Evaluation of antibacterial activities from major bioactive constituents of the whole plant of *Hedyotis corymbosa*

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**KEYWORDS**

- Anthraquinone
- Antimicrobial activities
- Aromatics
- Bioactive compound
- Sterols
- Triterpenes

**ABSTRACT**

*Hedyotis corymbosa* is locally known as *rumput mutiara* from the Rubiaceae family, widely distributed in tropical regions of Asia. Researchers provided scientific evidences the beneficial impact of this plant for pharmacologic activity. This study aimed to isolate and evaluate the bioactive constituents based on their biological activities. In this study, the whole plant of *H. corymbosa* was extracted using methanol. Extract of *H. corymbosa* was sequentially partitioned using ethyl acetate and n-butanol. The ethyl acetate layer was further fractionated and isolated using chromatographic techniques to obtain the pure compounds. The bioactive compounds structure was determined using spectroscopic analysis especially the Nuclear Magnetic Resonance (NMR). The investigation of *H. corymbosa* resulted in the isolation of eight compounds, were identified as ursolic acid, 3β-hydroxyolean-11-en-28,13β-olide, β-sitosterol, stigmastane-3,6-dione, ferulic acid, scopoletin, 2-hydroxy-1-methoxyanthraquinone, 3-hydroxy-1,2-dimethoxyanthraquinone. The antimicrobial effect of the crude extract, partitions and fractions were evaluated using agar well diffusion for antimicrobial susceptibility test and for the pure compounds were evaluated using minimum inhibitory concentration. The ethyl acetate layer and crude extract displayed the higher antimicrobial activities than n-butanol and water layer. The Minimum Inhibitory Concentration (MIC) for the pure compound was shown that most of the compounds have the ability to inhibit human pathogenic bacteria with average 100 µg/mL. The antimicrobial activities showed by the crude extracts, fractions, and pure compounds of *H. corymbosa* can be used as a commercial product for antimicrobial agent against *S. aureus*, *S. enterica*, *E. coli* and *B. subtilis*.

**Introduction**

*Hedyotis corymbosa* (L.). Lam (syn. *Oldenlandia corymbosa*), is a flowering plant from the Rubiaceae family and commonly called diamond flower (or *rumput mutiara*). Most of the species from the Rubiaceae family possess medicinal properties and is used as ingredients in Chinese Herbal Medicine for treatment of cancer, anti-inflammatory and hepatoprotective in male rat has been reported (Lin et al., 2002)

Previous phytochemical studies on some of genus *Hedyotis* showed that the genus contained iridoids, flavonoids, anthraquinones, alkaloids, lignans, coumarins and triterpenes (Ahmad et al., 2006). Since then, glucan (Cui et al., 2006), naphthoquinones and cyclotides (Ding et al., 2014) have been obtained from the species of this genus. A comparative chemotaxonomy study of different species demonstrated that iridoids are the predominant and characteristic constituents of genus *Hedyotis* (Chen et al., 2005; Van Long et al., 2013).

Based on the traditional uses, researchers provided substantial scientific evidence revealing the health beneficial impact of this plant. This study aimed to isolate and evaluate the typical components based on the secondary metabolites of the whole plant of *H. corymbosa*.

**Research Methods**

**Plant material**

The whole plant of *H. corymbosa* were collected in around Department of Biological Science and Technology, NPUST, Pingtung county Taiwan,
fresh sample of *H. corymbosa* were transferred to cutting and drying process.

The total weight of the dried sample from the whole plant of *H. corymbosa* was 9.8 kg. The dried sample of *H. corymbosa* was macerated by methanol 95% for 10 days. The methanol as a result of maceration was evaporated using rotary evaporation at 50°C.

**Identification by Nuclear Magnetic Resonance (NMR)**

1H NMR and 13C NMR spectra were measured using Varian Mercury Plus 400 MHz, NMR. Proton or carbon nuclear test was conducted by dissolved samples in a deuterated solvent of chloroform-d or dimethylsulfoxide (DMSO), kept the sample in 5 mm NMR tube, determined by NMR which yield the peaks as electromagnetic wave absorption signal. Chemical shift is expressed in parts per million (ppm) unit relative to TMS as an internal standard (Lambert and Eugene, 2004)

**Minimum inhibitory concentration (MIC)**

The minimum inhibitory concentration (MIC) of the compound was determined using the microdilution technique with 96 well plates and resazurin as an indicator cell growth based on Sarker et al. (2007), was assessed on four pathogenic bacteria from both gram positive (*S. aureus* and *B. subtilis*) and gram negative bacteria (*E. coli* and *S. enterica*).

**Results and Discussion**

Eight compounds were isolated from ethyl acetate layer of the whole plant of *H. pinifolia* by repeated column chromatography with the appropriate solvent systems. Their structures were identified as β-sitosterol (S1), stigmastane-3,6-dione (S2), ferulic acid (AR1), scopoletin (AR2), 2-hydroxy-1-methoxyanthraquinones (An1), 3-hydroxy-1,2-dimethoxyanthraquinone (An2), ursoic acid (T1) and 3β-hydroxyolean-11-en-28,13β-olide (T2) by the analysis of their NMR, EI-MS spectra and compared with published data.

**β – sitosterol (S1)**

White needles; mp 136 °C; for C29H50O. 1H NMR (400 MHz, CDCl3) δH 5.33 (1H, br s, H-6), 3.50 (1H, m, H-3), 1.01 (3H, s, H-19), 0.91 (3H, d, J = 6.0 Hz, H-21), 0.84 (3H, t, J = 2.0 Hz, H-29), 0.81 (3H, d, J = 6.0 Hz, H-26), 0.79 (3H, d, J = 10 Hz, H-27), 0.68 (3H, s, H-18). EI-MS (70 eV) m/z (%): 412[M]+(100), 396 (81), 381 (70),303 (72), 213 (72),145 (83), 69 (100).

**Stigmastane-3,6-dione (S2)**

Colorless amorphous crystal, for C29H48O2. 1H NMR (400 MHz, CDCl3, J in Hz) 0.66 (3H, s, H-18), 0.93 (3H, s, H-19 ), 0.90(3H, d, J = 6.4, H-21), 0.81 (3H, d, J = 6.8, H-26), 0.78(3H, d, J = 6.8, H-27), 0.84 (3H, d, J = 6.4, H-29).EI-MS (70 eV) m/z (%): 428 [M]+ (65), 413 (7), 287 (29), 245 (14), 231 (26),149 (55), 137 (58), 98 (100).

**Ferulic acid (AR1)**

Green powder; mp 168-172 °C; for C10H10O4. 1H NMR (400 MHz, CDCl3): 6.31 (1H, d, J = 9.6 Hz, H-3), 6.82 (1H, d, J = 8.4 Hz, H-5), 7.01 (1H, d, J = 1.6 Hz, H-2), 7.60 (1H, d, J = 16.0 Hz, H-7). EI-MS (70 eV) m/z (%): 194 [M]+ (100), 179 (20), 177 (8), 133 (18), 77 (8), 51 (6).

**Scopoletin (AR2)**

Colorless needle; mp 206-207°C; for C15H11O5. 1H NMR (400 MHz CDCl3): 6.3.94 (3H, s, OCH3), 6.24 (1H, d, J = 9.6 Hz, H-3), 6.82 (1H, s), 6.89 (1H, s), and 7.60 (1H, d, J = 9.6 Hz, H-4).EI-MS (70 eV) m/z (%): 192 [M]+(100), 177 (56), 164 (24), 149 (44), 121 (20), 79 (14), 69 (26), 51 (12).

**2-hydroxy-1-methoxyanthraquinone (An1)**

Yellow powder, for C15H10O4. 1H NMR (400 MHz, CDCl3) δH: 7.34 (1H, d, J = 8.4 Hz, H-3), 8.12 (1H, d, 8.4 Hz, H-4), 8.25 (2H, m, H-5, H-8), 7.75 (2H, m, H-6, H-7), 4.02 (3H, s, OCH3), 6.71 (1H, s, OCH3). 13C NMR (100 MHz, CDCl3). δC: 146.6 (C-1), 155.6 (C-2), 120.3 (C-3), 125.7 (C-4), 127.1 (C-5), 133.9 (C-6, C-7), 126.8 (C-8), 182.7 (C-9), 182.1 (C-10), 132.9 (C-11), 134.4 (C-12), 125.7 (C-13), 127.5 (C-14), 62.3 (OCH3). EI-MS (70 eV) m/z (rel. Int.): 254 [M]+, 208 (100), 183 (24), 139 (24), 155 (16), 87 (6).

**3-hydroxy-1,2-dimethoxyanthraquinone (An2)**

Yellow-orange powder, for C16H12O3. 1H NMR (400 MHz, CDCl3) δH: 117.4 (1H, s, H-4), 7.83 (2H, m, H-5, H-7), 8.08 (2H, m, H-6, H-8), 3.83 (3H, s, 1-OCH3), 3.86 (3H, s, 2-OCH3). 13C NMR (100 MHz, CDCl3). δC: 155.2 (C-1), 147.1 (C-2), 156.4 (C-3), 111.0 (C-4), 126.7 (C-5), 133.7 (C-6), 134.6(C-7), 126.3(C-8), 182.3(C-9), 180.5(C-10), 132.2(C-11), 134.7(C-12), 119.4(C-13), 130.6(C-14), 60.8(C-1′), 61.4(C-2′). EI-MS (70 eV) m/z (rel. Int.): 284 [M]+(100), 269 (95), 241 (40), 223 (15), 195 (7), 170 (25), 139 (14), 114 (20), 97 (70), 57 (21).
Table 1. MIC determination using the resazurin assay

| Compounds | S. aureus | B. subtilis | E. coli | S. enterica |
|-----------|-----------|-------------|---------|------------|
| Streptomycin | 15 | 15 | 1.875 | 15 |
| S1 | 9.4 | 37.5 | 75 | 75 |
| S2 | 75 | 4.69 | 75 | 37.5 |
| An1 | 600 | 37.5 | 75 | 150 |
| An2 | 75 | 37.5 | 75 | 75 |
| T1 | 150 | 4.69 | 75 | 75 |

Ursolic acid (T1)

White amorphous powder, for C_{30}H_{48}O_{3}. 1H NMR (400 MHz, DMSO-d_{6}, J/Hz): 5.18 (1H, s, H-12), 2.31 (1H, s, H-3), 2.12 (1H, d, J= 1.72, H-18), 1.06 (3H, s, H-24), 0.92 (3H, s, H-29), 0.89 (3H, s, H-27), 0.86 (3H, s, H-23), 0.81 (3H, d, J = 16.8, H-30), 0.75 (3H, s, H-26), 0.64 (3H, s, H-25). EI-MS (70 eV) m/z (rel. Int.): 456 [M]+, 438 (100), 410 (47), 392 (69), 300 (42), 255 (30), 202 (46), 189 (8).

3β-hydroxyolean-11-en-28,13β-olide (T2)

Brownish, for C_{30}H_{48}O_{3}. 1H NMR (400 MHz, CDCl3) δH: 3.23 (1H, dd, J=5.2, H-3), (1H, dd, J=10.4, 3.20, H-11), 5.91 (1H, d, J=10.4, H-12), 0.99 (3H, s, H-23), 0.90 (3H, s, H-27), 1.03 (3H, s, H-29), 0.85 (2H, s, H-30). 13C NMR (100 MHz, CDCl3). δC: 38.9 (C-1), 27.7 (C-2), 78.8 (C-3), 38.2 (C-4), 54.7 (C-5), 17.7 (C-6), 36.3 (C-7), 41.6 (C-8), 53.0 (C-9), 38.1 (C-10), 128.8 (C-11), 133.4 (C-12), 89.7 (C-13), 41.6 (C-14), 25.5 (C-15), 26.9 (C-16), 45.0 (C-17), 53.0 (C-18), 36.3 (C-19), 31.1 (C-20), 33.2 (C-21), 30.0 (C-22), 27.7 (C-23), 17.9 (C-24), 14.9 (C-25), 17.7 (C-26), 18.8 (C-27), 180.0 (C-28), 32.0 (C-29), 22.7 (C-30).

Minimum Inhibitory Concentration (MIC)

The MIC of the compounds was determined using microdilution with resazurin as an indicator of cell growth. The data obtained through MIC revealed variability in the inhibitory concentrations for five pure compounds (S1, S2, An1, An2, T1) against pathogenic bacteria. The MIC values of the different compound against the gram negative bacteria and gram positive bacteria were found in the range of 4.69 – 600 μg/mL. The MIC value of each isolated compound was shown in Table 1.

According to Holetz et al. (2002), the isolated compound less than 100 μg/mL is good antimicrobial activity, from 100-500 μg/mL is moderate antimicrobial activity, whereas the MICs over than 1000 is inactive. From Table 13, results showed that almost of the isolated compounds were considered good antimicrobial activity against the test strains. Except, compound An1 and T1 showed moderated activity against S. enterica and S. aureus and compound An1 shown weak activity against S. aureus.

Conclusions

Eight pure compounds were isolated from ethyl acetate extract in various fractions, and identified as: ursolic acid, β-sitosterol (S1), stigmastane-3,6-dione (S2), ferulic acid (AR1), scopoletin (AR2), 2-hydroxy-1-methoxyanthraquinone (An1), 3-hydroxy-1,2-dimethoxyanthraquinone (An2), ursolic acid (T1) and 3β-hydroxyolean-11-en-28,13β-olide (T2). Ursolic acid (T1) has the biggest effect to inhibit B. subtilis with minimum inhibition concentration of 4.69 μg/mL, β-sitosterol (S1) has the biggest effect to inhibit S. aureus with minimum inhibition concentration of 9.4 μg/mL. All the compounds showed significant inhibition effect against E. coli with 75 μg/mL, minimum inhibition concentration. Stigmastane 3,6-dione (S2) has the biggest inhibition effect against S. enterica with 37.5 μg/mL minimum inhibition concentration.

Conflict of interest

The authors declare that there is no conflict of interest in this publication.

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