In Vitro Anti-microbial Activity of Hydroethanolic Extracts of *Ruellia tuberosa* L.: Eco-friendly Based-product Against Selected Pathogenic Bacteria

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**Abstract.** Search for new sources of antibacterial agents that are more environmentally-friendly to the environment and human health has emerged since synthetic antimicrobial drugs led to antibiotic resistance. South East Asia region is one of the world’s most biodiverse regions and provide reservoir of new and potentially useful molecules from plants. *Ruellia tuberosa* L., an indigenous species from South East Asia, is a plant that is grown in almost in every area in Indonesia. In the current study, anti-microbial activity of hydroethanolic extracts of *R. tuberosa* L. was screened against *S. aureus* and *E. coli*, using well diffusion method. Results showed that hydroethanolic extracts of *R. tuberosa* L. at concentrations of 5; 10; 20; 50; 75; and 100% (v/v) resulted in inhibition zones of 7; 7; 9.80; 11.50; 14.15; and 15.25 mm, respectively, against *S. aureus*, Gram-positive bacteria. The inhibition zones against *E. coli*, Gram-negative bacteria, resulted in 7; 7; 7; 10.75; 11.00; and 15.00 mm, for concentrations of 5; 10; 20; 50; 75; and 100% (v/v), respectively. The commercial antibiotics, chloramphenicol and ampicillin were used for the control, and resulted in inhibition zones of 30.20 and 25.70 mm for *S. aureus*; 29.85 and 25.40 mm for *E. coli*. These results indicated that both extracts and antibiotics were more potent to inhibit *S. aureus* than *E. coli*. This study contributes to the development of environmentally friendly based-product for diseases caused by bacteria.

**Keywords:** *R. tuberosa* L, anti-bacterial agent, *E. coli*, *S. aureus*, well diffusion

1. **Introduction**

Nature has provided a complete storehouse of remedy to cure all ailments of mankind and its related disease. In the past, almost all medicines used extracts from plants. In recent times, focus on plant research has increased all over the world an immense potential of medicinal plants used in various traditional system has been highlighted. Traditionally used medicinal plants produce a variety of compounds of known therapeutic properties. Plants have great importance due to their therapeutic value and they are the major sources of medicines which play an important role in human history [1]. Plants synthesize primary metabolites (protein, fats, nucleic acid and carbohydrates) by simple substances such as water, carbon dioxide, nitrogen and a number of inorganic salts in small amounts. These primary metabolites are transformed into secondary metabolites (alkaloid, steroid, terpenoid, saponin, flavonoids, tannin) that are used as drugs [2]. Recently, considerable attention been paid to utilize eco-friendly and bio-friendly plant based products for the prevention and cure of different diseases. Considering the adverse effects of synthetic drug and the newly evolved drug-resistant
version, people are in continual search for alternative remedies for their primary health care that proved to be safe and efficiency. Additionally, in vitro screening method have been applied to plant extract with useful properties for science investigation and validation as potential source of alternative medicine that could be of use to prevent and cure human diseases.

Phytochemical substances have been widely studied for their beneficial properties. LC-MS technique has been extensively used for the determination of various compounds. The fact that it provides extra sensitivity, specificity and good separation in complex samples, makes LC-MS/MS the ultimate tool in the determination of many types of chemical compounds, such as secondary metabolites. It becomes evident that the potential for their use as chelators, biocides, biofuels, biofertilizers, pharmaceuticals, food and feed.

Ruellia tuberosa L. (common name pletekan) belongs to family Acanthaceae is a Minnie root medicinal plant. It is a tropical plant that is widely distributed in South East Asia. Medicinal plants are rich with bio-resources of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. In folk medicine, it has been used as anti-inflammatory, antidiabetic, antioxidant, antibacterial and anticancer. Several groups of secondary metabolites such as alkaloid, terpenoid, tannin, flavonoids and steroid may be responsible for various potential medicinal properties attributed to the plant. Considering all these facts, the present study is carried out qualitative phytochemical analysis, LC-MS analysis and in vitro antibacterial activities present in root of R. tuberosa L. extracted with hydroethanolic solvents against selected pathogenic bacteria, S. aureus and E. coli. Furthermore, results from the current study provide the future for the sustainability of the R. tuberosa L as one of the indigenous plants in South East Asia region, in particular in Indonesia. Therefore, this contributes to environmental issues related to the plants conservatives.

2. Material and Methods

2.1. Chemicals and Instrumentation
All chemicals were purchased from Merck. The chemicals used were of analytical grade. ethanol (96%), chloroform, acetic acid anhydride, H₂SO₄, HCl, magnesium ribbon, FeCl₃, 1% gelatin NaCl, KMnO₄, nutrient agar. The growth media for pathogenic bacteria was nutrient broth. The determination of flavonoids was conducted using LC-MS (ACCELLA type 1250 Thermo Scientific). The mobile phase of LC-MS was formic acid in aqua-bidest (98%) and formic acid in acetic acid (99%), as well as methanol LC-MS grade.

2.2. Collection and Identification of Plants Materials
The plant specimens (roots) of R. tuberosa L. were collected from Materia Medica, Batu, East Java. The plant was identified and authenticated by a plant taxonomist of the herbarium unit, UPT Materia Medica, Batu. The fresh root parts of this species were washed under running tap water, shade dried at room temperature and crushed into powder.

2.3. Extract Preparation
Preparation extracts of the root R. tuberosa L. has been conducted by successive maceration. The hydroethanolic plant extracts were prepared by soaking 900 g of R. tuberosa L. powder (90 mesh) in 3 L of aqueous ethanol 96% (1:1) for 3 x 24 h. The extracts were then filtered using Whatman filter paper. The solvent was completely removed by rotary evaporator at 80 °C, 120 rpm to obtain dark gummy extracts (yield 31.6%). The extracts were collected in dark glass bottles, and stored at 4 °C for further use.
2.4. Phytochemical Screening
Phytochemical screening of root extracts of *R. tuberosa* L. was subjected to phytochemical screening to determine the presence of active secondary metabolites using standard procedures, for detection of terpenoids, steroids, flavonoids, phenolics, tannins, saponins, and ascorbic acids [7].

2.5. LC-MS of Hydroethanolic Extracts of *R. tuberosa* L.
The test sample was prepared by weighing the extract then dissolved with 1 mL of methanol. Then, sonication was carried out for 10 minutes, and the solution was filtered with 0.2-micron filter. The sample solution was put in a vial bottle for analysis with LC-MS / MS equipment. Columns used was Hypersil Gold specifications (50 mm x 2.1 mm x 1.9 µm). UHPLC ACCELLA brand type 1250 made by Thermo Scientific which consists of vacuum degasser, quartener pump, thermostatic auto sampler controlled by personal computer through X-calibur program 2.1. The mobile phase A consisted of 0.1% formic acid in aqua-bidest, phase B consisted of 0.1% formic acid in Acetonitrile. A linear gradient with a flow rate of 300 µl/min was applied. The column was controlled at 30 °C, and the auto sampler compartment was set to 16 °C.

Electrospray ionization (ESI) source was operated in negative ion detection mode. The conditions for negative ion detection mode (controlled by Software TSQ Tune) were as follow: spray voltage of 3 kV; evaporation temperature of 300 °C; capillary temperature of 300 °C; nitrogen as gas sheath with pressure at 40 psi; and auxiliary gas pressure at 10 psi with argon gas. LC-MS spectra were obtain using UHPLC ACCELLA type 1250 with mass spectrophotometer TSQ QUANTUM ACCESS MAX.

2.6. Antibacterial Activity
Antibacterial activity root extract of *R. tuberosa* L. was determined by well diffusion method [8]. The bacterial cultures were maintained in nutrient broth. Bacterial culture was spread over nutrient agar plates using sterile cotton buds. The well of 7 mm size was punctured on plates with help of gel puncture (sterilized) and various concentrations (100%, 75%, 50%, 20%, and 10%) of the samples were added into each well. Chloramphenicol and ampicillin was used as a positive control. The bacterial cultured plates were incubated for overnight at 37 °C. After incubation period, the zone of inhibitions was measured in mm.

3. Result and Discussion
3.1. Qualitative Analysis of Phytochemical Compounds in the Extracts
The phytochemical screening test conducted to investigate the presence of bioactives or secondary metabolite compounds such as terpenoids, steroids, flavonoids, phenolic, tannin, saponins, ascorbic acid. However, only steroids, flavonoids, phenolic and ascorbic acid were detected in hydroethanolic root extracts of *R. tuberosa* L. Table 1 shows the result of qualitative phytochemical analysis of hydroethanolic root extracts of *R. tuberosa* L. These tests were based on the visual observation of colour modification or precipitation formation after the addition of specific reagents.

| Phytochemicals       | Detection |
|----------------------|-----------|
| Terpenoids           | -         |
| Steroids             | +         |
| Flavonoids           | +         |
| Phenolic             | +         |
| Tannin               | -         |
| Saponins             | -         |
| Ascorbic acid        | +         |
These secondary metabolites are reported to have many biological and therapeutic properties [9], thus these species is expected to have many medicinal uses such as antimicrobial, anti-hyperglycemic and anti-inflammation. The extraction yield calculated for hydroethanolic extract plant showed that extracts resulted in relatively high percentage of yield (28.44%). It may be due to high polarity of hydroethanolic solvent which can draw high variety of plant constituents.

3.2. LC-MS Analysis of Hydroethanolic Root Extracts of R. tuberosa L

The LC-MS method was used for the determination of bioactive compound in this extract. The presence of these compounds were confirmed based on the comparison of \( m/z \) values from the LC-MS spectra with the literature. A negative ionization mode was used for the identification of the compound in the extract. Electrospray in negative mode is, by far the most generalized ionization source employed, usually providing the deprotonated molecule \([M-H]\) [10].

Four compounds were identified in LC-MS chromatogram hydroethanolic root extracts of \( R. \) tuberosa \( L. \). LC-MS results indicated that flavonoid compounds, including sorbifolin (1), cirsimaritin (2), cirsimarin (3), and cirsiliol 4'-glucoside (4), as indicated in Figure 1 are contained in the extracts.

![Figure 1. Structure of Compounds 1-4](image)

The LC-MS of compound 1 is present in Figure 2a. The peak of protonated ion \([M+H]\) occurred at \( m/z \) 299, indicating molecular weight of 300, and a fragment ion peak at \( m/z \) 169 (100% abundance) corresponding to loss of one hexose unit. Therefore, the \( m/z \) signal at 299 would be a typical fragment ion detection in the spectrum of sorbifolin. The LC-MS of compound 2 is presented in Figure 2b, the peak of protonated ion \([M+H]\) occurred at \( m/z \) 313, indicating molecular weight of 314, Therefore, the \( m/z \) signal at 313 would be a typical fragment ion detection in the spectrum of cirsimaritin. The LC-MS of compound 3 is presented in Figure 3c, the peak of protonated ion \([M+H]\) occurred at \( m/z \) 475, indicating molecular weight of 476 and a fragment ion peak at \( m/z \) 315 (100% abundance) corresponding to the loss of one hexose unit, suggesting that compound 2 is an O-glycoside. Therefore, structure of 3 was identified as cirsimarin. The LC-MS of spectrum Figure 2d shows a \([M+H]\) ion at \( m/z \) 491, indicating a molecular weight of 492, and a fragment ion peak at \( m/z \) 329 (100% abundance) corresponding to loss of one hexose unit, suggesting that compound 4 is also O-glycoside. Thus, the structure of 4 was identified as cirsiliol 4'-glucoside. Table 2 summarizes the results of LC-MS analysis hydroethanolic root extracts of \( R. \) tuberosa \( L. \).

Identification of main component of the root extract on LC-MS spectra show a compound with \( m/z \) 299, 313, 475, and 491 which is evidenced by the abundance read 100% at the retention time 2.51, 2.6, 2.53 and 2.62 respectively (Figure 2).
Table 2 LC-MS profile of hydroethanolic root extracts of *R. tuberosa* L.

| Analyte                  | t<sub>(min)</sub> | m/z  | Dwell time (s) | Cone Voltage (V) |
|--------------------------|-------------------|------|----------------|------------------|
| Sorbifolin               | 5.0               | 299  | 0.1            | 20               |
| Cirsimaritin             | 5.0               | 313  | 0.1            | 20               |
| Cirsimarin               | 5.0               | 475  | 0.1            | 20               |
| Cirsiliol 4’-glucoside   | 5.0               | 491  | 0.1            | 20               |

Figure 2. LC-MS results of hydroethanolic root extracts of *R. tuberosa* L: (a) sorbifolin; (b) cirsimaritin; (c) cirsimarin; and (d) cirsiliol 4’-glucoside.

Identification of peaks of sorbifolin, cirsimaritin, cirsimarin, and cirsiliol 4’-glucoside and in root extract of *R. tuberosa* L were carried out by comparing mass spectra of the standard data and literature. The results are consistent with research conducted by Chwan-Fwu Lin et al.[11]. It can be concluded that identification of root extract from *R. tuberosa* L compound by LC-MS were successful for identifying qualitative analysis of four major flavonoids (sorbifolin, cirsimaritin, cirsimarin, and cirsiliol 4’-glucoside).
3.3. Antibacterial Activity

The well-diffusion method was used to provide evidence for the antibacterial activity of hydroethanolic root extracts of *R. tuberosa L.* against *E. coli* and *S. aureus.* Figure 3. shows the antibacterial activity hydroethanolic root extracts of *R. tuberosa L.* was indicated by the formation of the clear zone and the clear zone was measured as mm in diameter.

![Figure 3](image_url)

**Figure 3.** Antibacterial activity of hydroethanolic root extracts of *R. tuberosa L.* in various concentrations of: a) hydroethanolic extracts of *R. tuberosa L.* 100 %, 75 %, 50 % against *E. coli*.; b) hydroethanolic extracts of *R. tuberosa L.* 20 %, 10 %, 5 % against *E. coli*.; c) hydroethanolic extracts of *R. tuberosa L.* 100 %, 75 %, 50 % against *S. aureus*; d) hydroethanolic extracts of *R. tuberosa L.* 20 %, 10 %, 5 % against *S. aureus*.; d) antibacterial activity of ampicillin; and e) chloramphenicol, against *E. coli* and *S. aureus*.

The clear zone diameter of inhibition of each extract concentration was measured in % as shown in Table 3. The inhibition zones produced by the antibiotic references, chloramphenicol and ampicillin was larger than those produced by the root extracts. The root extracts at six different concentrations 100, 75, 50, 20, 10, 5 % showed significant zones of inhibition against Gram-positive bacteria, *S. aureus* at 15.25; 14.15; 11.50; 9.80; 7; and 7 mm; and Gram-negative bacteria, *E. coli* at 15.00; 11.00; 10.75; 7; 7 and 7 mm. Overall, Gram-positive strains were more sensitive than Gram-negative bacteria to the extracts.

These results indicate that the extracts from the root of *R. tuberosa L.* was positively inhibited the bacterial growth. The inhibition was observed in accordance with the performance of clear zone around the well. The clear zone was formed by the active compound contain in the well-disc that diffuses into the agar medium containing the bacteria and inhibited their growth. The bioactive compounds inhibits the synthesis of cell wall, nucleic acid, and protein, disturb the metabolism of bacteria or change the cell membrane permeability [12]. In this study, we verify that root of *R. tuberosa L.* contain flavonoid that is effective to inhibit the growth of *S. aureus* and *E. coli*.

The root extracts showed the highest inhibition toward *S. aureus* than *E. coli*. *S. aureus* is Gram-positive bacteria that has simple cell wall structure, single-layered with low lipid content, and enables the bioactive compounds to enter the cells. On the other hand, *E. coli* are Gram-negative bacteria with much more complex cells, three-layer lipoprotein consisting of an outer layer, a middle layer of lipopolysaccharide which acts as a barrier to antibacterial bioactive material, and a coat of peptidoglycan with high lipid content and thus more difficult to be destroyed.
Antibacterial activities of the root extract depend on the nature of phytochemicals present in the extract. Researchers showed that presence of terpenoids, steroids, flavonoids, phenolic, alkaloids, and tannin as active antibacterial agent [11, 12]. Based on phytochemical analysis, the extracts contain terpenoids, steroids, flavonoids, phenolic, and ascorbic acid. Among these phytochemicals, flavonoids from a complex with extracellular and soluble protein and a complex with bacterial cell walls [5, 11]. This proves that the flavones impede the bacterial proliferation by inhibiting cell wall synthesis. Flavanones has been previously reported as antibacterial [5]. All others are associated with antibacterial action through different mechanisms [13]. It is clear that this antibacterial activity may be attributed to the terpenoids, steroids, flavonoids, phenolic, since those active secondary metabolites were detected in the extracts [14].

Table 3. Antibacterial activity of hydroethanolic root extracts of *R. tuberosa* L.

| Extract concentration (%) | Zone diameter of inhibition (mm) | E. coli | S. aureus |
|---------------------------|---------------------------------|---------|-----------|
| (v/v)                     |                                 |         |           |
| 100                       | 15.00                           | 15.25   |           |
| 75                        | 11.00                           | 14.15   |           |
| 50                        | 10.75                           | 11.50   |           |
| 20                        | 7.00                            | 9.80    |           |
| 10                        | 7.00                            | 7.00    |           |
| 5                         | 7.00                            | 7.00    |           |
| Chloramphenicol           | 29.85                           | 30.20   |           |
| Ampicillin                | 25.40                           | 25.70   |           |

4. Conclusion
The current study has demonstrated that hydroethanolic root extracts of *R. tuberosa* L. have antibacterial activity that could inhibit the growth of *S. aureus* and *E. coli*. The plant-based products that active as antibacterial agents may be developed as eco-friendly cure for bacteria-related diseases. Furthermore, the current study has highlighted the future for the conservation of *R. tuberosa* L., as the plant that native species in the Asian continent.

References
[1] J. P. Rosenthal, ‘Plants, People and Culture: the Science of Ethnobotany by M. J. Balick (New York Botanical Garden) and P. A. Cox (Brigham Young University). Scientific American Library, New York, NY. 1996. ix + 228 pp. 22 × 24 cm. $32.95. ISBN 0-7167-5061-9. - Journal of Natural Products (ACS Publications)’. [Online]. Available: https://pubs.acs.org/doi/abs/10.1021/np960626w. [Accessed: 14-Oct-2018].
[2] S.-L. Chen, H. Yu, H.-M. Luo, Q. Wu, C.-F. Li, and A. Steinmetz, ‘Conservation and sustainable use of medicinal plants: problems, progress, and prospects’, *Chin Med*, vol. 11, Jul. 2016.
[3] N. Ferlazzo et al., ‘Natural iron chelators: Protective role in A549 cells of flavonoids-rich extracts of Citrus juices in Fe3+-induced oxidative stress’, *Environmental Toxicology and Pharmacology*, vol. 43, pp. 248–256, Apr. 2016.
[4] F. Haque, S. Banayan, J. Yee, and Y. W. Chiang, ‘Extraction and applications of cyanotoxins and other cyanobacterial secondary metabolites’, *Chemosphere*, vol. 183, pp. 164–175, Sep. 2017.
[5] B. Arirudran, A. Saraswathy, and V. Krishnamurthy, ‘Antimicrobial Activity of Ruellia tuberosa L. (Whole Plant)’, *Pharmacognosy Journal*, vol. 3, no. 23, pp. 91–95, Jul. 2011.
[6] M. N. Samy, S. Sugimoto, K. Matsuani, H. Otsuka, and M. S. Kamel, ‘CHEMICAL CONSTITUENTS AND BIOLOGICAL ACTIVITIES OF GENUS RUELLIA’, *International Journal of Pharmacognosy*, vol. 2, p. 11.
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