INTRODUCTION

Bacterial infections remain as the main leading cause of death, particularly in developing countries [1]. Infection treatment with antibacterial has reduced the morbidity and improved patient’s survival with bacterial infections. However, in many cases, the increasing prevalence of strains from common pathogenic bacteria resistance to widely available and affordable antimicrobials is dangerously eroding their effectiveness [2]. Therapeutic options for this case are extremely limited; therefore, it is needed to develop the new antibacterial agent. Antibacterial screening from the plant is an alternative to start the invention of new antibacterial.

**Anredera cordifolia** (Ten.) v. Steenis leaves have been proven to own pharmacological activity such as gastro protector, antidiabetic, anti-obesity, antihyperlpidemic, vasodilator, and wound healer [3-8]. Ethanolic extract of **A. cordifolia** leaves was actively proven to inhibit the growth of some Gram-positive and Gram-negative bacteria such as Bacillus subtilis, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, and methicillin sensitive Staphylococcus aureus (MSSA) [9]. Aqueous extract of **A. cordifolia** leaves showed inhibition to ward B. subtilis, E. coli, S. aureus, and *P. aeruginosa* growth [10].

Antibacterial activity from **A. cordifolia** leaves extract, and its fractions have not been reported by the previous researcher. Therefore, in this study, antibacterial activities of **A. cordifolia** extract and its fractions in different solvents were determined toward some pathogen bacteria. This study could show active compounds of **A. cordifolia** leaves as an antibacterial agent.

MATERIALS AND METHODS

**Materials**

**Anredera cordifolia** dried leaves were collected from Lembang - West Java, Indonesia. Plant identification was confirmed by Herbarium Bandungense, Bandung Institute of Technology. Standard ursoinic acid (UA), oleanonic acid (OA), apigetrin, rutin, and antibiotic amoxicillin were obtained from Sigma-Aldrich (USA). Mueller Hinton Broth and Mueller-Hinton Agar were obtained from Oxoid.

The tested microorganisms were obtained from School of Pharmacy, Bandung Institute of Technology, Indonesia, which included five of Gram-positive bacteria such as *S. aureus* (ATCC 6538), *MSSA*, methicillin resistant *S. aureus* (MRSA), *B. subtilis* (ATCC 6633), *B. cereus* (ATCC 11778), *P. aeruginosa* (ATCC 9027), *E. coli* H7 (0156), and ESBL E. coli. Antimicrobial activity was evaluated using the disc diffusion method.

**Extraction**

First method of extraction: Crude drug of **A. cordifolia** leaves was extracted by reflux method using ethanol 96% as a solvent, then evaporated to obtain a thick extract (EE1). Ethanolic extract was added with boiling water and separated in separatory funnel by liquid-liquid extraction method using n-hexane and ethyl acetate solvents. This process produced three fractions at the end n-hexane (HF), ethyl acetate (EAF), and water fraction (WF).

The second method of extraction was using gradual extraction. Crude drug was extracted by reflux using n-hexane, ethyl acetate, and ethanol 96% solvents, respectively, so there were three extracts: n-hexane extract (HE), ethyl acetate extract (EAE), and ethanol extract (EE2). EE1, HF, EAF, HE, EAE, and EE2 were sample in this study.

**Methods**

Crude drug was extracted using two methods. First method was extraction by reflux using ethanol 96% and then fractionated by liquid-liquid extraction using n-hexane and ethyl acetate. Second method was gradually extraction by reflux using n-hexane, ethyl acetate and ethanol 96%, respectively. Phytochemical screening was applied to all extracts and fractions, followed by thin-layer chromatography using ursoinic acid, oleanonic acid (OA), apigetrin, and rutin as reference substances. A two-fold serial microdilution method was used to determine minimum inhibitory concentration (MIC) against *Staphylococcus aureus* (ATCC 6538), methicillin-susceptible *S. aureus*, methicillin-resistant *S. aureus* (MRSA), *Bacillus subtilis* (ATCC 6633), *Bacillus cereus* (ATCC 11778), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8939), *E. coli* H7 (0156), and ESBL E. coli.

**Results**

The ethanolic extract, n-hexane and ethyl acetate fractions of **A. cordifolia** from the first method had antibacterial activity against *S. aureus*, MRSA, *B. subtilis*, and *B. cereus* (MIC 256-512 μg/ml). However, n-hexane and ethyl acetate extract from the second method had broad spectrum of antibacterial activity, which could inhibit the growth of *S. aureus*, MRSA, *B. subtilis*, *P. aeruginosa*, and *E. coli* (MIC 256-512 μg/ml). Extracts and fractions showed bacteriostatic and bactericidal activities, but n-hexane extract has most bactericidal activity. Furthermore, steroid/triterpenoid, ursoinic, and OA were found in this extract.

**Conclusion**

The n-hexane extract from the second method showed the highest antibacterial activity.
Phytochemical screening

The following tests performed on extracts and fractions were to detect the presence of flavonoid, tannin, quinone, saponin, alkaloid, and steroid/triterpenoid, as detailed previously [10].

Detection of UA and OA

Extracts, fractions, UA, and OA in methanol were spotted on a thin-layer chromatography (TLC) plate. For pre-chromatographic derivatization, the plate was developed in a horizontal chamber with 1% iodine in chloroform at 1.2 cm distance. The plate was placed in darkness for 10 minutes and then dried in stream of warm air to remove the excess of iodine [11]. Then, the plate was developed with toluene ethyl acetate formic acid (35:15:1 v/v/v) as the mobile phase. After drying, the plate was sprayed using H$_2$SO$_4$ reagent and then heated. The visualized spots were documented under visible light.

Detection of apigetrin and rutin

Extracts, fractions, apigetrin, and rutin in methanol were spotted on a TLC plate. The plate was developed with ethyl acetate methanol water (7:1:1 v/v/v) as the mobile phase. After that, the dried plate was sprayed using citroborate reagent and then heated. Spots were documented in UV l 366 nm.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Extracts and fractions were dissolved in 10% dimethyl sulfoxide to obtain stock solutions. Standard solution (amoxicillin) was dissolved in sterile water. A two-fold serial microdilution method was used to determine MIC of samples. The lowest concentration that showed no visible growth was regarded as the MIC. Cells from the wells will show no growth of sub-cultured on MHA agar plate to determine the inhibition is reversible or permanent. The MBC was determined as the lowest concentration at which no growth occurred on the plate. The procedure was performed according to the Clinical and Laboratory Standards Institute guidelines [12].

Statistical analysis

MIC data of extracts, fractions, and amoxicillin against the same bacteria were analysis using Kruskal–Wallis test and continued with Mann–Whitney for pairwise comparison. $P < 0.05$ was considered significant.

RESULTS

Phytochemical screening

The result of phytochemical screening of extracts and fractions could be seen in Table 1.

Detection of UA and OA

TLC profile of extracts and fractions toward the existence of UA (Rf 0.61) and OA (Rf 0.72) showed that EE1, HF, and HE contained both of those acids (Fig. 1).

Detection of apigetrin and rutin

TLC resulted that qualitatively analyze the existence of apigetrin (Rf 0.50) and rutin (Rf 0.25) could be seen in Fig. 2. Based on the

| Table 1: Phytochemical screening of Anredera cordifolia extracts and fractions |
|-----------------------------------------------|
| Sample                               | Flavonoid | Tannin | Quinone | Saponin | Alkaloid | Steroid/triterpenoid |
|-----------------------------------------------|
| First extraction method                      |           |       |          |         |          |                     |
| Ethanolic extract (EE1)                     | +         | -     | -        | +       | +        | +                   |
| N-hexane fraction (HF)                      | -         | -     | -        | -       | +        | +                   |
| Ethyl acetate fraction (EAF)                | +         | -     | -        | -       | +        | +                   |
| Water fraction (WF)                         | +         | -     | -        | +       | +        | +                   |
| Second extraction method                     |           |       |          |         |          |                     |
| N-hexane extract (HE)                       | -         | -     | -        | -       | +        | +                   |
| Ethyl acetate extract (EAE)                 | +         | -     | -        | -       | +        | +                   |
| Ethanolic extract (EE2)                     | +         | -     | -        | +       | +        | +                   |
| +: Detected, -: Not detected                |           |       |          |         |          |                     |
observation, EE1, EAF, WF, and EAE contained apigetrin and rutin. Meanwhile, EE2 only contained apigetrin.

**Determination of MIC and MBC**

Antibacterial activities of the sample were presented in Table 2. Extracts and fractions had antibacterial potential if their MIC value was <1024 μg/ml.

**DISCUSSION**

Biologically active compounds commonly occur in low concentration in plants. An extraction technique is able to obtain an extract with high yield and with minimum changes in functional properties of the required extract. Several studies have reported variations in the biological activities of extracts prepared using different extraction techniques [13-15]. Antibacterial activity test from 1st extraction method (ethanol extract and its fraction) showed that they could only inhibit Gram-positive bacteria. HF could inhibit three microorganisms, *S. aureus* (MIC 256 μg/ml), *B. subtilis*, and *B. cereus* (MIC 512 μg/ml). Meanwhile, EE1 could only inhibit the growth of two microorganisms, *S. aureus* and MRSA (MIC 512 μg/ml). EE2 could inhibit three Gram-positive bacteria, *S. aureus* (MIC 256 μg/ml), MRSA, and *B. subtilis* (MIC 512 μg/ml) and two Gram-negative bacteria, *P. aeruginosa* (MIC 256 μg/ml) and *E. coli* (MIC 512 μg/ml). Only HE could inhibit Gram-negative bacteria, and its activity was significantly different compared to other extracts and fractions of *A. cordifolia* leaves toward MRSA. HE had the highest antibacterial activity among all extracts and fractions of *A. cordifolia* (p<0.05). Amoxicillin is shown to be effective against a wide range of infections caused by wide range of Gram-positive and Gram-negative bacteria [16]. Amoxicillin is still being drug of choice within the class because it has better pharmacokinetic profile than other β-lactam antibiotics for the treatment of infections due to susceptible organisms [17].

Based on MBC to MIC ratio of the sample which had antibacterial potential (MIC <1024 μg/ml), some sample were known to have bacteriostatic and bactericidal activity. Bacteriostatic activity has been defined as a ratio of MBC to MIC >4 and bactericidal activity had ratio MBC to MIC >4 [18]. In this study, EE1 and EAE had bacteriostatic activity, while HF and HE had bactericidal activity except its activity toward *B. cereus* and *P. aeruginosa*. EE1 only had bactericidal activity toward MRSA. HE had the highest antibacterial activity among all extracts and fractions of *A. cordifolia* leaves.

The chemical constituents in plants or extracts are known to be biologically active ingredients. Some chemical constituents are considered as secondary metabolites components. They are directly responsible for different activity. Saponin was found in AF and EE1 that was obtained from first and second extraction methods. Flavonoid and alkaloid were found in most samples, except in HF dan HE. All samples contained steroid/triterpenoid. Some research showed that flavonoid, saponin, alkaloid, and steroid/triterpenoid had antibacterial potential against the same bacteria (p<0.05); **Significant difference compared to extracts and fractions of *A. cordifolia* which had antibacterial potential against the same bacteria (p<0.05). *A. cordifolia*: Anredera cordifolia, MSSA: Methicillin sensitive Staphylococcus aureus, MRSA: Methicillin resistant Staphylococcus aureus, ESBL: Extended-spectrum beta-lactamases, E. coli: Escherichia coli, P. aeruginosa: Pseudomonas aeruginosa, B. cereus: Bacillus cereus, B. subtilis: Bacillus subtilis, S. aureus: Staphylococcus aureus.

**Table 2: Antibacterial activities of *A. cordifolia* extracts and fractions**

| Bacterial species (μg/ml) | 1st extraction method | 2nd extraction method | Amoxicillin |
|--------------------------|-----------------------|-----------------------|-------------|
|                          | EE1                   | HF                    | EAF         | WF          | HE         | EAE       | EE2         |             |
| *S. aureus*              |                       |                       |             |             |            |           |             |             |
| MIC                      | 512*                  | 256*                  | 512*        | 1024        | 256**      | 512*      | 4096        | 0.5**       |
| MBC                      | >4096                 | >4096                 | >4096       | >4096       | 256**      | >4096     | >4096       | 0.5         |
| MSSA                     |                       |                       |             |             |            |           |             |             |
| MIC                      | 2048                  | 2048                  | 4096        | >4096       | 1024       | 4096      | 4096        | 8           |
| MBC                      | >4096                 | 4096                  | >4096       | >4096       | 2048       | >4096     | >4096       | 8           |
| MRSA                     |                       |                       |             |             |            |           |             |             |
| MIC                      | 512*                  | 1024                  | 1024        | >4096       | 512*       | 512*      | 4096        | 32**        |
| MBC                      | >4096                 | 2048                  | 2048        | >4096       | 1024       | 1024      | >4096       | 32          |
| *B. subtilis*            |                       |                       |             |             |            |           |             |             |
| MIC                      | 2048                  | 512*                  | 1024        | >4096       | 512*       | 512*      | 4096        | 4**         |
| MBC                      | 4096                  | 4096                  | 4096        | >4096       | 1024       | >4096     | >4096       | 4           |
| *B. cereus*              |                       |                       |             |             |            |           |             |             |
| MIC                      | >4096                 | 512*                  | >4096       | >4096       | 1024       | 2048      | 4096        | 0.5**       |
| MBC                      | >4096                 | 1024                  | >4096       | >4096       | 2048       | >4096     | >4096       | 2           |
| *P. aeruginosa*          |                       |                       |             |             |            |           |             |             |
| MIC                      | 1024                  | >4096                 | >4096       | >4096       | 256**      | 1024      | 4096        | 16**        |
| MBC                      | >4096                 | >4096                 | >4096       | >4096       | 512*       | 4096      | >4096       | 32          |
| *E. coli*                |                       |                       |             |             |            |           |             |             |
| MIC                      | >4096                 | >4096                 | >4096       | >4096       | >4096      | >4096     | >4096       | >4096       |
| MBC                      | >4096                 | >4096                 | >4096       | >4096       | >4096      | >4096     | >4096       | >4096       |
| *E. coli H7* (O156)      |                       |                       |             |             |            |           |             |             |
| MIC                      | >4096                 | 2048                  | >4096       | >4096       | >4096      | >4096     | >4096       | >4096       |
| MBC                      | >4096                 | >4096                 | >4096       | >4096       | >4096      | >4096     | >4096       | >4096       |
| ESBL *E. coli*           |                       |                       |             |             |            |           |             |             |
| MIC                      | 4096                  | 1024                  | 2048        | >4096       | 2048       | 4096      | >4096       | >256        |
| MBC                      | 4096                  | 2048                  | 4096        | >4096       | 4096       | >4096     | >4096       | >256        |

Experiment was conducted triplicate. EE1: Ethanolic extract of *A. cordifolia* leaves from 1st extraction method, HF: n-hexane fraction, EAF: Ethyl acetate extract, EE1: Ethanolic extract from 2nd extraction method, MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration, *p*-value = 0.05. **Significant difference compared to other extracts and fractions of *A. cordifolia* which had antibacterial potential against the same bacteria (p<0.05); *p*<0.05. *A. cordifolia*: Anredera cordifolia, MSSA: Methicillin sensitive Staphylococcus aureus, MRSA: Methicillin resistant Staphylococcus aureus, ESBL: Extended-spectrum beta-lactamases, E. coli: Escherichia coli, P. aeruginosa: Pseudomonas aeruginosa, B. cereus: Bacillus cereus, B. subtilis: Bacillus subtilis, S. aureus: Staphylococcus aureus.
Screening of Uruguayan medicinal plants for antibacterial activity against S. aureus [27]. Rutin could inhibit growth of S. aureus, B. subtilis, and MRSA [28-29].

CONCLUSION

The HE had the highest antibacterial activity. Phytochemical screening of the extract showed the presence of steroid/triterpenoid. Ursolic acid and OA that have been known to have antibacterial activity from previous research were found in this extract. In addition, HE had more bactericidal activity compared to others samples. This study could be used to determine the active compounds of A. cordifolia as antibacterial agent for the future research.

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