Antibacterial activity study of *Attacus atlas* cocoon against *Staphylococcus aureus* and *Escherichia coli* with diffusion and dilution method

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Abstract. The aim of this study was to evaluate the antibacterial activity from *Attacus atlas* cocoon extract against Gram-negative bacteria (*Escherichia coli*) and Gram-positive bacteria (*Staphylococcus aureus*) with 3 different solvents polar, semi-polar and non-polar which was ethanol, ethyl acetate and chloroform, also to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the extract. Cocoon was extracted with maceration method using 3 solvents with ratio of sample and solvent 1:10. Antibacterial activity of the Extracts obtained was evaluated with Agar disk diffusion method. The best result was then continued to determine the MIC and MBC of the extract using broth macro-dilution method. The results show that each of the extracts have antibacterial activity with broad spectrum against two different type of bacteria at concentration of 1 g/mL with different clear zone between these extracts. Clear zone from the biggest to the smallest against *Escherichia coli* was ethyl acetate (10.5 mm), chloroform (9 mm) and ethanol (8 mm). While against *Staphylococcus aureus*, was obtained by chloroform (12.5 mm), ethyl acetate (10.5 mm) and ethanol (7 mm). The MIC value of extracts can not be determine. The smallest MBC value against both bacteria was obtained by ethyl acetate with concentration of 3.125% b/v as a bactericidal.

1. Introduction
Infectious diseases are one of the leading causes of death worldwide. During the past few decades, new infectious diseases have appeared and old ones previously thought to be controlled have reemerged [1] and thus, despite of many significant developments in the antibacterial therapy, many problems remain to be solved for most of the antibacterial drugs available [2]. Hence, discovery of novel antibacterial agents with better pharmacological profile is still highly desirable [3]. Antibacterial substance can be from chemical synthesis or substance from plants or animals [4].

Indonesia has a wide range of natural wealth of flora and fauna. Indonesia’s natural wealth, especially in fauna, many are not getting the interest from researchers. So far, the use of fauna for traditional medicine is very minimum, compare to flora. One of the natural ingredients that can be used for traditional medicine is *Attacus atlas* cocoon, also known as wild silkworms or *ulat keket* in Indonesia.

*Attacus atlas* common name is Atlas Moth, which is one of the saturniidae family usually can be found in citrus, cinnamon, guava trees [5]. *Attacus atlas* cocoon is widely used to produce silk, but not every cocoon can be further processed to produce silk, i.e unqualified cocoon. Usually, these cocoons will be removed. Silk worm cocoon also has benefits for health. But, it is still empirical. Previous
research was conducted against *Antheraeamylitta* cocoon, which is still in one family with *Attacus atlas*, is known to have antibacterial activity [6].

*Staphylococcus aureus* is a facultative anaerobic gram positive coccal bacteria. It is found on the skin as a part of normal skin flora and nasal passages. It can cause a range of infections include minor skin infections such as pimples, impetigo, boils, cellulitis, folliculitis, scaled skin syndrome and abscesses and life threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteremia and sepsis also can cause staphylococcal scaled skin syndrome, a severe disease, in infants [7]. *Escherichia coli*, a gram negative bacterium, can cause food poisoning. The diseases caused by *E. coli* include gastroenteritis, urinary tract infections, neonatal meningitis, septicemias and pneumonia [7].

Other research [8], concluded that in food which was wrapped with cocoon sheets could inhibit microbes grow. But, the research could not prove the antibacterial activity in *Attacus atlas* cocoon scientifically. The current study investigated the antimicrobial activity of *Attacus atlas* cocoon extracts, MIC and MBC concentration of the extracts.

2. Experimental

2.1 Materials

*Attacus atlas* cocoons were obtained from PT. Yarsilk, Yogyakarta, Indonesia. Cultures of *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were obtained from Microbiology lab, Faculty of Medicine, Universitas Sebelas Maret. Cultures were maintained in nutrient agar in refrigerator at 4°C. Ethanol 75%, Chloroform and Ethyl Acetate as solvents. Mueller Hinton Agar, Nutrient Agar and Nutrient Broth as medium for Antibacterial assay.

2.2 Cocoon extraction

Cocoon *Attacus atlas* included in this study were collected from local company PT. Yarsilk, Yogyakarta, Indonesia. The dried material was grounded into powder, 150 g of the grounded cocoon was macerated 24h in 1500 mL ethanol, ethyl acetate and chloroform to obtain 3 extracts and remacerated twice, then filtered with filter paper. The filtrates were evaporated under rotary vacuum evaporator. The extract yields were weighted, stored in tube in fridge at 4°C and their yield percentages were calculated using the following formula:

\[
\text{Extract yield (\%) = } \frac{\text{weight of extracted sample residues (g)}}{\text{weight of raw sample (g)}} \times 100
\]

2.3 Bacterial strains

The antibacterial potency of each plant extracts were evaluated using 2 bacterial strains. 1 strain of Gram positive (*Staphylococcus aureus*) and 1 strain of Gram negative (*Escherichia coli*). The bacterial strains were obtained from the culture collection of Sebelas Maret University, Indonesia.

2.4 Inoculums preparation

Each bacterial strain was subcultured overnight at 35°C in Nutrient agar. The bacterial growth was harvested, diluted in sterile saline water and adjusted the suspension turbidity with 0.5 McFarland Standard of 10^8 CFU/mL.

2.5 Antibacterial activity of cocoon extract

The disk diffusion method is used to evaluate antimicrobial activity of each extracts. The extract residues (1 g) were dissolved in 1 mL DMSO. MHA medium was poured into sterile petri dishes to create 4 mm depth, sterilized and swabbed with the bacterial suspension of 10^8 CFU/mL sterile filter paper discs loaded with extracts of 25 µL and placed on top of MHA plates. Filter paper discs loaded with 10 µg Gentamycin was used as positive control. The plates were incubated at 35 C for 18 h. the presence of inhibition zones were measured, recorded and considered as indication for antibacterial activity.
2.6 Determination of the minimum inhibitory concentration (MIC) of the extracts
MIC is defined as the lowest concentration the antimicrobial agent that inhibits the microbial growth after 18 h of incubation. The extract which exhibiting an antibacterial activity was continued to determine their MIC using macro-broth dilution method as recommended by CLSI guidelines. Extract diluted 1/10 in sterile NB and transferred 2 mL in a tube which had already contained 1 mL Nutrient broth and then two-fold dilution by transferring 1 mL from the first tube to the 5th tube which each had already contained 1 mL NB. Inoculum of bacterial suspension of 0.5 McFarland diluted 1/150 in Nutrient broth and transferred 1 mL in each tube, then incubated 35 C for 18 h. 3 additional tubes as Antibiotic control (1 mL medium and 1 mL antibiotic), Growth control (1 mL medium and 1 mL inoculum) and Medium control (2 mL medium).

2.7 Determination of the Minimum Bactericidal Concentration (MBC) of the extracts
Streaks were taken from the two lowest concentrations of the tubes exhibiting invisible growth (clear tube) and subcultured onto sterile MHA plates. The plates were incubated at 35C for 18 h, then examined for bacterial growth. MBC was taken as the concentration of plant extract that did not exhibit any bacterial growth on the freshly inoculated agar plates.

3. Results and Discussion
3.1 Cocoon extraction yield
The extract of 150 g dried material with ethanol yielded extract residues of 4% w/v, with ethyl acetate of 4.67% w/v and with chloroform of 5.33% w/v. the highest yield of cocoon extract was obtained with chloroform followed by ethyl acetate and ethanol.

3.2 Antibacterial activity of cocoon extract
*Attacus atlas* cocoon were investigated to evaluate its antibacterial activity against Gram positive bacteria (*S. aureus*) and Gram negative bacteria (*E. coli*) using disc diffusion method. Evaluation of antibacterial activity of these cocoon extracts was recorded in Figure 1. and Figure 2. The results revealed that all extracts were potentially effective in suppressing microbial growth of *S. aureus* and *E. coli* with different potency. Ethyl acetate extract was the most effective extract against *E. coli* and chloroform was the most effective extract against *S. aureus*. In positive control the inhibition zone was 28 mm and there was no inhibition zone in negative control.

![Figure 1. Antimicrobial screening test of cocoon extracts against *S. aureus* and *E. coli*.](path_to_image)
3.3 Minimum inhibitory concentration (MIC’s) of the effective cocoon extract
The MIC and MBC of the extracts were employed to evaluate their bacteriostatic and bactericidal properties. The inhibitory effect of extracts can not be evaluated because of biased results. The tubes were cloudy even before incubation because of the extracts color, so we can not determine the MIC and subcultured all of the concentration onto fresh MHA plate to determine the MBC.

3.4 Minimum bactericidal concentration (MBC’s) of the effective cocoon extracts
The MBC was confirmed by absence of bacterial growth of the tested strains streaked form inhibition zone corresponding to their lowest MIC’s, but we streaked all concentration because the MIC could not be determine. The MBC results were recorded in Table 1. and Figure 3-6. Ethyl acetate and chloroform extracts showed potentially bactericidal activity against the tested pathogenic bacteria (S. aureus and E. coli). Etyhl acetate had MBC 3.125% w/v for both bacteria while chloroform had MBC 6.25% w/v against S. aureus and 12.5% w/v against E. coli.

Table 1. Minimum Bactericidal Concentration of each extracts.

| Extracts    | Concentration (g/100 mL) | Bacterial growth | E.coli | S. aureus |
|-------------|--------------------------|------------------|--------|-----------|
| Chloroform  | 50                       | -                | -      | -         |
|             | 25                       | -                | -      | -         |
|             | 12.5                     | +                |        | +         |
|             | 6.25                     | +                |        | +         |
|             | 3.125                    | +                |        | +         |
| Ethyl Acetate| 50                       | -                | -      | -         |
|             | 25                       | -                | -      | -         |
|             | 12.5                     | -                | -      | -         |
|             | 6.25                     | -                | -      | -         |
|             | 3.125                    | -                | -      | -         |
| Ethanol     | 50                       | +                | +      | +         |
|             | 25                       | +                | +      | +         |
|             | 12.5                     | +                | +      | +         |
|             | 6.25                     | +                | +      | +         |
|             | 3.125                    | +                | +      | +         |

Figure 2. Growth inhibition of S. aureus (a) and E. coli (b) caused by Attacus atlas cocoon
Figure 3. MBC of ethyl acetate extracts against *S. aureus* (a) and *E. coli* (b)

Figure 4. MBC of chloroform extracts against *S. aureus* (a) and *E. coli* (b)

Figure 5. MBC of ethanol extracts against *S. aureus* (a) and *E. coli* (b)
Figure 6. Antibiotic control (A.C); Growth control (G.C) and Medium control (M.C)

4. Conclusion

*Attacus atlas* cocoon have antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* with agar disk diffusion method. MBC of the extracts against *S.aureus* from lowest to highest was ethyl acetate (3.125%), chloroform (6.25%) and against *E.coli* (3.125%), chloroform (12.5%), respectively. Ethyl acetate and chloroform was bactericidal, while ethanol bacteriostatic.

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