Phytochemicals screening and anti-oxidant activity of hydroethanolic extracts of *Ruellia tuberosa* L

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**Abstract.** *Ruellia tuberosa* L, also known as pletekan, pletikan, or ciplukan, is a plant that is found in almost every region in Indonesia. Assessment of the antioxidant activities of crude hydroethanolic extracts of *R. tuberosa* L. root was carried out to investigate their medicinal properties. The dried roots of *R. tuberosa* L were extracted using maceration technique, with ethanol and water, in 1:1 ratio. The phytochemical screening revealed the presence of flavonoids, triterpenoids, steroids, ascorbic acids, and phenolic compounds in those extracts. The chemical compositions of the extracts were analyzed by targeted LC-MS, showed presence of phytosterol compounds, representing about 60% of the extracts. The principal components identified included stigmasterol, campesterol, and β-sitosterol. These were also supported by results from FTIR spectroscopy that indicated the functional groups of the typical phytosterol compounds. *In vitro* antioxidant study using 2, 2-diphenyl-1-picrylhydrazyl scavenging assay showed that the crude hydroethanolic extracts expressed high free radical scavenging activity with the IC$_{50}$ value of 2.48 µg/mL. In a similar test, vitamin C was used for a reference, and has resulted in the IC$_{50}$ value of 3.18 µg/mL. Conclusively, the hydroethanolic roots extracts of *R. tuberosa* L. could be considered a remedy for diseases which are associated with free radicals.

**Keywords:** antioxidant, IC$_{50}$, phytosterols, *R. tuberosa* L, hydroethanolic extracts

1. Introduction

Indonesia has rich diversity of flora that can be used as a source of raw materials for traditional medicines. One of plants that can be utilized and contains bioactive compounds is from the family of Acanthaceae. One member of the Acanthaceae family is genus *Ruellia*. *Ruellia tuberosa* L. is a tropical plant that is widely grown in Asia, including Indonesia, Malaysia, and India [1, 2]. This plant is locally known as pletekan, pletikan, or ciplukan.

Previous phytochemical determination pointed out that ethanolic leaves extract from *R. tuberosa* L contained flavonoids, glycosides, saponins, phenols, and carotenoids [3]. In addition, those extracts also had nutrients such as tocopherol, ascorbic acid and lycopene [4]. Further research from Manikandan mentioned that leaves extract of *pletakan* has activity as anti-diabetes and anti-oxidant [4].

Based on our previous research, root extracts from *pletakan* has anti-diabetic activity by conducting an *in vivo* study [5]. These include reduce blood glucose levels, reduce malondialdehyde (MDA) levels, and repair on the kidney histopathologic profiles [5]. Moreover, *pletakan* root extracts also had anti-diabetic activity from their positive effects on the pancreatic of diabetic rats [6]. Phytochemical investigation of this study revealed that the *pletakan* roots extracted with n-hexane contained triterpenoid compounds [6]. However, no further characterizations for the extracts were conducted. Another study
mentioned that the content of triterpenoids and flavonoids in the leaves of methanolic extracts of pletekan had activity as a drug for diabetic rabbits [7].

In the current work, pletekan roots are extracted using maceration technique, followed by identification and characterization of the resulted extracts. Bioactive compounds such as terpenoids, flavonoids, alkaloids, saponins, and tannins are secondary metabolite compounds that usually dissolve in polar solvents, thus, in this study ethanol and water are used to extract pletekan roots. Characterization of hydroethanolic extracts of pletekan roots are carried out by FTIR spectrophotometric method to determine functional groups contained in secondary metabolites of the extracts. In addition, the LC-MS technique is also applied to characterize the resulted extracts. Considering the biological functions of the R. tuberosa L that has previously shown, the potential of anti-oxidant activity of hydroethanolic extracts of pletekan roots is determined by using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radicals scavenging assay.

2. Material and Methods

2.1. Materials and instruments
Materials used in this research included pletekan roots powder obtained from Materia Medica (Batu, East Java) enclosed with determination letter of the species, Ruellia tuberosa L. Other materials were purchased from Sigma-Aldrich: ethyl alcohol (pure, \(d\)=0.789 g/mL), glacial acetic acid (pharmaceutical secondary standard), HCl (37%, analytical grade), H\(_2\)SO\(_4\) (99.99%, analytical grade), FeCl\(_3\) (97%, reagent grade), NaCl (powder, ≥99.5%, analytical grade), KMnO\(_4\) (≥99.0%, reagent grade), magnesium (turnings powder, 98%, reagent grade), 2,2-diphenyl-1-picrylhydrazyl (DPPH, mp~135 °C, reagent grade). Instruments used were UV-Vis spectrophotometer (1601, Shimadzu), FTIR spectrophotometer (1600S, Shimadzu), UHPLC ACCELLA (1250, Thermo Scientific) coupled with mass spectrometer TSQ QUANTUM ACCESS MAX (Thermo Finnigan).

2.2. Preparation of hydroethanolic extracts of pletekan roots
A 900 g of pletekan root powder (90 mesh) was macerated with water:ethyl alcohol in 1:1 ratio for 3×24 h. The resulted extracts were filtered, and then concentrated using a rotary evaporator vacuum at 50°C, 120 rpm. The extracts were stored at 4°C for further characterization.

2.3. Phytochemical screening tests of hydroethanolic extracts of pletekan roots
Phytochemical qualitative tests were conducted based on standard phytochemical tests [8-10]. The extracts were tested for flavonoids, triterpenoids, steroids, phenolic compounds, saponins, tannins, and ascorbic acids.

2.4. Characterization of hydroethanolic extracts of pletekan roots using LC-MS and FTIR spectrometry
Liquid chromatography was conducted on UHPLC ACCELLA with injection volume of 2000 \(\mu\)L, isocratic volume of 400 \(\mu\)L, at a flow rate of 250 \(\mu\)L/min. Solvents used were: solvent A=water; solvent B=0.1 % formic acid in acetonitrile; solvent C=20 mmol of ammonium formic; solvent D=0.1 % formic acid in methanol. Liquid chromatographic separation was applied for 70 min, followed by mass spectrometric analysis using TSQ Quantum Access Max in ESI method and in positive ion mode. Typical experiments settings were as follow: sheath gas (N\(_2\)) pressure=50 psi; spray voltage=4.5 kV; capillary temperature=300 K; cone voltage=39 V; tube lens offset=15 V; and \(m/z\) range, 150-200. The extracts were dried and pressed into KBr pellet before analyzed by a FTIR spectrophotometer, at a range of 4000-400 \(m/z\).

2.5. Anti-oxidant activity test of hydroethanolic extracts of pletekan roots
Anti-oxidant activities were determined on the basis of their scavenging potential of the stable DPPH free radicals in quantitative assay, according to the method described earlier [11, 12]. Stock solution (1
mg/mL) of the extracts were prepared in ethanol from which serial dilutions were carried out to obtain concentrations of 2, 4, 6, 8, and 10 μg/mL. Diluted solutions (6 mL) were added to 4 mL DPPH solution (20 μg/mL), mixed, and incubated at 37°C for 20 min, for reaction to occur. The absorbance was determined at 516 nm, and from these values responding percentages of inhibitions were calculated. Then % inhibitions were plotted against log concentration and from the graph IC₅₀ was calculated. The experiment was performed in triplicate. Ascorbic acid was used for positive control using the same concentrations (2:4:6:8:10 μg/mL).

3. Results and Discussion

3.1. Identification and characterization of hydroethanolic extracts of pletekan roots
Table 1 shows secondary metabolites contained in the hydroethanolic extracts of pletekan roots from phytochemical tests. The test was based on the color changes after extracts were reacted with standard reagents for secondary metabolites detection. The positive color changes as shown in Fig. 1 are indication that the extracts contained steroids, flavonoids, phenolics, and ascorbic acids. The physicochemical properties of the hydroethanolic extracts resulted in the ash content of 10.54%, water content of 10%, and percentage yield of 28.44%. These results are in agreement with previous studies conducted on hydroethanolic leaves extracts of this species, which reported the presence of ascorbic acid, phenolic, tannin, lycopene, carotenoid, and tocopherol [3].

| No | Parameter      | Result* |
|----|----------------|---------|
| 1  | Flavonoid      | +       |
| 2  | Terpenoid      | -       |
| 3  | Steroid        | +       |
| 4  | Phenolic       | +       |
| 5  | Tannin         | -       |
| 6  | Saponin        | -       |
| 7  | Ascorbic acid  | +       |

*+ sign = presence; - sign = absence

The LC-MS and FTIR results are shown in Fig. 2 and 3, while interpretations of the results are listed in tables 2 and 3. Interpretations for the LC-MS are based on the library of the targeted components stored in the library. The targeted compounds contained in the extracts are phytoserol compounds, since previous study revealed that the major component of the pletekan root extract was lupeol, a plant sterol [3]. Compound 1 (Fig. 2a) separated at retention time 1.48 s, and its molecular weight is 383, predicted has empirical formula of C₂₈H₄₈O, and identified as campesterol. Compound 2 separated at RT-1.45 s and its MW is 395, projected has empirical formula of C₂₉H₄₈O, identified as stigmasterol. Finally, compound 3 separated at RT-1.59 s, and with LC-MS fragment, had a mass [M+H]+ 397, predicted as C₂₉H₅₀O, identified as β-sitosterol. From these results, it appeared that targeted LC-MS is a rapid and effective approach to obtain information on secondary metabolites content on the plant extracts.
Figure 1. The phytochemical qualitative test results of hydroethanolic extracts of *pletekan* roots: (a) steroid test using Lieberman-Burchard’s reagent; (b) flavonoid test; (c) phenolic test; (d) tannin test; (e) saponin test; and (f) ascorbic acid test. Positive test results are shown in (a), (b), (c), and (f).

Figure 2. Chromatogram results of hydroethanolic extracts of *pletekan* roots showing prediction of phytosterol compounds: (a) campesterol; (b) stigmasterol; and (c) β-sitosterol.
As shown in Fig. 3, there are five major absorption bands appeared in the FTIR spectra of hydroethanolic extracts of pletekan roots. Those areas are 3450-3100, 2950-2850, 1650-1640, 1460-1450, and 1180-1160 cm$^{-1}$. The bands assignment for those are O-H bending, C-H stretching from alkanes, C=C stretching alkenes or C=O stretching from carbonyl groups, C-C stretching from aromatic compounds, and C-O from secondary alcohol, respectively. Previous studies reported absorption peaks at 1172 cm$^{-1}$ for β-sitosterol myristate [13]. Another study by Hang and Dussault [14] also reported absorption spectra at 1730 cm$^{-1}$ for campesterol acetic ester. Other assignments for the appeared absorption bands are corresponded with those typical functional groups in the FTIR spectra for phytosterol compounds [15, 16]. Thus, these FTIR results are in good agreement with those LC-MS results, revealing that the extracts mainly composed of phytosterol compounds.

Table 2. Interpretation of chromatogram results.

| No | Retention time (s) | Ion fragmentation m/z | Prediction of compounds |
|----|-------------------|------------------------|-------------------------|
| 1  | 1.45              | 254.50-255.50          | Stigmasterol            |
| 2  | 1.48              | 160.50-161.50          | Campesterol             |
| 3  | 1.59              | 160.50-161.50          | β-sitosterol            |

**Figure 3.** FTIR spectra from hydroethanolic extracts of *pletekan* roots, inset numbers 1-5 showing major absorption bands.

Table 3 Interpretation of FTIR spectra for major absorption bands.

| No | Wavenumber (cm$^{-1}$) | Assignment          |
|----|------------------------|---------------------|
| 1  | 3450-3100              | -OH                 |
| 2  | 2950-2850              | -C-H- alkane        |
| 3  | 1650-1640              | -C=C- and -C=O      |
| 4  | 1460-1450              | -C-C- aromatic compounds |
| 5  | 1180-1160              | -C-O- from secondary alcohol |

3.2. Anti-oxidant activity of hydroethanolic extracts of *pletekan* roots
Free radicals are involved in many disorders like neurodegenerative diseases, cancer and diabetes. Antioxidants through their scavenging power are useful for the management of those diseases. DPPH
stable free radical technique is an easy, rapid and sensitive way to determine the antioxidant activity of a specific compound or plant extracts [17, 18].

The hydroethanolic extracts of pletekans roots shows high anti-oxidant activity with the IC$_{50}$ of 2.48 µg/mL (table 4). This value is comparable with the anti-oxidant activity of ascorbic acid, that has IC$_{50}$ of 3.18 µg/mL. Most of the secondary metabolite compounds such as flavonoids and phenolic compounds may be responsible for antioxidants properties of many plants [19]. In this study, anti-oxidant activity is due to the presence of flavonoids and ascorbic acids in the extracts as shown from phytochemical test (table 1 and Fig. 1). The presence of -OH groups in ortho position; and conjugated C=C double bonds contribute to antioxidant properties of flavonoids. When there are free radicals (R*), flavonoid compounds donate H atom(s) from the -OH groups, and bind to free radicals, forming R-H bond. This will produce phenoxyl flavonoid radicals (FIO*); FIO * will be stabilized by conjugated C=C double bonds in the flavonoid rings (Fig. 4).

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In addition, the phytosterol compounds (stigmasterol, campesterol, and β-sitosterol) contained in the extracts may also contribute to the high anti-oxidant activity. The phytosterol compounds generally act as modest free radical scavengers [20]. The proposed mechanisms of actions of phytosterol compounds acting as free radical scavenger is shown in Fig. 5. In that proposed mechanism of action, H atom from hydroxyl group (-OH) binds to –ONOO radicals from streptozotocin forming a more stable compound, while -O radicals from phytosterols forming a stabilized double bond. Since phytosterol compounds in the extracts consist of stigmasterol, β-sitosterol, and campesterol, thus, the subsequent reaction resulted in H• radicals that bind to H• from other compounds forming H$_2$ (Fig. 5).

Phytosterol compounds work synergistically in stabilizing free radicals. Therefore, the synergistic effects take into account when several phytosterol compounds presence in the extracts [21], and as a result expressed the high anti-oxidant activity. Results of the current study are in agreement with previous study that reported anti-oxidant activity of R tuberosa L roots extracted with various solvents, including methanol, water, and ethyl acetate [22].

**Table 4** Anti-oxidant activity of hydroethanolic extracts of pletekans roots.

| No | Sample concentration (µg/mL) | Inhibition activity (%) Extracts | Inhibition activity (%) ascorbic acid | IC$_{50}$ (µg/mL) Extracts | IC$_{50}$ (µg/mL) ascorbic acid |
|----|------------------------------|---------------------------------|--------------------------------------|-----------------------------|-----------------------------|
| 1  | 2                            | 49.62                           | 48.86                                |                             |                             |
| 2  | 4                            | 50.76                           | 50.38                                |                             |                             |
| 3  | 6                            | 52.27                           | 53.41                                | 2.48±0.75                   | 3.18 ± 0.77                 |
| 4  | 8                            | 53.03                           | 56.25                                |                             |                             |
| 5  | 10                           | 53.98                           | 57.77                                |                             |                             |

**Figure 4.** Proposed mechanism of action of flavonoids compound acting as free radicals scavenger [19].
4. Conclusion
The current study has demonstrated that hydroethanolic extracts of *pletekan* roots contained steroids, flavonoids, phenolics compounds, and ascorbic acids. The LC-MS results revealed that those extracts mainly composed of phytosterols compounds including stigmasterols, beta-sitosterols, and campesterol. The FTIR studies support these results and FTIR spectra show typical functional groups of phytosterol compounds such as –OH group at ~3366 cm\(^{-1}\), alkenes (C=\(\text{C}\)) at ~1645 cm\(^{-1}\), aromatic compounds at ~1426 cm\(^{-1}\), and –C–O from secondary alcohol at ~1032 cm\(^{-1}\). The hydroethanolic extracts of *pletekan* roots possess high anti-oxidant activity with the IC\(_{50}\) value of 2.48 µg/mL against DPPH free radicals. These results indicate that hydroethanolic extracts of *pletekan* roots have potential to be used as cure for diseases related to free radicals.

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