

## ANTIBACTERIAL EFFECT OF RED *PIPER BETLE* LEAF (*PIPER CROCATUM* RUIZ AND PAV.) ETHANOLIC EXTRACTS TO *LACTOBACILLUS ACIDOPHILUS* AND *LACTOBACILLUS BIFIDUS* GROWTH INHIBITION

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### ABSTRACT

**Objective:** This study was performed to determine the antibacterial concentration of red *Piper betle* leaf ethanolic extract that is safe for *Lactobacillus acidophilus* and *Lactobacillus bifidus* as normal flora of the vagina.

**Methods:** The phytochemical screening of the ethanolic extract of red *P. betle* leaf was performed using standard procedures. The extracts were tested for determining minimum inhibitory concentration value (MIC) using macrodilution method and minimum bactericidal concentration (MBC) done using the streak plate method. Evaluation of its antibacterial effect to vaginal normal flora growth inhibition was conducted using a plate count method with varying extract concentrations as follows: 0.2, 0.4, and 0.6% w/v. *L. acidophilus* FNCC-0051 and *L. bifidus* FNCC-0210 were used as tested normal flora vaginal bacteria.

**Results:** The phytochemical analysis of the red *P. betle* leaf extract revealed the presence of flavonoids, tannin, steroids, saponin, and polyphenolic compounds. The MIC and MBC concentrations of testing extracts against both *Lactobacillus* ranged from 0.625% w/v to 1.25% w/v. The results showed that the extract at 0.2% w/v was the minimum concentration to maintain the presence of *Lactobacillus* approaching the amount of normal flora in the vaginal ecosystem that is 73-75%. At a concentration of 0.2% w/v, it was obtained *L. acidophilus* survival of 78.43% while *L. bifidus* amounted to 76.39%.

**Conclusion:** It can be concluded that the red *P. betle* leaf ethanolic extract is a potential antibacterial and safe for normal flora of the vagina.

**Keywords:** Red *Piper betle*, *Lactobacillus acidophilus*, *Lactobacillus bifidus*, Survival, Minimum inhibitory concentration.

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### INTRODUCTION

Women's reproductive health is one of the important parts that should be taken seriously. According to the WHO data, women's reproductive health issues have reached 33% of all diseases suffered by women. This value is higher than male reproductive health problems, which only reached 12.3%. One of the reproductive problems in women is predominantly fluor albus. According to the International data, 75% of women had experienced a minimum of fluor albus once in her life [1].

Most women consider fluor albus as a common problem. However, not all discharges are normal condition. In normal vaginal discharge (physiological), its liquid production is influenced by several factors such as hormonal factors, physical exhaustion, and psychological. However, abnormal vaginal discharge (pathological) is usually caused by infection of *Neisseria gonorrhoeae* bacterium, *Candida albicans* fungal, and *Trichomonas vaginalis* protozoan [2]. A research study reported that the prevalence of vaginal infections in women caused by bacteria (38%), *Trichomonas* (3.7%), and *Candida* (52.5%) [1].

As a non-specific defense against infectious microorganisms, the normal flora in the vagina available to maintain the balance of the vaginal ecosystem by inhibiting the excessive growth of pathogen microorganisms. In a normal vaginal ecosystem, there is some normal vaginal flora, such as *Lactobacillus* (73-75%), *Escherichia coli* (20-28%), and *Staphylococcus epidermidis* (41-57%) [3-5]. Based on these data, it is known that *Lactobacillus* is the main normal flora in the vagina.

Physiologically, the increasing of estrogen levels can cause the releasing of epithelial cells, especially in the superficial. The died cells rupture and produce glycogen. The existence of *Lactobacillus* in the vagina may convert glycogen to lactic acid that protects the vagina from infection

with an acidic pH ranging 4.5-5. Therefore, it is not allowed to change the acidity of the substances that can disturb the pH balance of the vagina [6]. The importance of the normal flora in the vagina causes its existence must be maintained considering that a lot of commercial antiseptics does not pay attention to its effect to the existence of the normal flora in the vagina. Therefore, we need an antiseptic that can kill pathogenic microorganisms but does not disturb the balance of the existing normal vaginal flora.

### MATERIALS AND METHODS

#### Materials

The tested plant used in this study is a red *Piper betle* leaf (*Piper crocatum* Ruiz and Pav.). Fresh plant materials were collected from Bogor, Indonesia. Plant samples were determined in Plant Taxonomy Laboratory of Biology Major, Faculty of Mathematics and Natural Science, Universitas Padjadjaran.

The tested bacteria used in this study were *Lactobacillus acidophilus* FNCC-0051 and *Lactobacillus bifidus* FNCC-0210 obtained from Universitas Gajah Mada, Yogyakarta, Indonesia.

The chemicals used are ethanol 70% (brataco), de Man Rogosa Shape agar (MRSA-Oxoid) medium and de MRS broth medium (MRSB-Oxoid, London, UK), dimethyl sulfoxide (DMSO-Sigma Aldrich, UK), chloroform (Merck, Germany), mercury (Merck, Germany), ether (Merck, Germany), sulfuric acid (Merck, Germany), amyl alcohol (Merck, Germany), bismuth potassium iodide (Merck, Germany), gelatin (Merck, Germany), iron (III) chloride (Merck, Germany), magnesium powder (Merck, Germany), hydrochloric acid (Merck, Germany), ammonia (Merck, Germany), potassium hydroxide (Merck, Germany), vanillin (Merck, Germany), and aqua dest.

### Preparation of samples

The leaves of red *P. betle* were cleaned and chopped, then dried at room temperature on a laboratory bench, and grounded to a coarse powder using a manual grinder. The powdered material was weighed using an electronic balance. The plant material (300 g) was macerated with 70% ethanol for 72 hrs successively with intermittent shaking every 2 hrs. The extracts were evaporated using a rotary evaporator at 40-50°C and then continued to evaporate on a water bath until dried extract with constant weight was obtained (51.56 g). The extract was stored in a refrigerator at 4°C until time of use. The percentage yields (w/w) of the extracts were calculated using the formula below [7]:

$$(\text{Weight of extract/weight of starting plant material}) \times 100\%$$

### Phytochemical screening

The ethanolic extract of the red *P. betle* leaf was subjected to a preliminary phytochemical screening. The phytochemical screening was done using a standard method to determine contain of alkaloids, flavonoids, tannins, quinones, phenolics, saponins, steroids, triterpenoids, monoterpenoids, and sesquiterpenoids in both simplisia and ethanol extracts of *P. betle* leaf [8].

### Confirmation of testing bacteria

Identification of bacteria includes observation of bacterial colony morphology, microscopic observation of bacterial cells consisting of Gram-staining and coloring spores, as well as biochemical test that consists of a test motile and catalase test [9]. The identification was performed according to Bergey's manual of determinative of bacteriology [10].

### Gram-staining

The bacteria colony was examined using the Gram-staining method according to Collins *et al.* [11]. One dose of bacteria colony was taken and then suspended in sterile normal saline (0.9%). One dose of bacterial suspension was swabbed on a sterile glass slide. Microbial swabs were imbued with carbolic gentian violet dye and allowed for 1 minute. Excess dye was dumped and then rinsed with running water. Then, spread microbes were flooded with lugol and allowed for 1 minute. Lugol excess was dumped, washed with 95% alcohols until the dye dissolves, and then rinsed with water. Then, the smear was flooded by fuchsin and left to stand for 1 minute. Excess dye was dumped, rinsed with water, and then dried using a filter paper. The smear was spilled with oil immersion and then examined under a microscope at a magnification of  $\times 1000$ . Gram-negative bacterial cell will be red while Gram-positive bacteria in purple.

### Spores staining

Spores staining method were conducted with a standard procedure using different staining reagents. As with the acid-fast stain, heat is required to penetrate the endospore coat. To perform endospore, air tap in beaker glass was boiled on a hot plate. A clean glass slide was obtained and prepared for a bacterial smear. The slide was placed on top of the beaker of water and steam for 5 minutes. Carbol fuchsin was applied on the preparat and allowed to sit for 2-3 minutes. The slide was removed with a clothes pin and placed on the staining tray to cool. The slide was tilted and rinse with distilled water. The slide was covered with methylene blue and allowed to sit for 5 minutes. The slide was tilted and rinsed with distilled water. The slide was dried with filter paper and observed it using oil immersion under a microscope. Endospores will appear as red or clear circles or ovals, whereas vegetative cells will be blue [12].

### Catalase test

To perform this test, a single isolated colony was streaked on a glass slide and one drop of 3% hydrogen peroxide (Merck, Germany) was added on to it. The effervescence of oxygen indicated the positive response of the bacteria to catalase test [13].

### Preparing bacterial suspension

The bacterial suspension was prepared by transferring a loopful of inoculum into normal saline (0.9%) under aseptic conditions from

the stock culture maintained at 4°C. The density of each microbial suspension was adjusted to equal that of  $10^6$  CFU/ml (standardized by 0.5 McFarland standard) [14].

### Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination

Determination of MIC of red *P. betle* ethanolic extract was done using macrodilution method. MIC of red *P. betle* against both bacteria was assessed by serial dilution method. The ethanolic extracts of red *P. betle* leaf were dissolved in DMSO and then diluted with MRSB medium until achieved concentration as follows: 0.675%, 1.25%, 2.5%, and 5% w/v. The volume of 10  $\mu$ l standardized cell bacterial suspensions was put into each tested concentration. The liquid media were incubated for 20 hrs at temperature 37°C. MIC was determined from the smallest concentration which did not show any turbidity. MBC was determined from the MIC range using the spread plate method. MRSA media in Petri dishes were subcultured from tubes without growth and incubated at 37°C for 24 hrs. The Petri dishes were observed macroscopically. The highest dilution that yielded no bacterial colony on a solid medium was taken as MBC [15].

### Calculations of bacterial numbers

The antibacterial effect of extracts on *Lactobacillus* growth was observed by calculating the percentage of the surviving bacteria number after contact with the extracts during the incubation period. Type of treatment of tested bacterial cell suspensions can be seen in Table 1.

Each treatment in a test tube was homogenized and diluted to reach  $10^{-4}$  dilutions. Each dilution was pipetted to 10  $\mu$ l and dropped on the surface of the MRSA media and then spread using a spreader. The plates were then incubated at temperature 37°C for 24 hrs. The survival of *Lactobacillus* colonies was calculated to determine the number of bacterial colonies with and without treatment (L0). The percentage of surviving bacteria (S) was calculated according to the following equation [16]:

$$S (\%) = (N - N_0) / N_0 \times 100$$

Where:

$N_0$  = Plate count of *Lactobacillus* without treatments

N = Plate count of *Lactobacillus* after treatments.

## RESULTS AND DISCUSSION

### Yield of the extract

Extraction method used in this study was maceration method. Maceration was done by immersing the sample in an organic solvent. Organic solvents will penetrate the cell wall and into the cavity of the cell that contains the active substance so that the active substance will dissolve. Due to the difference between the solution concentrations of active substance in the cell, the solution is pushed out [17]. The ethanol leaf extract of *P. betle* yielded 17.18% w/w. The characteristic of the extract is brownish green, is sticky, and has a leafy smell.

### Phytochemical screening results

Ethanol extracts of red *P. betle* leaves had shown the presence of flavonoids, polyphenolics, steroids, quinones, and saponins. The result of phytochemical screening can be seen in Table 2. Based on the data in Table 2, it can be seen that in the extracts, simplisia of red betel leaf contains the same compounds. This proves that the extraction

Table 1: Type of treatment

Type	Treatment
L0	Cell bacterial suspension
L1	0.2% w/v extracts in cell bacterial suspension
L2	0.4% w/v extracts in cell bacterial suspension
L3	0.6% w/v extracts in cell bacterial suspension

process which was carried out did not change the content of secondary metabolites present in simplisia.

The secondary metabolites can affect the growth of normal vaginal flora, when formulated as an antiseptic vaginal [18]. This is evidenced in another study which revealed that ethanol extracts of *Camellia sinensis* is bactericidal against *L. acidophilus*. The presence of major natural chemical groups in the ethanolic extract of *C. sinensis*, such as saponins, flavonoids, phenols, terpenoids, alkaloids, quinones, and steroids, was analyzed [15]. These compounds were detected in the ethanolic extract of red *P. betle*.

#### Bacterial identification results

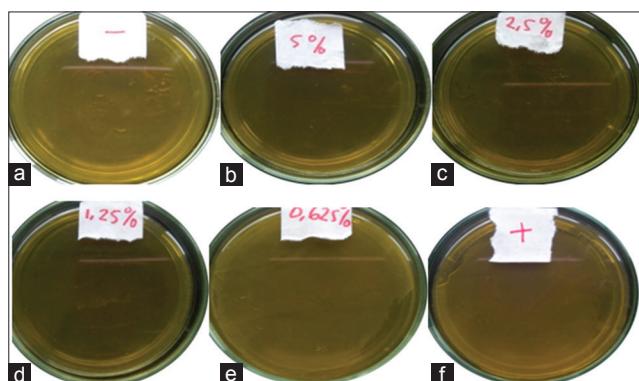
Identification of the bacteria was conducted to determine the level of taxa by comparing the character; it has the character of test bacteria which are known in the literature. The results of the identification of *L. acidophilus* and *L. bifidus* can be seen in Table 3.

Bacterial identifications were done by comparing the observations colony morphology, microscopic observation, and biochemical tests with the information contained in the book "Bergey's Manual of Determinative Bacteriology" [10]. The identification results showed that both cells were *Lactobacillus* species.

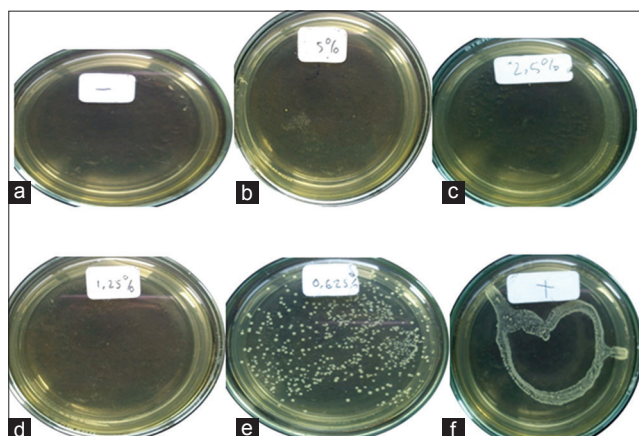
#### The result of MIC and MBC determination

Red *P. betle* ethanolic extract exhibited antibacterial effect on both of *L. acidophilus* and *L. bifidus*. The results can be seen in Figs. 1 and 2.

From the figures, it can be seen that the MIC of ethanolic extract of red *P. betle* leaf against both test bacteria lies in the concentration



**Fig. 1:** Growth inhibition of red *Piper betle* leaf extracts against *L. acidophilus*. (a) Negative control, concentration of extract in % w/v (b=5, c=2.5, d=1.25, e=0.625), (f) positive control



**Fig. 2:** Growth inhibition of red *Piper betle* leaf extracts against *L. bifidus*. (a) Negative control, concentration of extract in % w/v (b=5, c=2.5, d=1.25, e=0.625), (f) positive control

of 0.625% w/v. The MBC value for both *Lactobacillus* is 1.25% w/v. Another study revealed that MIC of green tea extract on *L. acidophilus* was found to be 0.3% and the MBC was found to be 0.9% [15]. The smaller MIC and MBC values against *Lactobacillus* were safer for the growth of *Lactobacillus* as normal vaginal flora.

#### The result of bacterial numbers calculation

Probiotics improve the values of human life since the intestinal tract is a diverse microenvironment where more than 500 species of bacteria thrive. A single layer of epithelium is all that separates these microorganisms and pathogens from the underlying immune cells, and thus, epithelial barrier function is a key component in the defense mechanisms required to prevent infection and inflammation [19].

Vaginitis used to be considered a mere annoyance but now is being examined for a role in serious conditions including pelvic inflammatory disease, pregnancy-related complications, and increased susceptibility to AIDS infection. Vaginitis can be caused by several different organisms, and in many cases, the causative agent may not be identified. What is known is that *Lactobacilli* predominate in the healthy vagina, and a lack of *Lactobacilli* is a risk factor for vaginitis [19]. Therefore, *Lactobacilli* are thought to be maintained in the vaginal ecosystem.

The use of an antiseptic substance concentration should be examined to prevent disruption of the normal vaginal flora. Antibacterial activity of extracts against *Lactobacillus* will decrease the number of *Lactobacillus* in the vaginal ecosystem. Therefore, this study was conducted to determine the minimum concentration of red *P. betle* extracts which is safe for the presence of the normal vaginal flora. In a normal vaginal ecosystem, *Lactobacillus* must remain a minimum of 73-75% to be able to inhibit the growth of other pathogens [3]. The analysis result of the effects of the extracts on the decreasing percentage of *Lactobacillus* can be seen in Tables 4-5.

**Table 2: Phytochemical screening of extract and simplisia**

Compounds	Extract	Simplisia
Alkaloids	-	-
Flavonoids	+	+
Tannins	-	-
Polyphenolics	+	+
Saponins	+	+
Monoterpenoids sesquiterpenoids	-	-
Triterpenoids	-	-
Steroids	+	+
Quinones	+	+

(+)=Present; (-): Absence

**Table 3: Characteristics of tested *Lactobacillus***

Criteria	<i>L. acidophilus</i>	<i>L. bifidus</i>
Morphology	Opaque, convex	Large round
Cell shape	Rod	Rod
Gram	+	+
Spore	-	-
Motility	-	-
Catalase	-	-

**Table 4: The number of colonies of *Lactobacillus* per 10  $\mu$ l**

Treatment	<i>L. acidophilus</i>	<i>L. bifidus</i>
0	$51 \times 10^4$	$14.4 \times 10^4$
1	$40 \times 10^4$	$11 \times 10^4$
2	$27.9 \times 10^4$	$80 \times 10^4$
3	$17.3 \times 10^4$	$47 \times 10^4$

Treatment consists of *Lactobacillus* suspension with a certain extract concentration=(0) without extract, (1) 2% w/v, (2) 4% w/v and (3) 6% w/v. *L. acidophilus*: *Lactobacillus acidophilus*, *L. bifidus*: *Lactobacillus bifidus*

**Table 5: The effects of extracts on the decreasing percentage of *Lactobacillus* colonies number**

Treatment	Decreasing (%)		Survival (%)	
	LA	LB	LA	LB
0	0.00	0.00	100	100
1	21.57	23.61	78.43	76.39
2	45.29	44.44	54.71	55.56
3	66.08	67.36	33.92	32.64

LA=*L. acidophilus*; LB=*L. bifidus*

The data indicated that at the maximum concentration of 2% w/v, ethanolic extract of red *P. betle* leaf can be used as an active ingredient in the vaginal antiseptic preparation which is safe for normal flora.

## CONCLUSION

It can be concluded that the red *P. betle* leaf ethanolic extract is a potential antibacterial and safe for normal flora of the vagina.

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