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 Seleka 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 arousal, 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 preterm
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 nerve fiber (Turovskiy, 2012 dan Anukam, 2008).

About 68% Gardnerella 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. (Nagapraga, 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, antiulcer, anticonvulsant, antidepressant, antimalarial, antimitugenic, 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). Annonoaceous acetogenin disrupt mitochondrial function via inhibition of mitochondrial complex I and ubiquinon-link NADH oxysdase, and bound the matrix on third loop in NDI sub unit within NADH-ubiquinone oxoreductase 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 hiodgen bond. In low concentration a phenol-protein complex formed with a weak bond, 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 cytoadherenbyme 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, Erlemeyer tube, osc nafi, microscope, Beaker glass, cotton, gauze, aluminium foil, autoclave, object glass with cover, filter paper, stirrer, hand glove, 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 millimeters (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 Analysis of Varian (ANOVA). If significant, then Post Hoc 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 between Decembe to July 2014.

**ETHICAL ASPECT**
Ethical aspect in this study were coherent with ethical principle of using stored bioologic 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

<table>
<thead>
<tr>
<th>No</th>
<th>Active substance</th>
<th>Aqueous extract</th>
<th>Ethanol extract</th>
<th>Ethyl acetate fraction</th>
<th>N-hexane fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkaloid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Flavonoid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Quinon</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Saponin</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Tannin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Steroid and Triterpenoid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Note: + = Detected  
- = Not detected

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

![Figure 1 Kirby-Bauer Diffusion test](image-url)

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Repeat</th>
<th>Concentration (mm)</th>
<th>Control (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20%</td>
<td>40%</td>
<td>60%</td>
</tr>
<tr>
<td>I</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>III</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IV</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Averagea</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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)

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

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 Peptone-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 (Clindamycin 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

<table>
<thead>
<tr>
<th>Repeat</th>
<th>Ethanolic extract concentration Of soursop leaves(mm)</th>
<th>Control (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20%</td>
<td>40%</td>
</tr>
<tr>
<td>I</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>III</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IV</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Average</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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 Gardnerella 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%)

Average diameter of ethyl acetate fraction inhibition zone is shown in table 4.

Figure 6 Negative and positive control
Note: no 1 (negative control aquades), 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

<table>
<thead>
<tr>
<th>Group</th>
<th>Repeat</th>
<th>Ethyl acetate fraction concentration (%)</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>80 %</td>
<td>60 %</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
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 Purwatesrnsa 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, 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 drug resistance. Gardnerella vaginalis ATCC 14018 resistant to metronidazol. Aqueous extract, ethanolic extract, and ethyl acetate fraction of soursop leaves could not inhibit the Gardnerella vaginalis ATCC 14018 growth.

CONCLUSION

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

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