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GARDNERELLA VAGINALIS ATCC 14018 RESISTANT TO METRONIDAZOL AND SOURSOP LEAVES (*ANNONA MURICATA LINN*) PREPARATION

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ABSTRACT

Bacterial vaginosis is a common cause of vaginal discharge caused by polymicrobial agent, mainly Gardnerella vaginalis, which many strain has known to be resistant to metronidazole. Soursop leaves (Annona muricata Linn) had empirically used for genital cleansing to prevent and cure vaginal discharge. Its active substances: tannin, saponin, alkaloid, steroid, flavonoid and annonaceous acetogenin had previously known to have antibacterial effect. This study was aimed at finding out the active substances in aqueous extract of soursop leaves and assess its antibacterial effect on Gardnerella vaginalis ATCC 14018. The study was an in vitro preclinical test conducted with pure experimental methods. Samples were pure culture Gardnerella vaginalis ATCC 14018, obtained from Biofarma laboratory. Materials were aqueous extract, ethanol extract and acethyl acetat fraction of leaves of soursop (Annona muricata Linn) with concentrations of 20%, 40%, 60%, 80%, and clindamycin as positive control. Antibacterial effect on Gardnerella vaginalis ATCC 14018 were tested using the Kirby-Bauer method on peptone starch dextrose blood agar media by measuring inhibition zone, performed four times. Results of phytochemical screening showed that the aqueous extract of soursop leaves contains active substances such as flavonoids, tannins, saponins, alkaloids, quinones, and steroids. The result of antibacterial test showed no inhibition zone formation at either concentration of 20%, 40%, 60%, and 80%. The conclusion was aqueous extract, ethanol extract and acethyl acetat fraction of leaves of soursop has no antibacterial effects on Gardnerella vaginalis ATCC 14018. This result was probably due to the influence by species of plant, demography, and processing the material to concentration of active substance in leaves of soursop.

KEYWORDS: Annona muricata Linn, bacterial vaginosis, clindamycin, Gardnerella vaginalis ATCC 14018, metronidazole.

INTRODUCTION

Vaginal discharge or leucorrhea or fluor albus is a common complain of female in any age. Vaginal discharge is a discharge from vagina beside blood, could be mucus, transudate, or exudate from genital tract lesion (Kapita Selekta Kedokteran, 2001). There are many cause of vaginal discharge, which could be pathological or non pathological condition. Physiologically vaginal discharge occurred previous to or after menstruation, during sexual arrousal, pregnancy and physical or psychological stress. Pathologically vaginal discharge may occurred due to fungal, bacterial, protozoal and gonorrhoe (Manuaba, 2001)

The most common microorganism causing vaginal discharge is bacteria or bacterial vaginosis (>50%), parasite or trichomoniasis (5-74% in female, 5-29% in male), and fungal or candidiasis (70-75%) (Manuaba, 2008). Almost 75% of female population in the world experienced this symptom, more than 75% Indonesian female experienced vaginal discharge at least once in her lifetime while 45% twice or more. (Monalisa, 2012)

Bacterial vaginosis (BV) is the most common cause of a fishy odor vaginal discharge, but more than 50% female with BV were asymptomatic. BV is a polymicrobial syndrome characterized with the displacement of lactobacili as vaginal normal flora with anaerobic bacteria, mostly *Gardnerella vaginalis.*(Gerbaring, 2005 dan Dempa 2006).

Female with BV has a higher risk of Human Papillomavirus (HPV) infection, Human Immunodeficiency Virus (HIV), genital herpes virus *Herpes Simplex Virus* 2 (HSV-2), and pelvic inflammatory disease. Pregnant woman with BV has a risk of developing endometritis and pelvic infection post delivery or post abortion, and also could induce prematur

labor which increasing the risk of prenatal death, and intra amniotic infection (Gillet, 2011)

Drugs of choice for BV recommended by *Center for Disease Control and Prevention* are metronidazole and clindamycin. The drugs only effective on 60% case which in turn cause the high treatment failure and increase the drug resistance. Nevertheless, there is some side effect of the drug, ranging from mild to severe on disturbing peripheral nerveifer (Turovskiy, 2012 dan Anukam,2008).

About 68% G*ardnerella vaginalis strain were resistant to metronidazole*, while 78% *Gardnerella vaginalis* strain sensitive to clindamycin. In this case we need the alternative drug which more effective and safe, and herbal medicine is the choice. (Nagapraja, 2014).

Soursop plant (*Annona muricata Linn*) frequently used as herbal medicine among Indonesian people. According to Agricultural Bureau of West Java there are many area in West Java which become the excellent place for producing soursop plant: Garut, Tasikmalaya, Subang, Sumedang. All part of the plant has benefit, as antibacterial, anticancerous, antimicrobial, antiparasite, antifungal, anticonvulsant, antidepressan, antimalarial, antimutagenic, cardiodepressant etc. Soursop leaves have many chemical compound such as *annonaceous acetogenin, tannin, flavonoid, saponin* etc. Those compounds has antibacterial, antifungal, antiinflammation, antimicrobial, anticancerous effect. (Tropical plant graviola, 2012). *Annonaceous acetogenin* disrupt mitochondrial function via inhibition of mitochondrial complex I and ubiquinon-link NADH oxydase, and bound the matrix on third loop in NDI sub unit within *NADH-ubiquinone oxidoreductase mitochondrial*.(Nakanishi, 2011) *Tannin* inhibit microbial growth via gene expression, so that disrupt the microbial DNA synthesis. *Flavonoid* disturb the bacterial membrane by interacting with bacterial cell via adsorption process linkage with hidrogen bond. In low concentration a phenol-protein complex formed with a weak bound, and rapidly disseminated followed by phenol penetration into cell and thus causing protein precipitation and denaturation. At a high level of phenol could cause cell protein coagulation and lysis of cytoplasm. *Saponin* inhibit cytoadherent by membrane cell and cytoskeleton activation of bacteria, mimicking detergent which disrupt bilayer lipid of bacterial cell membrane(Arabski dan Eka P, 2012).

In this study we use uncontaminated *Gardnerella vaginalis* ATCC 14018 which grow appropriate with the growth curve. The study was aimed at finding out the active substance and the antibacterial effect within aqueous, methanol and ethyl acetate extract of soursop leaves (*Annona muricata Linn*) on *Gardnerella vaginalis* (ATCC 14018).

MATERIAL AND METHOD

We used *Gardnerella vaginalis* (ATCC 14018) from Biofarma Laboratory. Inclusion criteria: bacteria which grow appropriate with the growth curve. Exclusion criteria: contaminated bacteria. Materials were aqueous, methanol and ethyl acetate extract of soursop leaves (*Annona muricata Linn.*), *peptone-starch-dextrose broth* for *Gardnerella vaginalis suspension***,** Clyndamycin 50 µg *disk***,** metronidazole 50 µg *disk.* Equipments: incubator, *laminar air flow cabinet* (nuaire)**,** petri disk**,** Erlenmeyer tube, ose nail**,** microscope, Beaker glass, cotton, gauze**,** aluminium foil**,** *autoclave,* object glass with cover**,** filter paper**,** stirrer, *hand gloves*, sucking pipet**,** ruler and caliper.

Study design were in vitro pure experimental method by testing the soursop leaves preparation on *Gardnerella vaginalis* (ATCC 14018) culture. Independent variable in this study were concentrations of soursop leaves preparation, and the dependent variable was antibacterial effect of soursop leaves preparation on *Gardnerella vaginalis* (ATCC 14018) culture, while controlled variable were *Gardnerella vaginalis incubation time*, *Gardnerella vaginalis growth medium*, clindamycin concentration, metronidazole concentration, *peptone-starch-dextrose broth* and incubation temperature 37°C.

Antibacterial effect were examined by observing the soursop leaves preparations activity in inhibiting *Gardnerella vaginalis* (ATCC 14018), indicated with formation of inhibition zone (mm). Inhibition zone is the clear zone formed around the disk or pit which diameters could be measured with caliper in milimeters (mm), indicating level of sensitivity of certain drug or substance in inhibiting bacterial growth..

Group treatment *Gardnerella Vaginalis culture:*

1) *Gardnerella vaginalis* culture on agar media + solvent (negative control)
2) *Gardnerella vaginalis* culture on agar media + soursop leaves preparation in 80% concentration.
3) *Gardnerella vaginalis* culture on agar media + soursop leaves preparation in 60% concentration.
4) *Gardnerella vaginalis* culture on agar media + soursop leaves preparation in 40% concentration.
5) *Gardnerella vaginalis* culture on agar media + soursop leaves preparation in 20% concentration.
6) *Gardnerella vaginalis* culture on agar media + clindamycin
7) *Gardnerella vaginalis* culture on agar media + metronidazole

Sample size was 28 using Frederer formula, with r= 4.
Soursop leaves were taken from Padjadjaran University, Jatinangor plantation. Extraction and fractionation process took place in PAU laboratory ITB according to their standard operating procedure. *Gardnerella vaginalis culture were developed in* Biofarma laboratory, then underwent identification microscopically and biochemically. The growth curve of the bacteria was then

drew prior to inoculation. Agar diffusion test was done to find the antibacterial effect of soursop leaves preparations compared with metronidazole as resistency test control and clindamycin as positive control. Inhibition zone formed were measured with caliper in millimeters.

Data then tested with Saphiro Wilk then analyzed with An*alysis of Varian* (ANOVA). If significant, then *Post Hoct Test* will be done to find out which group is the most significant.

All the extract were processed at Pusat Antar Universitas laboratory ITB Bandung (LAB PAU ITB), and *Gardnerella vaginalis* suspension were taken from Biofarma Microbiological laboratory. The study took place at Biofarma Microbiological laboratory enelitian ini dilakukan di laboratorium mikrobiologi Biofarma between Deecmber to July 2014.

ETHICAL ASPECT

Ethical aspect in this study were coherent with ethical principle of using stored biologic material. Each aspect including collecting, storing, using and destroying were ethically responsible.

RESULTS

Phytochemical screening test result

Phytochemical screening test was done qualitatively as an initial step to find out the active substance within the soursop leaves (*Annona muricata* Linn.) preparation. Table 1 showed that ethyl acetate fraction of soursop leaves contain many active substances such as alkaloid, flavonoid, quinon, tannin, steroid and triterpenoid.

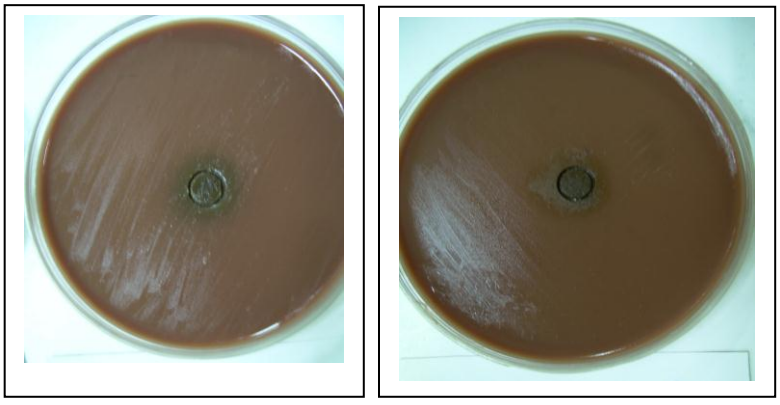
Table 1. Phytochemical screening test result soursop leaves preparation

No	Active substance	Aqueous extract	Ethanolic extract	Ethyl acetate fraction	N-hexane fraction
1	Alkaloid	+	+	+	+
2	Flavonoid	+	+	+	+
3	Quinon	+	-	+	-
4	Saponin	+	+	-	-
5	Tannin	+	+	+	+
6	Steroid and Triterpenoid	+	+	+	+

Note : + = Detected
- = Not detected

Antibacterial test

Effectivity test result of soursop leaves (*Annona muricata Linn*) aqueous extract on *Gardnerella vaginalis* ATCC 14018 displayed on figure 1,2 and table 2.



A.20% concentration | B. 40%concentration

Figure 1 Kirby-Bauer Diffusion test

Note : A= Kirby-Bauer Diffusion test 20% concentration
B= Kirby-Bauer Diffusion test 40% concentration

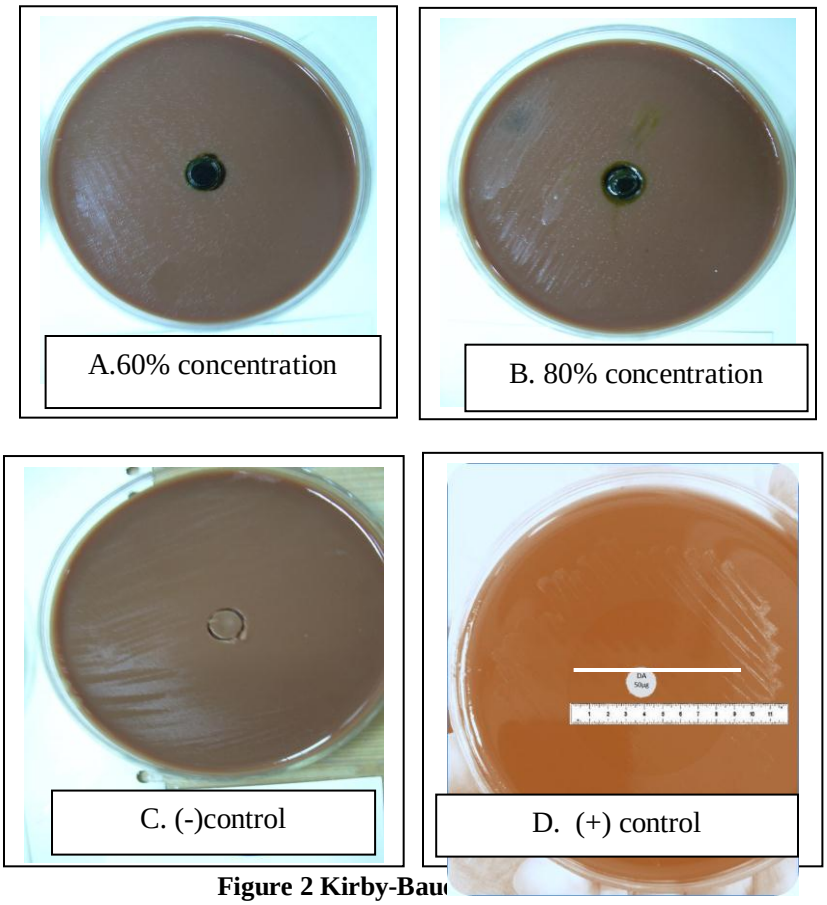


Figure 2 Kirby-Bau

Note: A= Kirby-Bauer Diffusion test 60% concentration
B= Kirby-Bauer Diffusion test 80% concentration
C= Kirby-Bauer Diffusion test negative control(aquadest)
D= Kirby-Bauer positive control (clyndamicin)

From figure 1 and 2 we found that there are no inhibition zones measurable from all concentration of soursop leaves aqueous extract (20%, 40%, 60%, 80%). In positive control with clindamycin disk 50 µg there was an inhibition zone (clear zone) around the disk with 48 mm in diameters, while in negative control with aquadest there were no inhibition zone either. The control positive result (clindamycin) showed that *Gardnerella vaginalis* (ATCC 14018) was sensitive to clindamycin, and the negative result from soursop leaves preparation had no antibacterial effect on *Gardnerella vaginalis* (ATCC 14018).

Tabel 2. Average Diameter of Inhibition Zone Aqueous Extract of soursop leaves (*Annona muricata Linn*) on *Gardnerella vaginalis* ATCC 14018

Group Repeat	Concentration (mm)				Control (mm)	
	20%	40%	60%	80%	Clindamycin	Aquadest
I	0	0	0	0	48	0
II	0	0	0	0	48	0
III	0	0	0	0	48	0
IV	0	0	0	0	48	0
Average[a]	0	0	0	0	48	0

Note:
a. Clindamycin sensitivity criteria according to *Clinical and Laboratory Standard Institute* (CLSI): > 9mm: Sensitive; <9 mm: resistant
Average diameter of ethanolic extract inhibition zone is shown in figure 3 and 4

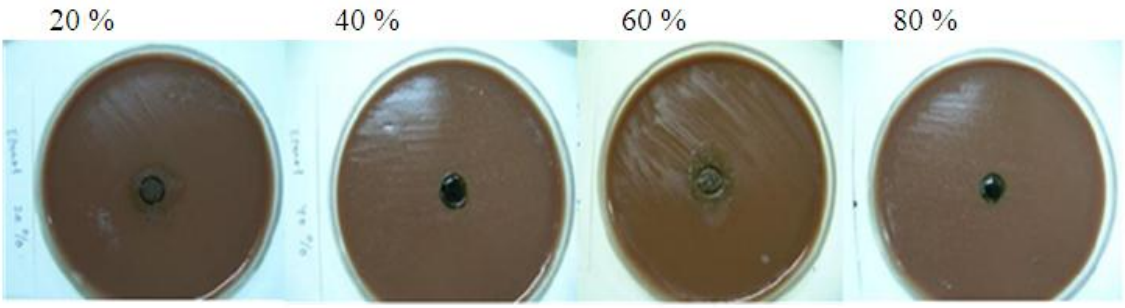


Figure 3 P*eptone-starch-dextrose blood agar* with well filled with ethanolic extract of soursop leaves in 20 %, 40%, 60%, and 80% concentration.

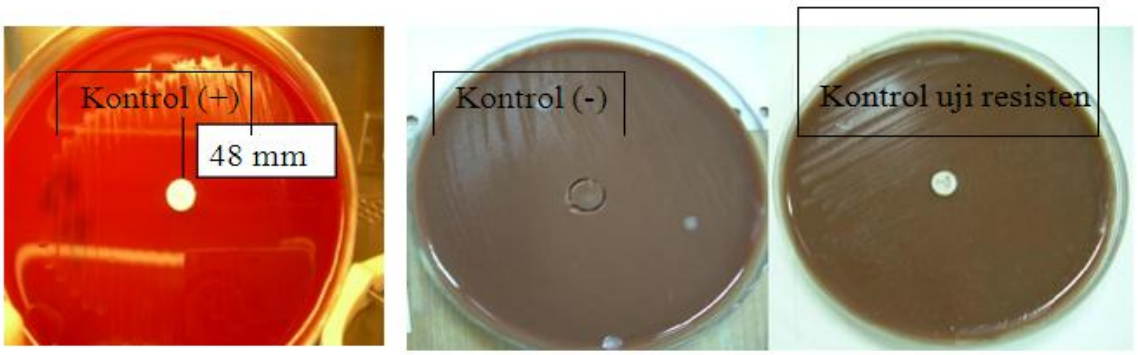


Figure 4 *peptone-starch-dextrose blood agar* contained positive control (Clindamisin 50 µg disk), negative control (solvent) and resistency test control (Metronidazole 50 µg disk).

Figure 3 and 4 showed that there were no inhibition zone measurable from all concentration of soursop leaves aqueous extract (20%, 40%, 60%, 80%). In positive control with clindamycin disk 50 µg there was an inhibition zone (clear zone) around the disk with 48 mm in diameters, resistancy test control containing metronidazole 50 µg showed no inhibition zone either. The control positive result (clindamycin) showed that *Gardnerella vaginalis* (ATCC 14018) was sensitive to clindamycin, and the negative result from soursop leaves preparation had no antibacterial effect on *Gardnerella vaginalis* (ATCC 14018). Average diameter of ethanolic extract inhibition zone and clindamycin as positive control were shown in table 3.

Table 3 Average Diameter of Inhibition Zone Ethanolic Extract of soursop leaves (*Annona muricata Linn*) on *Gardnerella vaginalis* ATCC 14018

Repeat	Ethanolic extract concentration Of soursop leaves(mm)				Control (mm)		
	20%	40%	60%	80%	Clindamycin (+)	Metronidazole (Resistancy test)	Solvent (-)
I	0	0	0	0	48	0	0
II	0	0	0	0	48	0	0
III	0	0	0	0	48	0	0
IV	0	0	0	0	48	0	0
Average[a]	0	0	0	0	48	0	0

Note: a. Metronidazole sensitivity criteria according to Clinical and Laboratory Standard Institute (CLSI): > 26mm : Sensitive; 20-26: moderate; < 20mm

Antibacterial test result of ethyl acetate fraction of soursop leaves on Gardn*erella vaginalis* ATCC 14018.

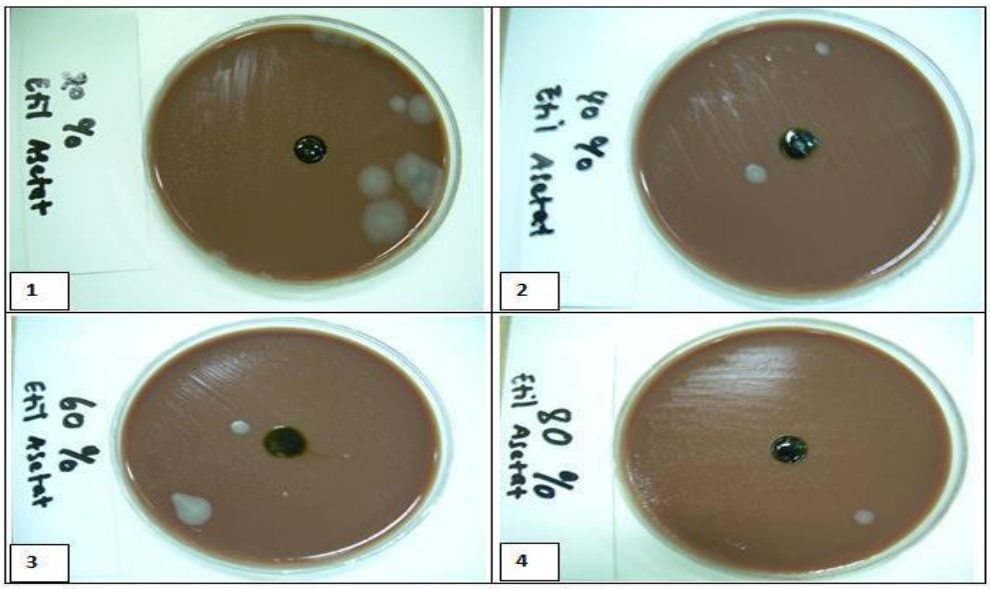


Figure 5 Ethyl acetate fraction diffusion test result

Note : no 1 (ethyl acetate fraction 20%), no 2 (ethyl acetate fraction 40%), no 3 (ethyl acetate fraction 60%), no 4 (Ethyl acetate fraction 80%)

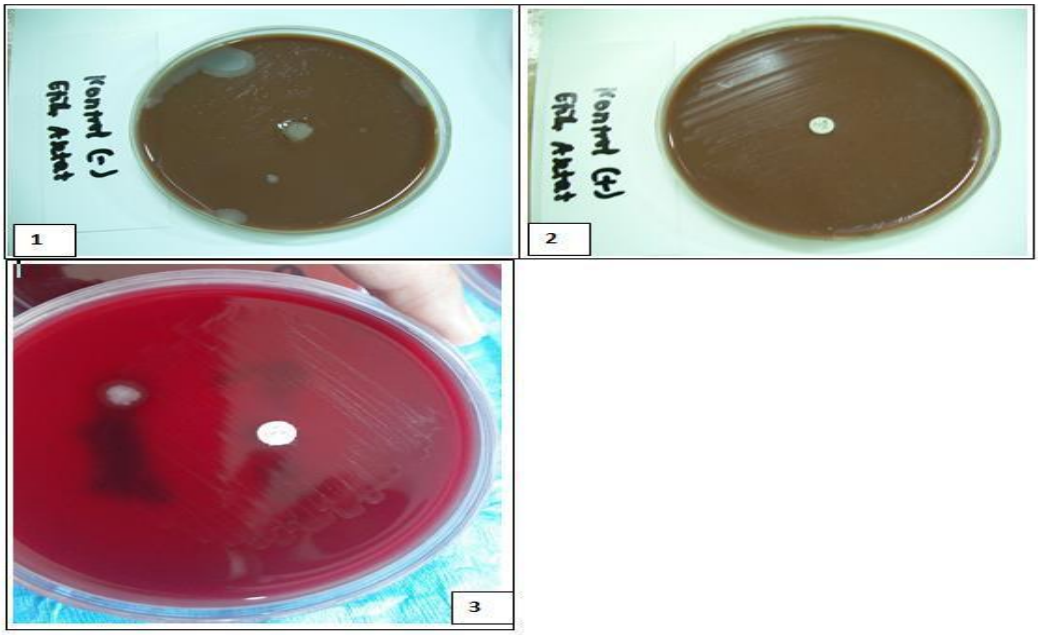


Figure 6 Negative and positive control

Note : no 1 (negative control aquadest), no 2 (positive control metronidazol), no 3 (positive control clindamycin)
Average diameter of ethyl acetate fraction inhibition zone is shown in table 4.

Table 4 Diffusion test result of Ethyl acetate fraction, metranidazole, and clindamycin

Group Repeat	Ethyl acetate fraction concentration				Control		
	80%	60%	40%	20%			
	Diameter inhibition zone (mm)				Metronidazole	Clindamycin	Aquadest
1	0	0	0	0	0	48 mm	0
2	0	0	0	0	0	48 mm	0
3	0	0	0	0	0	48 mm	0
4	0	0	0	0	0	48 mm	0

Note : Clinical breakpoint Metronidazol = 16 mm ; Clindamycin = 9 mm

The table above showed no inhibition zone on contact test using diffusion method Kirby-Bauer modification in any concentration 20%, 40%, 60%, nor 80%. Positive control test with 50µg clindamycin disk showed inhibition zone – a clear zone around the disk -, while on positive control with metronidazole 50µg there was no inhibition zone.

DISCUSSION

Phytochemical test showed that substance contained within aqueous extract of soursop leaves were *flavonoid, tannin, saponin*. This result was coherent with Eka Purwatresna in 2012 who found that aqueous extract of soursop leaves contain substances that could be detected qualitatively: *flavonoid, tannin, saponin*, dan *alkaloid.* (Eka ,2012).

Phytochemical screening test on ethyl acetate fraction of soursop leaves showed that the contain was alkaloid, flavonoid, tannin, quinon, triterpenoid and steroid. There were no previous study concerning phytochemical screening test of ethyl acetate fraction of soursop leaves. Screening on ethanolic extract showed the flavonoid, saponin, alkaloid, tannin and steroid content. This result relevant with study by Vimala et al which said that ethanolic extract of soursop leaves has secondary metabolite such as flavonoid, tannin, alkaloid, saponin and steroid (Vimala,2014).

Screening test on n-hexane fraction showed that saponin, alkaloid, terpenoid and flavonoid. According to Watson et al, compound which could bound in n-hexane fraction were tannin, saponin, alkaloid, terpenoid and flavonoid. Differences of content might occur due to treatmen of the plant, the soil and the physical condition. Soursop plant grew well in lowland tropical area, treated and fertilized, watered, and prevented from plant disease. (Mironova,2008).

Antibacterial effectivity test showed that clindamycin was more sensitive to *Gardnerella vaginalis* (ATCC 14018) than to soursop leaves preparation, in aqueous and ethanolic extract and also in ethyl acetate fraction. Based on previous study by SO *et* al. in 2009 that active substance *tannin, flavonoid, saponin,* dan *annonaceous acetogenin* isolated from soursop leaves has antibacterial effect. This difference might due to the aqueous extract in this study were from the rough extract. Pharmacologically, the active substances within a rough aqueous extract were secondary metabolites which had interaction as toxin to bacterial cell and could damaged protein membrane or inhibit specific protein attached to RNA/DNA, change its structure then causing sinergistic or antagonistic pharmacological reaction. However, the reaction depends on the concentration of the active substance(Mironova,2008)

Chee Kent Lim *et al* (2013) found that *tannin* had antibacterial effect at 160 µg concentration. Bacterial resistant to *tannin* coud happen if the structure of tannin changed due to bacterial wall role. (Vimala JR, 2013). Michal Arabski *et al* (2011) found that *saponin* has an antibacterial agent if given at 15 µg concentration, but not significant for negative and positive Gram variable. This mechanisme influenced by saponin structure, concentration, membrane permeability and lipid bilayer thickness of bacterial cell.(Arabski M, 2012). T.P. Tim Cushnie (2003) found that fl*avonoid* had antibacterial effect at 3,9 dan 15,6 µg concentration, but no effect at 128 µg concentration. This difference might due to the concentration of substance react with flavonoid, which caused the transformation in flavonoid structure (Thomas, 2011).

Nigel W McLean et al in 1996 presumed that drug decreasing activity could be due to drug's decreasing ability to cross the bacterial membrane, or change in bacterial metabolism. Density of outer membrane bacterial wall in several Gardnerella vaginalis strain has made it impenetrable for drugs or active substance which leads to drug resistance. Gardnerella vaginalis 14018 strain has a thick and dense wall, which make drug or substance with same antibacterial property had different effect, might be no antibacterial effect at all(McLean 1996)

Active substances in ethanolic extract of soursop leaves have the similar antibacterial activity with metronidazole, by inhibiting nucleic acid synthesis and disrupt bacterial membrane wall. This could be a factor causing the same result between soursop leaves preparation and metronidazol which was no inhibition zone on *Gardnerella vaginalis* strain 14018.

There were many physical factor affecting the concentration of active substance within a herbal medicine: plantation source, climate, soil condition, and other demographic factors. Quality and quantity of active substance might also affected by time of harvesting. The negative inhibition zone not only depends on active substance mechanism, drug mechanism of action and bacterial morphology (Mironova, 2008).

CONCLUSION

Active substances within soursop leaves (*Annona muricata Linn*) were *flavonoid, tannin, saponin, alkaloid, kuinon*, and *steroid. Gardnerella vaginalis* ATCC 14018 resistant to metronidazole. Aqueous extract, ethanolic extract, and ethyl acetate fraction of soursop leaves could not inhibit the *Gardnerella vaginalis* ATCC 14018 growth.

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