eight isolates of endophytic bacteria from Purwoceng (*Pimpinella pruatjan* Molkend) have been screened for their potential in preventing growth of pathogens, including *Staphylococcus aureus* and *Pseudomonas aeruginosa*. In the present study four isolates (GP11, GP12, RP6 and DG1) and another three isolates (GP2, GP14 and DG1) showed the activity to prevent the growth of *S. aureus* and *P. aeruginosa ATCC 27853*, respectively. Bioactive compounds were extracted from culture broth using ethyl acetate, followed by Thin layer chromatography (TLC) for metabolic profiling to identify the chemical class of compounds. Taxonomic status of the isolates was determined by BLAST analysis based on 16S rRNA sequences. The result of inhibition zone from 500 mg · mL^{-1} crude extract DG1, GP11, GP12 and RP6 against *S. aureus* respectively are 0.78 cm, 1.18 cm, 0.95 cm and 1.2 cm, whereas for 25 mg · mL^{-1} crude extract DG1, GP2 and GP14 against *P. aeruginosa* respectively are 0.144 cm, 0.151 em and 0.095 cm. TLC assay showed that endophytic bacteria produce alkaloid compound and from the BLAST analysis based on 16S rRNA sequences isolates closely related to *Bacillus subtilis* (GP11, GP12, GP14 and DG1), *Bacillus cereus* (RP6) and *Enterococcus faecalis* (GP2).

Keywords: Antibacterial, endophytic bacteria, *Pimpinella pruatjan* Molkend, *Pseudomonas aeruginosa, Staphylococcus aureus*, TLC.

INTRODUCTION

Purwoceng (*Pimpinella pruatjan* Molkend) is a commercial herbaceous plant contains secondary compounds such as coumarin, sterol, alkaloid, saponin and several kinds of oligosaccharide on the root [1]. Secondary metabolites produced by Purwoceng have an antibacterial, anticoagulant and anti-inflammatory activity [1, 2].

Staphylococcus aureus and *Pseudomonas aeruginosa* are pathogenic bacteria that often found as a pathogenic bacteria that major cause of infectious diseases in the society [3, 4, 5]. *S. aureus* is a major cause of lower respiratory tract infections and the second leading cause of nosocomial pneumonia, bacteremia and cardiovascular infection [5]. The ability of pathogenicity *S. aureus* is caused by combination of extracellular and toxic factors along with natural invasive spread of *S. aeureus* [6]. Reported an increasing number of cases of *S. aureus* infections worldwide. In the United States reported 400 000 cases of infection per year in 2003 [3]. In 1999 to 2005 there was an increase of 62 % cases of pathogenic infection due to Meticillin-Resistant *S. aureus* [5].

Towards the sustainable use of biodiversity in a changing environment: From basic to applied research
AIP Conf. Proc. 1744, 020063-1–020063-7; doi: 10.1063/1.4953537
Published by AIP Publishing. 978-0-7354-1401-3/$30.00

P. aeruginosa has been known many years as causal agent of serious infectious diseases such as eyes, ears, chronic respiratory, pneumonia, urinary tract, blood vessels and skin infection. Pathogenicity mechanism of *P. aeruginosa* caused by toxic compound produced by this bacteria. Antibiotics treatment can be done to overcome the infection caused by *P. aeruginosa* [4]. Sometimes the treatment with antibiotics is failed. *P.aeruginosa* has the resistance ability to antibiotics by several mechanisms such as the low permeability of its outer membrane, efflux pump and antibiotic-inactivating enzyme production (e.g., chepalosporinases) [7]. This resistance mechanism requires the new source of antibiotics to treat the infection. The antibacterial activity of secondary metabolites from Purwoceng can be used to suppress the growth of pathogenic bacteria.

The utilization of secondary metabolites from Purwoceng as raw material for the manufacture of drugs has significant constraints. One of them is the limited number of raw materials such as plants that will be extracted. One way to overcome these obstacles is to use the endophytic bacteria residing in the plant tissue [8, 1]. The endophytic bacteria can produce the similar secondary metabolites to the host plant and also has antibacterial activity to the pathogenic bacteria [9]. Endophytic bacteria's ability to produce the similar secondary metabolites to the host plant has the potential to ease the production of secondary metabolites that can use as antibacterial [10]. The last few years there is many studies about the potency of bioactive compounds from endophytic bacteria that could be used as antibacterial. However, there is just a view study about the endophytic bacterial and its potency from Purwoceng. This recent study has a focus on the potency of antibacterial compounds from Purwoceng endophytic bacteria against *S. aureus* and *P. aeruginosa*.

MATERIALS AND METHODS

Endophytic Bacteria Isolates

The twenty eight isolates in this research have been obtained from the previous research. Endophytic bacteria isolates that stored in glycerol solution were grown in the TSB (Tryptic Soy Broth) and then subcultured into TSA (Tryptic Soy Agar).

Optimation of Incubation Time

The optimation of incubation time has done by creating a growth curve by growing the endophytic bacteria isolates in a TSB (Tryptic Soy Broth) to observe the stationary phase. The growth of isolates was observed every 2 h for 48 h and continue every 12 h until the death phase. The observations performed using turbidimetry method by measuring the Optical Density (OD) of the isolates culture using a spectrophotometer ($\lambda_{600\ nm}$).

Screening of Antibacterial Activity Endophytic Bacteria Isolates

The antibacterial activity of endophytic bacteria isolates was screened using modification of *agar well diffusion* method. The 100 µl endophytic bacteria liquid culture containing 10^8 cell · mL^{-1} was inoculated into TSA media and incubated for 24 h. The target bacteria culture of *S. aureus* and *P. aeruginosa ATCC 27853* containing 10^8 cell · mL^{-1} were cultivated in TSA medium using spread method. A hole was made in the target bacteria petri disc with cork borrer (6 mm in diameter). A piece of agar from 24 h endophytic bacteria isolates was inoculated in TSA media containing the target bacteria and incubated at 37 °C. The clear zone must be observed from early stationary phase to determine the inhibition of bacterial targets growth. The time needed for formation of clear zone was used to determine as a time for production of secondary metabolite compounds

Production and Extraction of Secondary Metabolite Compounds from Endophytic Bacteria

Six Endophytic bacteria were inoculated into 300 mL TSB and incubated at 37 °C to produce the secondary metabolite compounds. The bacteria culture was centrifuged and filtered using sterile Whatman filter paper to obtain a cell-free supernatant. Secondary metabolite compounds of endophytic bacteria were prepared by extraction of cell-free supernatant using 70 % of ethyl acetate (1:1 v/v),

followed by the separation using fractionation column resulting the three layers : supernatant, secondary metabolite and ethyl acetate. The secondary metabolite and ethyl acetate were evaporated to obtain a crude extract of endophytic bacteria secondary metabolites.

Antibacterial Assay of Secondary Metabolites Crude Extract

Antibacterial assay of secondary metabolites crude extract was screened by modification Kirby-Bauer Disc-Diffusion Assay. The target bacteria culture containing 10^8 cell · mL^{-1} in a total volume of 100 µL was inoculated into TSA medium by spread plate method. Filter paper discs were cut approximately 6 mm in diameter and soaked into 20 µL of aquadest containing (25, 50, 100 and 200) mg · mL^{-1} of the secondary metabolites crude extract of DG1, GP2, GP11, GP12, GP14 and RP6 isolates. The paper discs were put into the previously prepared agar plates of target bacteria and incubated at 37 ºC. After 16 h to 24 h of incubation, the diameter of clear zone was measured to examine the inhibition zone. The penicilin and amoxycilin were used as positive control of *S. aureus* and *P. aeruginosa*, respectively.

Identification of Secondary Metabolites Using Thin Layer Chromatography (TLC)

Identification of secondary metabolites using Thin Layer Chromatography (TLC) was performed by dissolving the crude extracts into distilled water and then spoting it into Silica Gel 60 F254 plate. The plates were developed using optimized mobile phase (ethyl acetate : n-hexane 4:6 v/v) through development chamber. The plates were removed, dried at 100 °C and sprayed with Dragendorff reagent and then were scanned with visible light and UV-254 nm.

Molecular Identification of Bacterial Strains

Six endophytic isolates (DG1, GP2, GP11, GP12, GP14 and RP6) were identified based on 16S rDNA genes. The total genomic DNA of endophytic bacteria were isolated and purified by using the methods as described previously [11]. 16S rDNA genes were amplified from bacterial genomic DNA using bacterial universal primers (forward primer: 5'-TGGCTCAGAACGAACGCTGGCGGC-3', reverse primer: 3'-TACCTTGTTACGACTTCACCCCAGTC-5'). The sequence obtained above was aligned by using BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST) and the phylogenetic tree was constructed by MEGA 6 [12].

RESULTS

Screening of Endophytic Bacteria Isolates Antibacterial Activity

The screening result indicated six isolates of endophytic bacteria, namely DG1, GP2, GP11, GP12, GP14 and RP6 had an antibacterial activity. The DG1 isolate showed antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa.* Isolates GP11, GP12 and RP6 only indicated that they had an antibacterial activity against *S. aureus* and isolates GP2 and GP14 only had an antibacterial activity against *P. aeruginosa* .

Extraction of Secondary Metabolites

Secondary metabolites bioactive compounds of endophytic bacteria obtained from the fermentation medium using ethyl acetate extract method. Ethyl acetate was chosen due to a semi-polar solvent that can attract compounds that were polar or non-polar, have low toxicity and easily evaporated [13, 14, 15]. It was observed that ethyl acetate extract of endophytic bacteria was yellowish white in color.

Antibacterial Assay of Secondary Metabolites Crude Extract

The results of antibacterial assay of secondary metabolites crude extract against *S. aureus* and *P. aeruginosa* were shown in Table 1 and Table 2.

TABLE 1. Antibacterial Assay of Secondary Metabolites Crude Extract Against *S. aureus* and *P. aeruginosa*

Extract (500 mg · mL^{-1})	Inhibition zone (cm)			Average (cm)
	I	II	III	
DG1	0.75	0.80	0.80	0.78
GP11	1.15	1.15	1.25	1.18
GP12	1.15	1.35	3.55	0.95
RP6	-	-	1.2	1.2
Control ([a]Amp. 30 mg · mL^{-1})	1.90	2.1	1.80	1.93

[a]Amp. : Ampicilin

TABLE 2. Antibacterial Assay of Secondary Metabolites Crude Extract Against *P. aeruginosa*

Extract (25 mg · mL^{-1})	Inhibition zone (cm)			Average (cm)
	I	II	III	
DG1	0.128	0.122	0.183	0.144
GP2	0.155	0.149	0.149	0.151
GP14	0.098	0.093	0.093	0.095
Control ([a]Amox. 20 mg · mL^{-1})	0.220	0.164	0.172	0.185

[a]Amox. : Amoxycilin

Identification of Secondary Metabolites Using Thin Layer Chromatography (TLC)

In the present study, the ethyl acetate extract of endophytic bacterias DG1, GP11, GP12 and RP6 after they were grown in the fermentation medium showed a potential to inhibit *S. aureus*. The identification using TLC showed that the alkaloids compound was produced by the four isolates (Fig. 1). The alkaloids compound was considered as the bioactive compound because of its potential against the clinical pathogen.

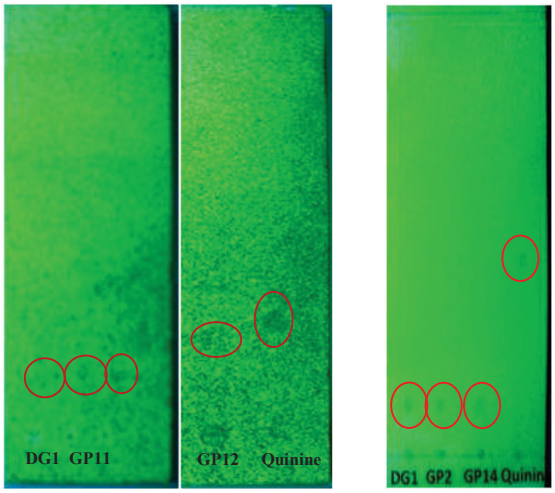

FIGURE 1. (a) Alkaloid assay of DG1, GP11, Rp6 and GP12 secondary metabolites crude extract againts *S. aureus* after sprayed using Dragendorff reagent under UV-254 nm and (b) alkaloid assay of secondary metabolites crude extract of DG1, GP2 and GP14 against *P. aeruginosa* after sprayed using Dragendorff reagent under UV-254 nm.

Identification of Endophytic Bacterial Isolates

Based on the results of 16S rRNA gene sequencing, six potential isolates were subjected for the BLASTn (Basic Local Alignment Search Tool) at NCBI (National Center for Biotechnology Information) for searching the closest phylogenetic relatives. On the basis of BLAST analysis, they were closely related to *Bacillus subtilis, Bacillus cereus* and *Enterococcus faecalis*. Isolate DG1 showed 97 % similarity with the *Bacillus subtilis* strain 262XY2', isolate GP11 had 97 % similarity with the *Bacillus subtilis* strain XJ-P89, isolate GP12 had 97 % similarity with the *Bacillus subtilis* strain T2-shier-5. Furthermore, GP14 showed 100 % similarity with the *Bacillus subtilis* strain JCM 1465, RP6 had 97 %

similarity with the *Bacillus cereus* strain S8SB3 and GP2 had 98 % similarity with the *Enterococcus faecalis* strain ATCC 19433.

FIGURE 2. The relationships of endophytic bacterial isolates from Purwoceng . The consensus tree of the bacterial strain based on 16S rRNA gene sequence in this study were downloaded from GeneBank at NCBI. The type strains were selected from ATCC and other databases

DISCUSSION

The antibacterial assay suggested that GP11, GP12 and RP6 isolates can prevent the growth of *S.aureus* by producing alkaloid as antibacterial compound as well as GP2 and GP14 which can prevent the growth of *P. aeruginosa*. The topology of the phylogram in Fig. 2 demonstrated that the endophytic bacterias in this study were closely related to the *Bacillus subtilis, Bacillus cereus* and *Enterococcus faecalis*.

The potency of isolates DG1, GP11, GP12 and RP6 as antibacterial to inhibit the growth of *S. aureus* was similar to the host plant purwoceng which has been known as a medicinal plant producing antimicobial compounds. According to reference[16] and reference [17] endophytic microbes capable of producing bioactive compounds that are similar to the host plants through the process of coevolution. Endophytic isolates from plant roots purwoceng (*P. pruatjan* Molkend) which showed no antibacterial activity against *S. aureus* was likely to have other potential compounds. Some studies indicated that microbial endophyte produces antifungal substances [18], antioxidants [19], anticancer [20] and IAA hormone [21].

The antibacterial activity of endophytic bacteria isolates in this study can not be separated from its ability to produce secondary metabolites during the fermentation process. Bioactive compounds (antimicrobial) were produced by microorganisms in the late stationary phase of growth [22]. The synthesis of secondary metabolites begins when some of the nutrients in the growth medium of microorganisms have been exhausted. Limitations of these nutrients cause the accumulation of secondary metabolites and enzyme inducers release of the genes for the synthesis of secondary metabolites [23].

The secondary metabolites of endophytic bacteria were obtained from the fermentation medium and were extracted by ethyl acetate solvent. Some studies demonstrated that the antibacterial compounds can be extracted from varies of solvent, e.g. n-butanol, n-hexane and ethyl acetate and showed a significant

difference. Extracts of endophytic actinomycetes fermentation of solvent ethyl acetate and ethyl acetate fraction showed the greatest inhibition against gram-positive bacterium *Bacillus subtilis* compared with n-butanol fraction. Metabolites endophyte of *Aglaia odorata* extracted with ethyl acetate demonstrated antimicrobial activity against *E. coli* and *S. aureus* [24].

The antibacterial activity against *P. aeruginosa* showed that a little amount (25 mg · mL^{-1}) of ethyl acetate crude extract from GP2, GP14 and DG1 isolates can suppress the growth of *P. aeruginosa*. It has been reported that ethyl acetate extract from *Pimpinella anisum* can suppress the growth of *P.aeruginosa* [25]. Even though the inhibition not as strong as ethanol extract, it still can suppress the growth of *P. aeruginosa*.

After identification, the main bioactive substance from extract fermentation of endophytic bacteria (DG1, GP2, GP11, GP12, GP14 and RP6) was confirmed to be an alkaloid compound. Alkaloids are chemical compounds of plant secondary metabolism that results can be found in the leaves, young buds, roots, the sap produced in tubes of sap in the epidermis and cells directly beneath the epidermis as in the cortex. Therefore, the roots, leaves, fruit, seeds and bark were used for preparing the simplicia containing alkaloid [26]. The mechanism of alkaloid compound as an antibacterial was demonstrated by interfering the peptidoglycan component of the bacterial cell *S. aureus*. Therefore, the cell wall layers are not fully formed and caused the death of these cells [27]. Moreover, according to reference [28], the alkaloid contain basic groups which will react with nitrogen compounds amino acids that make up the cell walls of bacteria and bacterial DNA. This reaction resulted in changes in the structure and composition of amino acids that will cause genetic changes in the balance of the DNA chain that will damage leads to a bacterial cell lysis which will cause cell death in bacteria. However, the detailed membrane damage mechanism of the alkaloid compound is still unknown. Conclusively, this study indicated that the sixth endophytic bacterial isolates had a potency as antimicrobial by producing alkaloids as bioactive compounds. Further study to identify the specific compounds potential for antimicrobial activity and the optimum condition for producing this compound need to be explored.

CONCLUSIONS

Conclusively, this study indicated that the sixth endophytic bacterial isolates had a potency as antimicrobial by producing alkaloids as bioactive compounds. Further study to identify the specific compounds potential for antimicrobial activity and the optimum condition for producing this compound need to be explored.

ACKNOWLEDGMENTS

This research partly supported by the funding from Faculty of Biology, Universitas Gadjah Mada through BOPTN 2015 to YAP and LS.

REFERENCES

1. I. Darwati and I. Roostika, Bul. Plas. Nut. **12**(1), 9–15 (2006). [Bahasa Indonesia].
2. T. Widayat and A. E. S. Soetarto, Health Sci. Indones. **28**(1), 31–36 (2012).
3. H. W. Boucher and G. R. Corey, CID. **46**(5), 344–349 (2008).
4. A. R. Hauser and E. A. Ozer, *Pseudomonas aeruginosa*, 2011 [Internet cited 2014 April 24]. Available from: http://www.nature.com/nrmicro/posters/pseudomonas/index.html.
5. E. Klein, D. L. Smith and R. Laxminarayan, Emerg. Infect. Dis. **13**(12), 1840–1846 (2007).
6. G. F. Brooks, J. S. Butel and S. A. Morse. *Medical Microbiology 23rd Edition* (McGraw-Hill Companies Inc., New York, 2004), pp. 317-327.
7. R. Vaisvila, R. D. Morgan, J. Posfai and E. A. Raleigh, Mol. Microbiol. **42**, 587–601 (2001).
8. A. Elita, S. Saryono and J. Christine, J.Indo.Che.Acta. **3**(2), 56–62 (2013).
9. N. Malfanova, B. Lugtenberg and G. Berg, "Bacterial Endophytes: Who and Where and What are They Doing There?" in *Molecular Microbial Ecology of the Rhizosphere*, edited by Frans J. de Bruijn (Wiley-Blackwell , 2013), pp. 15–37.
10. M. Radji, Maj.Ilmu Kefarmasian. **2**(3), 113–126 (2005). [Bahasa Indonesia].
11. W. Wilson, "Bakteri endofit tanaman Purwoceng (*Pimpinella pruatjan* Molk.) berdasarkan karakter morfologis, biokimiawi dan molekular," ["Endophytic bacteria of Purwoceng (*Pimpinella pruatjan*

Molk.) based on morphological, biochemistry and molecular characters,"]. thesis, Fakultas Biologi Universitas Gadjah Mada Yogyakarta, 2014 [Bahasa Indonesia].
12. K. Tamura, G. Stecher, D. Peterson, A. Filipski and S. Kumar, Mol. Biol. Evol. Letters **30**(12), 2725–2729 (2013).
13. USP Convention, USP 30/ NF 25. Twinbrook Parkway: United States Pharmacopeial Convention (2007).
14. R. C. Rowe, P. J. M. Sheskey and E. Quinn. *Handbook of Pharmaceutical Excipients* (Lexi-Comp: American Pharmaceutical Association, Inc., Washington DC, 2009), pp. 418, 685.
15. L. K. Wardhani and N. Sulistyani. J.Ilmiah Kefarmasian. **2**(1), 1–16 (2012). [Bahasa Indonesia].
16. R. X. Tan and W. X. Zou, Nat. Prod. Rep. **18**, 448–459 (2001).
17. G. Strobel and B. Daisy, Microbiol.` Mol. Rev. **67**(4), 491–502 (2003).
18. A. Souza, J. C. Cruz, N. R. Sousa, A. R. L. Procopio and G. F. Silva, Genet. Mol. Res. **13** (4), 8661–8670 (2014).
19. D. C. Savi, C. W. I. Haminiuk, G. T. S. Sora, D. M. Adamoski, J. Kenski, S. M. B. Winnischofer and C. Glienke, IJPCBS **5**(1), 347–356 (2015).
20. J. Xiao, Q. Zhang, Y. Q. Gao, J. J. Tang, A. L. Zhang, J. M. Gao, J. Agric. Food Chem. **62**(16), 3584–90 (2014).
21. M. W. Siregar, "Isolasi dan uji kemampuan bakteri endofit penghasil hormon IAA (indole acetic acid) dari akar tanaman padi (*Oryza sativa* L.)," ["Isolation and test of the ability of IAA (Indole Acetic Acid) hormone-producing endophytic bacteria on the roots of the rice plant (*Oryza sativa* L.),"]. thesis, Universitas Sumatera Utara, 2009 [Bahasa Indonesia].
22. Pelczar, Michael and E. C. S. Chan, *Dasar-Dasar Mikrobiologi* [Fundamentals of Microbiology] (UI-Press, Jakarta, 1986), p. 101 [Bahasa Indonesia].
23. S. T. Pratiwi, *Mikrobiologi Farmasi* [Pharmaceutical Microbiology] (Penerbit Erlangga, Yogyakarta, 2008), pp. 23, 106–108, 111–117, 142. [Bahasa Indonesia].
24. N. E. N. Sugijanto, B. Yodianto, M. N. Kusumajaya and N. C. Zaini, Period. Sci. Pharma. Chem. **3**(1), 22–25 (2014).
25. S. A. A. M. Huda, S. A. Warda and Z. I. A. Aisha, J. Adv. Res. **3**(1), 359–367 (2015).
26. M. Sirait, *Penuntun Fitokimia dalam Farmasi* [Guidance Phytochemicals in Pharmacy] (Penerbit ITB, Bandung, 2007), pp. 158–159. [Bahasa Indonesia].
27. F. R. Juliantina, *Manfaat Sirih Merah* (Piper crocatum) *Sebagai Agen Anti Bakterial Terhadap Bakteri Gram Positif dan Gram Negative* [Benefits of Red Betel (*Piper crocatum*) as Antibacterial Agents Against Gram Positive and Gram Negative Bacteria] (UI-Press, Jakarta, 2008), p. 101 [Bahasa Indonesia].
28. A. W. I. Gunawan, "Potensi buah Pare (*Momordica charantia* L.) sebagai antibakteri *Salmonella typhimurium*," ["Potential fruit Pare (*Momordica charantia* L.) as antibacterials against *Salmonella typhimurium*,". thesis, Universitas Mahasaraswati Denpasar , 2009 [Bahasa Indonesia].