UPLC-PDA-ESI-MS/MS Profiling of Clerodendrum inerme and Clerodendrum splendens and Significant Activity Against Mycobacterium tuberculosis

Hesham I. Elaskary, Omar M. Sabry*, Asmaa M. Khalil, Soheir M. El Zalabani

INTRODUCTION
Antibiotic resistance like that of the multi-drug resistant Mycobacterium tuberculosis (TB), can be minimized through increasing awareness about the proper use of antibiotics and development of new antibacterial agents effective against this strain.1

UPLC brings dramatic improvements in sensitivity, resolution and speed to analytical procedures of plant metabolites.2 In UPLC-MS, the high sensitivity of MS detection provides an important tool for detection and measurement of minor metabolites in complex plant extract samples.3,4 Several studies have applied this technology to look at metabolite profiles in closely allied plant taxa, different cultivars of individual taxa, or plants at different stages of development.3,4

The aim of our work is to evaluate the antimycobacterial activity of the leaves of Clerodendrum inerme and Clerodendrum splendens and to define and compare their phytochemical composition using UPLC-PDA-ESI-MS/MS technique.

MATERIAL AND METHODS

Chemicals

For UPLC-PDA-ESI-MS/MS analysis

Solvents used for UPLC-PDA-ESI-MS/MS analysis viz., water, acetonitrile and formic acid were of LC-MS grade and acquired from Fluka, Sigma-Aldrich chemicals (Germany).

For antibacterial activity

Alamar Blue was purchased from Sigma Co. (USA) while isoniazid was purchased from Sigma Aldrich Co. (St. Louis, Mo, USA).

Plant samples

Leaves of Clerodendrum inerme L. and Clerodendrum splendens G. were collected, during the year 2018, from plants growing in the Medicinal, Aromatic and Poisonous Plants Experimental Station (MAPPES), Faculty of Pharmacy, Cairo University, Giza, Egypt. Plant material was kindly authenticated by Dr. Mohamed Gibali, senior botanist and consultant at Orman Botanic Garden, Giza, Egypt. Voucher specimens (No. 19-3-2018 I and 19-3-2018 II) were

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deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University. The leaves were air-dried then finely powdered.

**Investigation of chemical composition by UPLC-PDA-ESI-MS/MS analysis**

UPLC-PDA-ESI-MS/MS system was used for profiling of secondary metabolites. It consisted of an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, Darmstadt, Germany) coupled to an UPLC-PDA-ESI-MS/MS system (Dionex Ulti Mate 3000, Thermo Fisher Scientific), equipped with RP-C18 column (particle size 1.8 μm, pore size 100 Å, 150 mm x 1 mm i.d., Acquity HSS T3, Waters, USA) and a photodiode array detector (220–600 nm, Thermo Fisher Scientific) and operated in the negative ion mode. A mobile phase consisting of water/formic acid, 99.9/0.1 v/v (A) and acetonitrile/formic acid, 99.9/0.1 v/v (B) was used for the separation. The following binary gradient was applied: 0 to 1 min: isocratic 95% A, 5% B; 1 to 11 min: linear from 5 to 100% B; 11 to 19 min: isocratic 100% B and 19 to 30 min: isocratic 5% B. The column temperature was maintained at 40 °C. The injection volume was 2 μL and the flow rate was 150 μL/min. The apparatus was externally calibrated by the Pierce ESI negative ion calibration solution (product No. 88324) from Thermo Fisher Scientific. The data were evaluated using the software X-calibur 2.2 SP1. Metabolites were characterized by their UV–VIS spectra (220–600 nm), mass spectra and comparison to phytochemical dictionary of natural products database CRC Press and reported literature.

**EXTRACTION PROCEDURE**

For anti-mycobacterial activity

Samples (10 g, each) of the air-dried powdered leaves were separately extracted using n-hexane (60–80 °C) and the defatted mark macerated with ethanol (70%) until exhaustion. The extraction process was aided by sonication (Elma Terrasonic TS 540, Germany). The obtained ethanol extracts were filtered then dried under vacuum (rotatory evaporator Büchi, G. Switzerland).

For UPLC-PDA-ESI-MS/MS analysis

Samples (5 mg, each) of the air-dried powdered leaves were separately homogenized with 1.5 mL 80% methanol containing 10 µg/mL umbelliferone (internal standard) using a Turrax mixer (11,000 rpm) for five 20 seconds periods. To prevent heating, a period of 1 min separated each mixing period. Extracts were then vortexed vigorously and centrifuged at 3000 g for 30 min to remove plant debris. An amount of 3μL of the supernatant was used for UPLC-PDA-ESI-MS/MS analysis. Chromatographic conditions and mass spectrometry parameters followed that described in previous reports.

**ANTI-MYCOBACTERIAL ACTIVITY**

**Principle of the Assay**

The ethanol extracts (EEs) of *C. inerme* and *C. splendens* were investigated for antibacterial activity against *Mycobacterium tuberculosis* (TB) using reported methods using the Microplate Alamar Blue Assay (MABA). The latter is based on measuring the change in colour intensity of the blue colour of Alamar Blue solution by the activity of *Mycobacterium tuberculosis*. The colour intensity is measured at 590 nm using ELISA microplate reader. The percentage inhibition of mycobacterial growth by different concentrations of each sample was calculated as the average of triplicate measurements. The concentrations required to inhibit 90% of mycobacterial growth (MIC 90) and the lowest concentration that prevented colour change of Alamar Blue solution (MIC) were determined from the dose–response curve. Isoniazid was used as a reference drug.

**Procedure**

The assay was performed according to (Franzblau et al., 1998) in a black, clear-bottomed, 96-well microplate to minimize background effect. The outer perimeter wells were filled with sterile water to prevent dehydration in experimental wells. A serial dilution (0.06 – 125 μg/mL) of each of the tested extracts and the reference drug, isoniazid, dissolved in DMSO, were prepared in the microplate then 0.1 mL of *Mycobacterium tuberculosis* inoculum (10⁶ CFU/mL) was added to the wells. Wells containing bacteria only were used as negative control. Plates were incubated at 37 °C for 4 days, then 20 μL of Alamar Blue solution and 12.5 μL of 20% Tween 80 were added to the entire plate. The plates were re-incubated at 37 °C for 24 hours then the colour intensity was measured at 590 nm using ELISA microplate reader. The percentage inhibition of mycobacterial growth was calculated from the following formula:

\[
\text{% inhibition} = 1 - \left( \frac{\text{mean of test well}}{\text{mean of B well}} \right) \times 100
\]

Where mean of test well and mean of B well are the averages of triplicate determinations of the absorbance of both sample and negative control at 590 nm, respectively. MIC 90 and MIC were determined for each sample and for standard isoniazid from the dose-response curve as the concentrations required to inhibit 90% of mycobacterium growth and the lowest concentration that prevented colour change of Alamar Blue solution indicating no mycobacterium activity, respectively.

**RESULTS**

**Anti-mycobacterial activity against Mycobacterium tuberculosis (TB)**

The methanol extracts of the leaves of *C. inerme* and *C. splendens* were evaluated for anti-mycobacterial activity against *Mycobacterium tuberculosis* using the Microplate Alamar Blue Assay (MABA). Results are represented by Figure 1 and recorded in Table 1. From table 1, the methanol extract of the leaves of *C. inerme* exerted significant antibacterial activity against *Mycobacterium tuberculosis*, with MIC 90 and MIC values of 7.2 μg/mL and 15.63 μg/mL, respectively. *C. splendens* showed moderate activity with MIC 90 and MIC values of 30.8 and 62.5 μg/mL, respectively. The MIC 90 and MIC values of the reference drug, isoniazid were 0.04 and 0.24 μg/mL, respectively.

**UPLC-PDA-ESI-MS/MS metabolic analysis of the methanol extracts of the leaves**

A non-targeted UPLC-PDA-ESI-MS/MS metabolite profiling of methanolic extracts derived from leaf samples of *C. inerme* and *C. splendens* was conducted to assess for differences in their metabolite composition. Results are represented by Figure 2 and recorded in Table 2.

**Identification of phenyl-propanoid glycosides**

Two types of phenyl-propanoid glycosides were detected in the methanol extract of the leaves of *C. inerme*. The first type, represented by verbascoside, magnoloside A or D (peaks 14 and 15). The second type, represented by markhamioside B (peak 20). The methanol extract of the leaves of *C. splendens* was found to contain one type of phenyl-propanoid glycosides, represented by markhamioside C (peak 13). The MS/MS data and UV absorbance of the detected phenyl-propanoid glycosides were compared to reference literature for confirmation of their identities.

**Identification of iridoid glycosides**

Melitoside (peak 2), inerminoside A1 (peak 5), p-coumaroyl melitotside (peak 12), inerminoside B (peak 17) and inerminoside A (peak 18) were detected in the methanol extract of the leaves of *C. inerme* only. Teucardioside (peak 6), was found in the methanol extract...
Table 1: *In-vitro* anti-mycobacterial activity of the EEs of the leaves of *C. inerme* and *C. splendens* compared to standard isoniazid.

<table>
<thead>
<tr>
<th>Sample/Standard</th>
<th>MIC 90 µg/mL</th>
<th>MIC µg/mL</th>
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<tr>
<td><em>C. inerme</em> EE</td>
<td>7.2</td>
<td>15.63</td>
</tr>
<tr>
<td><em>C. splendens</em> EE</td>
<td>30.8</td>
<td>62.5</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>0.04</td>
<td>0.24</td>
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</table>

Table 2: Metabolites identified in the methanol extracts of the leaves of *C. inerme* and *C. splendens* via UPLC-PDA-ESI-MS/MS in the negative ionization mode.

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Rt (min)</th>
<th>UV</th>
<th>Mol. ion m/z (-)</th>
<th>Mol. Formula</th>
<th>MS⁺ ions m/z (-)</th>
<th>Identification</th>
<th>C.I.</th>
<th>C.S.</th>
</tr>
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<td>377.0853</td>
<td>C₁₈H₁₈O₉</td>
<td>341.1089, 215.0329</td>
<td>Dihydroxy caffeic acid hexoside</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>1.44</td>
<td>ND</td>
<td>523.1668</td>
<td>C₂₁O₁₅</td>
<td>523.1668</td>
<td>Melittoside</td>
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<td>+</td>
</tr>
<tr>
<td>3</td>
<td>1.95</td>
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<td>2-Hydroxy-2-methyl butyric acid</td>
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<td>-</td>
</tr>
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<td>4</td>
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<td>Coumaric acid hexoside</td>
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<td>-</td>
</tr>
<tr>
<td>5</td>
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<td>C₁₉H₁₄O₁₄</td>
<td>463.1819, 375.1295</td>
<td>Inerminoside A</td>
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<tr>
<td>6</td>
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<td>281.0621, 251.0523</td>
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<td>303.1869, 259.2072</td>
<td>Trihydroxy abietatrienoic acid</td>
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<td>+</td>
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<table>
<thead>
<tr>
<th>No.</th>
<th>Rt  (min)</th>
<th>% Inhibition</th>
<th>Retention Time (min)</th>
<th>Molecular Formula</th>
<th>Mass (Da)</th>
<th>Metabolite Name</th>
<th>Presence/ Absence</th>
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<td>837.4135</td>
<td>C_{24}H_{36}O_{14}</td>
<td>287.2018</td>
<td>Monolinolein tri hexoside</td>
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<td>C_{28}H_{43}O_{11}</td>
<td>555.2835, 299.0443, 259.2274, 249.2228, 155.1443, 119.0803</td>
<td>Hispidulin methoxy tetradecenoate</td>
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<td>295.2274, 249.2228, 155.1443, 119.0803</td>
<td>Hydroxy octadecadienoic acid</td>
<td>-</td>
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</tbody>
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C. I., Clerodendrum inerme; C. S., Clerodendrum splendens
(+ and (-) indicate presence and absence of a metabolite, respectively; ND, not detected; Rt, retention time.

**Figure 1:** Line graphs representing concentration-dependent percentage inhibition of *Mycobacterium tuberculosis* growth of the ethanol extracts of the leaves of *C. inerme* and *C. splendens* compared to standard isoniazid.

**Figure 2:** Representative UPLC-MS total ion chromatograms (TIC) of the methanol extracts of the leaves of *C. inerme* and *C. splendens* in the negative ionization mode.
of the leaves of C. splendens only. The identities of the detected iridoid glycosides were confirmed through comparison with MS/MS data of reference literature.16–17

Identification of flavonoids

Apigenin (peak 24), picotinolinagenin (peak 30), hydroxy trimethoxy flavone (peak 31), 4'-methyl scutellarein (peak 27), Narigenin (peak 29), 4'-methyl scutellarein-7-O-hexonide (peak 21) and acacetin-7-O-hexonide (peak 23) were identified in the methanol extract of the leaves of C. inerme. On the other hand, rhamnadin-3-O-rutinoside (peaks 11) and nitensoside A (peaks 16) were identified in the leaves of C. splendens only. For confirmation of the identities of the compounds, their MS/MS data and UV absorbances were compared to reference literature.18–20

Identification of diterpenoids

Trihydroxy abeta-trienoic acid (peak 32), was detected only in the methanol extract of the leaves of C. inerme. Marrubigenin (peak 34) was detected in both methanol extracts of the leaves of C. inerme and C. splendens. For confirmation, the MS/MS data of the identified diterpenes was compared to reference literature of their identities.21

Identification of phenolic acid derivatives

Coumaric acid hexoside (peak 4), coumaric acid pentosyl hexoside (peak 7), p-Coumaroyl melitotioside (peak 12) and galloyl hexoside acetate derivative (peak 25) were detected only in the methanol extracts of the leaves of C. inerme. Caffeoyl shikimic acid (peak 10), rosmarinic acid (peak 19) and caffeic acid derivative (peak 22) were detected only in the methanol extracts of the leaves of C. splendens. For confirmation of the identities of the identified compounds, their MS data and UV absorbances were compared to reference literature.22–24

Identification of fatty acid derivatives

2-Hydroxy-2-methyl butyric acid hexoside (peak 3), tri hydroxy octadecenoic acid (peak 26), monolinolein tri hexoside (peak 35), 2-hydroxy-2-methyl butyric acid hexoside (peak 36) were detected only in the methanol extracts of the leaves of C. splendens.

DISCUSSION

From the previous results, the methanol extract of the leaves of C. inerme showed significant anti-mycobacterial activity against Mycobacterium tuberculosis, with MIC 90 and MIC values of 7.2 µg/mL value of 15.63 µg/mL, respectively. The methanol extract of C. splendens showed moderate anti-mycobacterial activity against Mycobacterium tuberculosis with MIC 90 and MIC values of 30.8 and 62.5 µg/mL, respectively. The UPLC-PDA-ESI-MS/MS analysis revealed a total of 36 metabolites detected and tentatively identified in the two species under investigation. Twenty-eight chromatographic peaks, belonging to various metabolite classes, were assigned and identified in C. inerme, whereas 14 chromatographic peaks were assigned and identified in C. splendens. The main classes of secondary metabolites detected were phenyl-propanoid and iridoid glycosides, flavonoids, diterpenoids, phenolic acid and fatty acid derivatives.

CONCLUSION

The methanol extract of the leaves of C. inerme exerts significant antibacterial activity against Mycobacterium tuberculosis, with MIC 90 and MIC values of 7.2 µg/mL and 15.63 µg/mL. Comparative UPLC-PDA-ESI-MS/MS metabolic analysis of C. inerme showed a chromatographic profile wealthier in components compared to that of C. splendens. Thus, the leaves of C. inerme can be utilized for treatment of Mycobacterium tuberculosis infections after further investigations and can be considered a richer source of plant metabolites compared to C. splendens.

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DISCLOSURE STATEMENT

The authors have no conflicts of interest to declare.

ABBREVIATIONS

C. I.: Clerodendrum inerme; C. S.: Clerodendrum splendens; ME: Methanol Extract; MIC 90: The concentration required to inhibit 90% of bacterial growth; MIC: The lowest concentration that prevented colour change of Alamar Blue solution; ND: Not Detected; Rt: Retention time.

REFERENCES


GRAPHICAL ABSTRACT

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Elaskary et al.: UPLC-PDA-ESI-MS/MS Profiling of Clerodendrum inerme and Clerodendrum splendens and Significant Activity Against Mycobacterium tuberculosis

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