Comprehensive Analysis of *Hedera Helix* Leaves: Chemical Composition and its Antibacterial and Antidiabetic Potentials

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Abstract: This research aimed to study on chemical constituents and investigate bioactivities of *Hedera helix*, including antibacterial and antidiabetic. From the methanol extract of *H. helix* leaves collected in Da Lat province (Vietnam), four triterpene saponins were isolated and determined as α-hederin (1), hederaicoside D (2), hederaicoside B (3), and hederaicoside C (4). Their chemical structures were elucidated by NMR. The fractions and isolated compounds of *H. helix* leaves showed low antibacterial activity against gram-positive (*Staphylococcus aureus*) and gram-negative (*Pseudomonas aeruginosa*) bacteria. In addition, these samples were found to have low-glucosidase inhibitory activity at tested concentration.

Keywords: *Hedera helix*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, α-glucosidase, triterpene saponins.

1. Introduction

*Hedera helix* L. is a dioecious evergreen woody liana, one of the 15 species of the genus *Hedera* in the Araliaceae family. The leaves are coriaceous, measuring between 4 and 10 centimeters in length and width, with a cordate base. Palmately, the lamina has three to five lobes. The upper surface is dark green with lighter, radiating venation, whereas the lower surface is more grayish-green and the venation is clearly elevated. The flowering stems are composed of ovate-rhomboidal to lanceolate, 3 to 8-cm long leaves [1]. The flowers produced from summer to late autumn are tiny, greenish-yellow umbels 3-5 cm in diameter, and the fruits are small, black berries that ripen in winter. *H. helix* is native to Western, Central, and Southern Europe, and has been introduced to North America and Asia. It is a widespread favorite in many nations [2].

*Hedera helix* L. is not only noted as an attractive plant, but also has potentially dangerous effects such as bloody diarrhea, gastrointestinal irritation, contact dermatitis, and death caused by the leaves and fresh fruits. However, the medical field treat begins warts due to the antioxidant, antilergic, and antispasmodic properties. Several authors have reported that dry extracts positively affect respiratory functions in children with chronic bronchial asthma, as well as antibacterial, antihelminthic, leishmanicidal, and anti fungal properties [3, 4]. Early studies analyzed the antifungal [5] and antibacterial [6] activities of *H. helix* saponins.

Besides, multiple studies have indicated a complex chemical composition for ivy leaves: phenolic acids (caffeic acid, neochlorogenic acid, chlorogenic acid), flavonoids (quercetin, kaempferol, isoquercitrin), phytosterols (stigmasterol, sitosterol), polyacetylenes (falcarniol), hederagenin, oleanolic acid, and *Hedera* saponins [3, 7, 8]. Triterpene saponins are potent ingredients for herbal plant medical uses that has been reported as the primary components in the crude extract of ivy plants. α-Hederin, hederaicoside B, hederaicoside C, and hederaicoside D were medicinally useful active constituents of ivy leaf [9].

Although extensive research has been conducted on the medicinal properties of *Hedera helix*, not many studies has examined the efficacy of the extract’s ivy saponins against naturally occurring bacterial strains and its antidiabetes activity. Therefore, the present study aimed to isolate triterpene saponins, and evaluate the antidiabetic and antibacterial activities of *Hedera helix*.

2. Materials and Methods

2.1. Chemicals and reagents

Deionized water; HPLC grade acetonitrile, methanol, chloroform; and analytical grade formic acid (≥98%) were obtained from Scharlau (Barcelona, Spain). α-glucosidase (Lot 0000209485), p-Nitrophenyl-α-D-glucopyranoside (PNPG), acarbose, chloramphenicol, DMSO, disodium hydrogen phosphate dodecahydrate (Na₂HPO₄·12H₂O), and sodium dihydrogen phosphate dihydrate (NaH₂PO₄·2H₂O) were purchased from Sigma-Aldrich Chemical Co. (Singapore).

2.2. Sample preparation

*Hedera helix* was collected from Da Lat province, Vietnam, and identified by botanist Tran Huu Dung, Southern Institute of Ecology (VAST). A voucher specimen (Code:...
NaPro.33.1019) was deposited in the Center for Research, Testing Pharmaceutical Chemistry (IAMS-VAST). After collection, the leaves were cleaned, let air dry, and then chopped into pieces in preparation for extraction.

2.3. Extraction and isolation

Air-dried powder (4.9 kg) of ivy leaves was extracted with methanol for 24 hours. Extracted solution was filtered throughout filter paper, and the solvent was evaporated to dryness using on rotary evaporator system (BÜCHI R-300, Switzerland). After five times of extraction, crude extract was directly immersed and partitioned effectively with n-hexane and ethyl acetate to obtain yielding n-hexane (81.5 g), EtOAc (30.0 g), residue (495.0 g) extracts, respectively. The residue extract was subjected to fractionation over silica gel eluting with MeOH in EtOAc (0-100%, step-wise) to give 3 fractions MA (15.0 g), MB (158.0 g), and MC (210.0 g). Fraction MC was separated by column chromatography (CC) over silica gel with a solvent mixture of CHCl₃-CH₂Cl₂-MeOH-H₂O (20: 50: 30: 0.5, v/v/v) to yield 5 fractions: MC1 (2.5 g), MC2 (49.8 g), MC3 (15.1 g), MC4 (4.3 g), and MC5 (5.0 g).

The fraction MC2 (25.0 g) was subjected to silica gel CC and eluted with CHCl₃-MeOH-H₂O (70: 30: 5, v/v/v) to obtain 5 sub-fractions: MC2a (0.1 g), MC2b (0.43 g), MC2c (2.46 g), MC2d (4.39 g), and MC2e (3.63 g). Compounds 1 and 4 were obtained from fraction MC2b and MC2c, respectively, by recrystallization. Fraction MC2b and MC2c were subjected to separation on HPLC (Hewlett Packard series 1100), with Phenomenex LUNA C18 (2), 250x10mm, 5μm (Phenomenex, USA), flow rate of 5mL/min, sample concentration 5%, inject volume 200µL, gradient of 65%-100% MeOH in water for 35 min, to yield compounds 2 and 3. Chemical structures of the isolated compounds (Figure 1) were determined by NMR.

2.4. Antibacterial activity

2.4.1. Preparation of test organism cultures

Bacterial strains: The antibacterial effectiveness of the extracts was evaluated using two bacterial strains Staphylococcus aureus ATCC 6538 and Pseudomonas aeruginosa ATCC 27853.

2.4.2. Inoculums preparation

The agar plates were incubated at 37°C for 24 hours. A meat peptone broth (MPB) was used for Staphylococcus aureus and Pseudomonas aeruginosa. Then, determine the bacterial density by optical densitometry at 660 nm and adjust to the bacterial density in the range of 10⁶-10⁷ CFU/ml.

2.4.3. Qualitative antibacterial activity by agar well diffusion assay

Antibacterial activity of the extract was performed using the agar well diffusion method. Resistant bacterial strains were inoculated on meat peptone broth. Each culture was swabbed on the surface of sterile nutrient agar plate. In agar plate, ten wells were prepared with the help of sterilized cork borer of 8 mm diameter. The antibacterial ability of the substance is tested by aspirating 20-50 µl of sample solution of different concentrations into the wells on the agar plate covered with the test bacteria. Chloramphenicol (10mg/ml) and DMSO were used as positive and negative control, respectively. Every plate used according to the aforementioned procedure was performed in triplicate for statistical average. The active ingredient will diffuse into the agar if it has antibacterial activity, it will inhibit the growth of bacteria and cause the appearance of an antibacterial ring [6].

2.5. Antidiabetic activity

α-Glucosidase activity was assessed by monitoring the formation of p-nitrophenol (PNP) from p-nitrophenyl-α-D-glucopyranoside (PNPG). Acarbose was used as positive control. Sodium phosphate buffer (0.1 M, pH 6.8) was used as reaction buffer. The samples were dissolved in DMSO 10% at a concentration of 1000 ppm. In each microplate well, 20 µL of test sample (or 20 µL of solvent for the negative controls), 20 µL of α-glucosidase (0.33U/mL) in water, and 140 µL of buffer were incubated at 37°C for 20 min. Then, 20 µL of PNP substrate (2.5 mM) in water (or 20 µL of water for the blanks) was added to each well to initiate the reaction. After incubating at 37°C for 30 min, athermostatically controlled PowerWave HT microplate spectrophotometer (BioTek, USA) was used to measure the absorbance at 405 nm. The percent inhibition was calculated as follows:

α-glucosidase inhibitory activity (%) = ((A0-A1))/A0×100%

Where A₀ is the absorbance of the control and A₁ is the absorbance of the sample. The IC₅₀ value was defined as the sample concentration required to inhibit 50% α-glucosidase activity [10].

2.6. Data analysis of bioactivities

Data were processed using Rstudio (version 1.4.1717) software. The results were compared by one-way ANOVA and Tukey's HSD post hoc test. A difference was considered statistically significant if p≤0.05.

3. Results and Discussion

3.1. Structural determination of isolated compounds

3.1.1. Compound 1

¹H-NMR (DMSO-d₆; 500 MHz) spectrum of compound 1 digital compound for 3 signals of CH₂ group at δH 0.57 (s); 1.07 (s); 1.08 (s), 3 CH₃ groups give 1 δH 0.87 (s) signal, 1 H-23 oxymethylene group at δH 3.12 and 3.28, 1 oxymethine H-3 group at δH 3.49 (1H, m). The ¹H-NMR spectrum also shows the presence of an olefin proton signal at δH 5.16 that characterizes H-12 in the structural framework of the 5-ring triterpene.

The ¹³C-NMR and DEPT spectroscopy of compound 1 exhibit resonant signals of 41 carbons, 30 of which are consistent with published data for the structure of the triterpenoid framework as hederagenin including the signal of carbon olefins C-12 at δC 121.5 corresponding to carbon
methine and tetracarbon C-13 at δc 143.8 also confirms the presence of double bonds in the ring in the position C-12 position, and also C-28 signaling at δc 178.6 of the carboxyl group. In addition, the remaining 11 carbon signals showed the presence of 2 sugar units with resonant signals of 2 anomeric carbons C-1' at δc 102.9 and C-1'' at δc 99.9.

The protons in the ring of sugar units are assigned precisely by means of COSY, HSQC and HMBC spectroscopy starting from the anomeric protons that have been identified. The chemical displacement of C-3 at δc 79.3 on the 13C-NMR spectrum suggests that sugar circuits are attached to this position. Binding to sugar units was confirmed by HMBC interactions between the anomeric proton H-1' of arabinose δh 4.19 (1H, d, 6.6) with C-3 at δc 79.8, H-1'' of glucopyranose δh 5.22 (1H, d, 7.8) with C-28 at δc 175.2, H-1''' of glucopyranose δh 4.27 (1H, d, 7.8) with C-6'' at δc 67.5, and H-1''''' of rhamnose δh 4.70 (1H, d, 3.6) with C-4'''' at δc 76.8. Thus, compound 2 was determined as hederaacide D.

3.1.3. Compound 3

The 1H-NMR (DMSO-d6, 500 MHz) spectrum of compound 3 gives 4 signals of the methyl group at δh 0.68 (s); 0.75 (s); 0.93 (s); 1.08 (s), 3 methyl groups give 1 δh 0.86 (s) signal, 1 oxymethine H-3 group at δh 3.00 (1H, m). The 1H-NMR spectroscopy also shows the presence of an olefin proton signal at δh 5.17 that characterizes H-12 in the structural framework of the five-ringed triterpene.

The 13C-NMR and DEPT spectroscopy of compound 3 show synergistic signals of 59 carbons, 30 of which are consistent with published data for the structure of the triterpenoid framework as oleanolic acid including signals of olefin C-12 carbon at δc 121.6 corresponding to methine carbon and C-13 tetracarbon at δc 143.4 also confirming the existence of double bonds in rings at the C-12 position, and also the C-28 signal at δc 175.2 of the carboxyl group. In addition, the remaining 29 carbon signals showed the presence of 5 sugar units with resonant signals of 5 anomeric carbons C-1' at δc 103.7, C-1'' at δc 99.9, C-1'''' at δc 93.9, C-1''''' at δc 102.5, C-1'''''' at δc 100.5; 19 oxymethine group signals, 3 oxymethylene group C-5'' signals at δc 63.7, C-6'' at δc 67.8, C-6'''' at δc 59.9 and 2 methyl group signals C-6'''' at δc 17.7 and C-6''''' at δc 17.7.

The 1H-NMR and HSQC spectroscopy also confirmed that there are five corresponding anomeric protons interacting with five anomic carbons: H-1' at δh 4.30 (1H, d, 5.4), H-1'' at δh 5.03 (1H, m), H-1'''' at δh 5.22 (1H, d, 7.8), H-1''''' at δh 4.27 (1H, d, 7.8) and H-1'''''' at δh 4.68 (1H, brs). The 1H-NMR signaling spectroscopy of 2 methyl groups at δh 1.08 indicates the existence of 2 deoxy pathways in the structure of compound 3.

The protons in the ring of sugar units are assigned precisely by means of COSY, HSQC and HMBC spectroscopy starting from the anomeric protons that have been identified. The chemical displacements of C-3 at δc 79.8 and C-28 at δc 175.2 on the 13C-NMR spectrum suggest that sugar circuits are attached to these sites. Binding to sugar units was confirmed by HMBC interactions between the anomeric proton H-1' of arabinose δh 4.30 with C-3 at δc 87.7, H-1'' of rhamnose δh 5.03 with C-2' of arabinose at δc 74.4, anonomic proton H-1'''' of glucopyranose δh 5.22 (1H, d, 7.8) and C-28 at δc 175.2, anonomic proton H-1''''' of glucopyranose δh 4.27 (1H, d, 7.8) with C-6'' at δc 67.8, anonomic proton H-1'''''' of rhamnose δh 4.68 (1H, br, s) with C-4'''' at δc 75.1. As a result, compound 3 was determined as hederaacide B.

3.1.4. Compound 4

The 1H-NMR (DMSO-d6, 500 MHz) spectrum of compound 4 gives 3 signals of the methyl group at δh 0.57 (s); 0.68 (s); 1.06 (s), 3 methyl groups give 1 δh 0.87 (s) signal, 1 oxymethene H-23 group at δh 3.14 and 3.27, 1 oxymethine H-3 group at δh 3.35 (1H, m). The 1H-NMR spectroscopy also shows the presence of an olefin proton signal at δh 5.18.
that characterizes H-12 in the structural framework of the 5-ring triterpene.

The $^{13}$C-NMR and DEPT spectroscopy of compound 4 exhibit resonant signals of 59 carbons, 30 of which are consistent with published data for the structure of the triterpenoid framework as hederagenin including the signal of carbon olefins C-12 at $\delta_C$ 121.8 corresponding to carbon methine and carbon tetravalent C-13 at $\delta_C$ 143.5 also confirms the existence of double bonds in the ring in position C-12 position, and also C-28 signaling at $\delta_C$ 175.4 of the carboxyl group. In addition, the remaining 29 carbon signals showed the presence of 5 sugar units with resonant signals of 5 anomeric carbons C-1’ at $\delta_C$ 102.9, C-1’’ at $\delta_C$ 99.9, C-1’’’ at $\delta_C$ 94.0, C-1’’’’ at $\delta_C$ 102.5, C-1’’’’’ at $\delta_C$ 100.6.

The $^1$H-NMR and HSQC spectroscopy also confirmed that there are five corresponding anomeric protons interacting with five anomeric carbons: H-1’ at $\delta_H$ 4.33 (1H, d, 7.2), H-1’’ at $\delta_H$ 5.06 (1H, brs), H-1’’’ at $\delta_H$ 5.21 (1H, d, 9.6), H-1’’’’ at $\delta_H$ 4.27 (1H, d, 9.0) and H-1’’’’’ at $\delta_H$ 4.87 (1H, d, 5.4). The $^1$H-NMR signaling spectroscopy of two methyl groups at $\delta_H$ 1.1 indicates the existence of two deoxy pathways in the structure of compound 4.

The protons in the ring of sugar units are assigned precisely by means of COSY, HSQC and HMBC spectroscopy starting from the anomeric protons that have been identified. The chemical displacements of C-3 at C-3 at $\delta_C$ 79.3 and C-28 at $\delta_C$ 175.4 on the $^{13}$C-NMR spectrum suggest that sugar circuits are attached to these sites. Binding to sugar units is confirmed by HMBC interactions between anomeric protons (H-1’) of arabinose $\delta_H$ 4.63 with C-3 at $\delta_C$ 79.3, H-1’’ of rhamnose at $\delta_H$ 5.06 with C-2’ of arabinose $\delta_C$ 74.2, anomeric proton H-1’’’ of glucopyranose $\delta_H$ 5.21 (1H, d, 9.6) and C-28 at $\delta_C$ 175.4, anomeric proton H-1’’’’ of glucopyranose at $\delta_H$ 4.27 (1H, d, 9.0) with C-6’’ at $\delta_C$ 67.6, anomeric proton H-1’’’’’ of rhamnose at $\delta_H$ 4.87 (1H, d, 5.4) with C-4’’’’ at $\delta_C$ 78.5.

HMBC spectroscopy shows that the correlation of protons H-24 at $\delta_H$ 0.57 and H-3 at $\delta_H$ 3.35 co-interacting with C-23 at $\delta_C$62.5 helps determine the OH group attached to the C-23 position. Therefore, compound 4 was determined as hederacoside C.

![Figure 1: Chemical structures of isolated compounds from H. helix.](image-url)
3.2. Antibacterial activity

The antimicrobial activity of the extracts from Hedera helix leaves was investigated by the agar well diffusion method to determine the antibacterial ability of the extracts against some bacterial strains associated with respiratory diseases. Mean zones of inhibition were expressed in mm ± standard error of mean (Table 1).

From the results in Table 1, hederacoside B, C, and D had lower antibacterial activity against Staphylococcus aureus and Pseudomonas aeruginosa than the fractions, indicating that the bioactive compounds were not these saponins. The result was similar when evaluating the activity of α-hederin against Staphylococcus aureus, but when investigated on Pseudomonas aeruginosa, α-hederin showed slightly higher activity than the other three saponins and was comparable to the fraction used to isolate it (MC2b). Thus, it can be inferred that α-hederin is the active compound against Pseudomonas aeruginosa isolated from MC2b. However, this result is still very low.

Antibiotic resistance remains a concern in a number of developing and industrialized countries, posing a significant hazard to the global health sector. Due to the inefficacy of presently available antimicrobial for the treatment of infectious diseases, numerous researchers have turned to natural products as potential sources of novel bioactive compounds [11, 12]. In this investigation, the antibacterial activity of the samples was not against Pseudomonas aeruginosa and Staphylococcus aureus. Therefore, the pharmacological activity of Hedera helix could not be confirmed. These findings are inconsistent with previous studies. It is possible that the growing conditions affect the chemical composition, which causes the antibacterial performance of the samples to change. However, it is also possible that the active ingredient with antibacterial activity in Hedera helix is different from the four isolated phytochemicals. To ascertain their effectiveness in inhibiting the development of the bacteria, additional research is required.

Table 1: Antibacterial activity of the samples from Hedera helix

<table>
<thead>
<tr>
<th>Sample</th>
<th>(mg/well)</th>
<th>Staphylococcus aureus ATCC 6538 Zone of Inhibition (zone diameter-mm)</th>
<th>Pseudomonas aeruginosa ATCC 27853 Zone of Inhibition (zone diameter-mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC2</td>
<td>1.52</td>
<td>8.6 ± 0.1*</td>
<td>12.0 ± 0.3**</td>
</tr>
<tr>
<td>α-hederin</td>
<td>1.50</td>
<td>8.5 ± 0.2**</td>
<td>12.2 ± 0.3**</td>
</tr>
<tr>
<td>Hederacoside D</td>
<td>1.52</td>
<td>8.5 ± 0.3**</td>
<td>9.2 ± 0.2**</td>
</tr>
<tr>
<td>Hederacoside B</td>
<td>1.51</td>
<td>8.5 ± 0.2**</td>
<td>9.1 ± 0.3**</td>
</tr>
<tr>
<td>Hederacoside C</td>
<td>1.52</td>
<td>8.2 ± 0.2**</td>
<td>9.0 ± 0.2**</td>
</tr>
<tr>
<td>MC2a</td>
<td>1.51</td>
<td>9.5 ± 0.3**</td>
<td>12.0 ± 0.3**</td>
</tr>
<tr>
<td>MC2b</td>
<td>1.50</td>
<td>11.0 ± 0.4**</td>
<td>12.3 ± 0.4**</td>
</tr>
<tr>
<td>MC2c</td>
<td>1.53</td>
<td>14.0 ± 0.4</td>
<td>12.0 ± 0.4**</td>
</tr>
<tr>
<td>MC2d</td>
<td>1.50</td>
<td>9.2 ± 0.5**</td>
<td>10.0 ± 0.3**</td>
</tr>
<tr>
<td>MC2e</td>
<td>1.51</td>
<td>12.0 ± 0.3**</td>
<td>11.0 ± 0.5**</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>22.0 ± 0.5</td>
<td>21.0 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

(Mean values followed by the same uppercase letter in each group are not significantly different from each other at p<0.05 according to Tukey’s HSD test).

3.3. α-glucosidase inhibitory activity

To evaluate antidiabetic potential of Hedera helix species, α-glucosidase inhibitory activity of the fractions and isolated compounds from this species was performed. The results were shown in Table 2. As a result, fraction MC2 of H. helix extract showed promising α-glucosidase inhibition (45.23%). Among the 4 isolated compounds, hederacoside C exhibited the strongest α-glucosidase inhibition activity (35.48%). The other three isolated saponins including hederasaponin B, hederasaponin D, and α-hederin showed low inhibitory activity (16.99, 14.45, and 11.34%, respectively). The results indicated that triterpene saponins in H. helix species were not the active phytochemicals with inhibitory activity of α-glucosidase enzyme from this medicinal species. In order to identify potential antidiabetic compounds from H. helix species, further studies are needed to isolate other compounds and test their α-glucosidase enzyme inhibitory activity.

Table 2: α-glucosidase inhibitory activity of H. helix fractions and isolated compounds at concentration of 100ppm

<table>
<thead>
<tr>
<th>Sample</th>
<th>Inhibitory activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC2</td>
<td>45.23 ± 0.18</td>
</tr>
<tr>
<td>α-hederin</td>
<td>11.34 ± 0.03</td>
</tr>
<tr>
<td>Hederacoside D</td>
<td>14.45 ± 0.07</td>
</tr>
<tr>
<td>Hederacoside B</td>
<td>16.99 ± 0.08*</td>
</tr>
<tr>
<td>Hederacoside C</td>
<td>35.48 ± 0.12</td>
</tr>
<tr>
<td>MC2a</td>
<td>13.84 ± 0.03</td>
</tr>
<tr>
<td>MC2b</td>
<td>17.09 ± 0.05 +a,b</td>
</tr>
<tr>
<td>MC2c</td>
<td>30.73 ± 0.11</td>
</tr>
<tr>
<td>MC2d</td>
<td>16.11 ± 0.04</td>
</tr>
<tr>
<td>MC2e</td>
<td>17.42 ± 0.04</td>
</tr>
<tr>
<td>Acarbose</td>
<td>85.39 ± 0.23</td>
</tr>
</tbody>
</table>

(Mean values followed by the same uppercase letter are not significantly different from each other at p≤0.05 according to Tukey’s HSD test).

4. Conclusion

This study was initiated with the aim of evaluating the antibacterial and inhibitory activity of α-glucosidase enzyme from H. helix species. In an effort to purify possible pure saponins compounds in H. helix species, four triterpene saponins including hederacose B, hederacose C, hederacose D, and α-hederin were successfully isolated. The results of antibacterial testing showed that fractions and active ingredients from H. helix species had low effectiveness against Pseudomonas aeruginosa and Staphylococcus aureus by agar well diffusion method. In addition, although the MC2 fraction had potential α-glucosidase inhibitory activity, the subfractions and four isolated compounds did not really show potential in the treatment of diabetes. In order to identify potential antidiabetic compounds from H. helix species, further studies are needed to isolate other compounds and test their α-glucosidase enzyme inhibitory activity.

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Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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