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

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ABSTRACT

Introduction: Antibiotic resistance is a major problem that is spreading and increasing while the development of new antibiotics is ceasing. As a result, some bacterial infections that were easily treated previously became untreatable. The antibacterial activity of *Clerodendrum inerme* and *Clerodendrum splendens* leaves were investigated against *Mycobacterium tuberculosis*, the widely known multi-drug resistant bacterium. UPLC-PDA-ESI-MS/MS is characterized by high sensitivity, resolution and speed for identification of plant metabolites even the minor ones. The chemical constituents of the leaves of *C. inerme* and *C. splendens* were investigated by UPLC-PDA-ESI-MS/MS metabolic analysis. **Methods:** The antibacterial activity of the ethanol extracts of the leaves of the two species under investigation was evaluated against the multi-ethanol drug resistant bacterium *Mycobacterium tuberculosis* using MABA assay. The methanol extracts of the leaves of *C. inerme* and *C. splendens* were subjected to comparative UPLC-PDA-ESI-MS/MS analysis. **Results:** The ethanol extract of *C .inerme* leaves showed significant antibacterial activity against *Mycobacterium tuberculosis*, while that of *C. splendens* showed moderate activity. The UPLC-PDA-ESI-MS/MS analysis revealed a total of 36 metabolites detected and tentatively identified in the two species under investigation, among them 28 chromatographic peaks were assigned in *C. inerme* while only 14 were assigned in these *C. splendens*. The main classes of secondary metabolites detected were Phenylpropanoid and, iridoid glycosides, flavonoids, diterpenoids, phenolic acid and fatty acid derivatives. **Conclusion:** The results of the antibacterial activity and UPLC-PDA-ESI-MS/MS analysis showed stronger activity and higher number of metabolites for *C. inerme* as compared to *C. splendens*.

Key Words: *Clerodendrum*, UPLC profiling, *Mycobacterium*, Phenyl-propanoids, Iridoids, Diterpenoids.

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.[5-8]

The aim of our work is to evaluate the anti-mycobacterial 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 Fischer Scientific, Darmstadt, Germany) coupled to an UPLC-PDA-ESI-MS/MS system (Dionex Ulti Mate 3000, Thermo Fischer Scientific), equipped with RP-C18 column (particle size 1.8 µm, pore size 100 Å, 150 mm × 1 mm i.d., Acquity HSS T3, Waters, USA) and a photodiode array detector (220–600 nm, Thermo Fischer 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 ⁰C) 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[5] was used for UPLC-PDA-ESI-MS/MS analysis. Chromatographic conditions and mass spectrometry parameters followed that described in previous reports[6].

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.[1] 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 (10^5 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;

% inhibition = 1 - (mean of test well/mean of B well) ×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).[9] 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.[10-15]

Identification of iridoid glycosides

Melittoside (peak **2**), inerminoside A1 (peak **5**), *p*-coumaroyl melittoside (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.

Sample/Standard	MIC 90 µg/mL	MIC µg/mL
C. inerme EE	7.2	15.63
C. splendens EE	30.8	62.5
Isoniazid	0.04	0.24

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.

Peak no.	Rt (min)	UV	Mol. ion m/z (-)	Mol. Formula	MS^n ions m/z (-)	Identification	C. I.	C. S.
1	0.67	249	377.0853	$C_{18}H_{18}O_9$	341.1089, 215.0329	Dihydroxy caffeic acid hexoside	+	+
2	1.44	ND	523.1668	$C_{21}H_{32}O_{15}$	523.1668	Melittoside	+	-
3	1.95	ND	279.1080	$C_{11}H_{20}O_{81}$	117.0541, 113.0235, 101.0231, 89.0235	2-Hydroxy-2-methyl butyric acid hexoside	+	-
4	8.82	350, 369	325.0924	$C_{15}H_{16}O_8$	163.0404, 119.0490	Coumaric acid hexoside	+	-
5	9.15	324, 369	507.1706	$C_{21}H_{32}O_{14}$	463.1819, 375.1295, 331.1397	Inerminoside A	+	-
6	9.35	343, 369	489.1602	$C_{21}H_{30}O_{13}$	281.0621, 251.0523, 179.0355, 161.0247	Teucardioside	-	+
7	9.54	334, 369	457.1343	$C_{20}H_{26}O_{12}$	325.0929, 205.0514, 163.0404, 119.0494	Coumaric acid pentosyl hexoside	+	-
8	9.67	329, 369	473.1292	$C_{20}H_{26}O_{13}$	341.0878, 179.0351, 135.0454	Caffeic acid pentosyl hexoside	+	+
9	9.75	329, 369	387.1656	$C_{18}H_{28}O_9$	369.1558, 207.1028, 163.1131	Coumaroyl hexoside oxinoformate	+	+
10	9.93	329, 369	335.0765	$C_{16}H_{16}O_8$	179.0353, 135.0453	Caffeoyl shikimic acid	-	+
11	10.07	325	637.1763	$C_{29}H_{34}O_{16}$	475.1462	Rhamnazin-3O-rutinoside	-	+
12	10.15	328	669.2020	$C_{30}H_{38}O_{17}$	507.1720	***p*-Coumaroyl melittoside**	+	-
13	10.32	285, 329	755.2395	$C_{34}H_{44}O_{19}$	623.1984, 593.2029, 461.1668	Markhamioside C	-	+
14	10.33	288, 332	623.1975	$C_{29}H_{36}O_{15}$	461.1669	Verbascoside	+	-
15	10.47	289, 327	623.1975	$C_{29}H_{36}O_{15}$	461.1669	Magnoloside A isomer	+	-
16	10.54	280, 331	607.1658	$C_{28}H_{32}O_{15}$	299.0563, 284.0329	**Nitensoside** A	-	+
17	10.58	268, 334	673.2704	$C_{31}H_{46}O_{16}$	507.1730, 489.1624	Inerminoside B	+	-
18	10.68	320	675.2857	$C_{31}H_{48}O_{16}$	507.1720, 489.1615	Inerminoside A	+	-
19	10.7	288, 329	359.0766	$C_{18}H_{16}O_8$	197.0467, 179.0351, 161.0246	Rosmarinic acid	-	+
20	10.84	286, 328	783.2702	$C_{36}H_{48}O_{19}$	651.2295, 607.2244, 589.2136	Markhamioside B	+	-
21	10.9	286, 331	475.0874	$C_{22}H_{20}O_{12}$	299.0560, 175.0248	4'-Methyl scutellarein-7-O- hexuronide	+	+
22	11.03	319	343.0814	$C_{18}H_{16}O_7$	223.0247, 197.0455, 179.0350, 161.0244, 145.0296, 135.0453	Caffeic acid derivative	-	+
23	11.13	269, 332	459.0925	$C_{22}H_{20}O_{11}$	283.0610, 175.0249	Acacetin-7-O-hexuronide	+	-
24	11.47	293	269.0456	$C_{15}H_{10}O_5$	269.0456, 225.0556, 201.0558, 149.0223	Apigenin	+	-
25	11.51	ND	581.2590	$C_{29}H_{42}O_{12}$	535.2559, 475.2331, 373.2021, 345.2106, 331.1913	Galloyl hexoside acetate derivative	+	-
26	11.58	ND	329.2300	$C_{18}H_{34}O_5$	314.0428, 311.2227, 293.2120, 229.1445, 213.1140, 171.1028	Tri hydroxy octadecenoic acid	+	-
27	11.69	297, 339	299.0558	$C_{16}H_{12}O_6$	284.0328, 153.0196, 119.505	**4'-Methyl scutellre**in	+	-
28	11.71	ND	287.2226	$C_{16}H_{32}O_4$	287.2226, 269.2126, 241.2175	**Di hydroxy hexadecenoic acid**	+	+
29	11.86	292	271.0606	$C_{15}H_{12}O_5$	256.0379, 119.0488	Naringenin	+	-
30	11.88	292, 334	313.0713	$C_{17}H_{14}O_6$	298.0485, 283.0249	Pictolinarigenin	+	-
31	11.99	293, 328	343.0816	$C_{18}H_{16}O_7$	328.0589	Hydroxy trimethoxy flavone	+	-
32	12.04	293	347.1858	$C_{20}H_{28}O_5$	303.1869, 259.2072, 243.1760	**Trihydroxy abietatrienoic acid**	+	-

33	12.28	ND	837.4135	$C_{39}H_{66}O_{19}$	----	Monolinolein tri hexoside	+	-
34	12.37	ND	331.1910	$C_{20}H_{28}O_4$	287.2018	Clerodermic acid	+	+
35	13.69	ND	555.2835	$C_{28}H_{43}O_{11}$	555.2835, 299.0443, 255.2332, 225.0073	Hispidulin methoxy tetradecenoate	+	-
36	13.88	ND	295.2274	$C_{18}H_{32}O_3$	295.2274, 249.2228, 155.1443,	Hydroxy octadecadienoic acid	-	+

C. I., *Clerodemdrum inerme*; C. S., *Clerodendrum splendens*
(+) and (-) indicate presence and absence of a metabolite, respectively; ND, not detected; Rt, retention time.

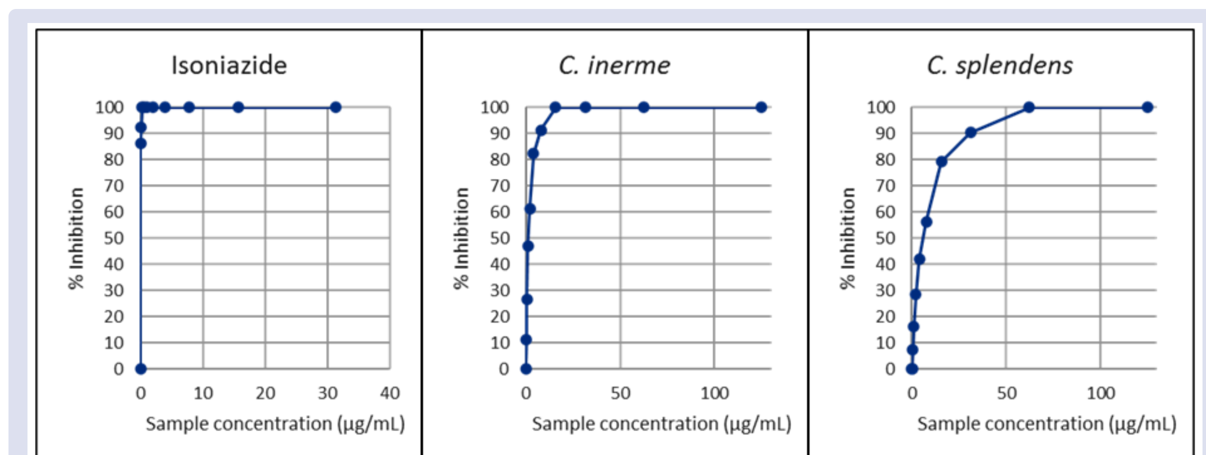

Figure 1: Line graphs representing concentration-dependent percentage inhibition of *Mycobacterium tuberculosis* growth of the ethanol extracts of the leaves of *C. inerme and C. splendens* compared to standard isoniazid.

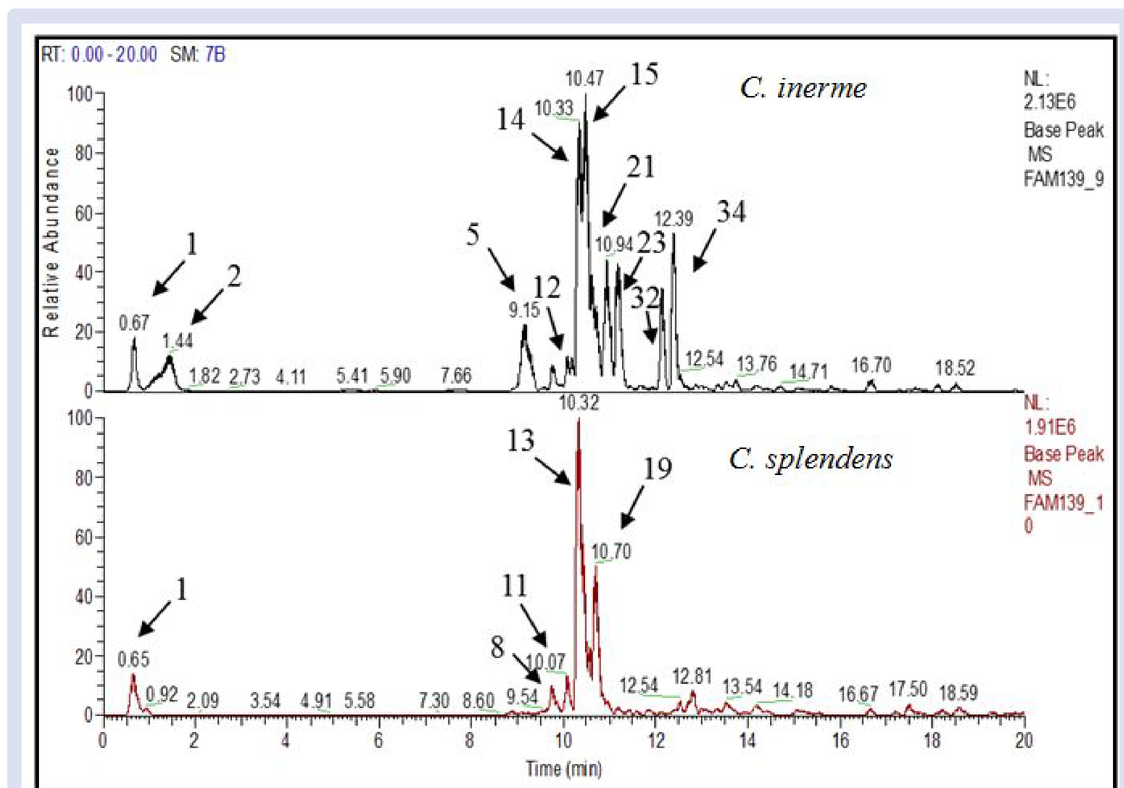

Figure 2: Representative UPLC-MS total ion chromatograms (TIC) of the methanol extracts of the leaves of *C. inerme* and *C. splendens* in the negative ionization mode.

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**), pictolinarigenin (peak **30**), hydroxy trimethoxy flavone (peak **31**). 4'-methyl scutellarein (peak **27**), Naringenin (peak **29**), 4'-methyl scutellarein-7-O-hexuronide (peak **21**) and acacetin-7-O-hexuronide (peak **23**) were identified in the methanol extract of the leaves of *C. inerme*. On the other hand, rhamnazin-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 abieta-trienoic acid (peak **32**), was detected only in the methanol extract of the leaves of *C. inerme*. Marrubiagenin (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 melittoside (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 **33**) and Hispidulin methoxy tetradecenoate (peak **35**) were detected only in the methanol extracts of the leaves of *C. inerme*. hydroxy octadecadienoic acid (peak **36**) was 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.: *Clerodemdrum 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.

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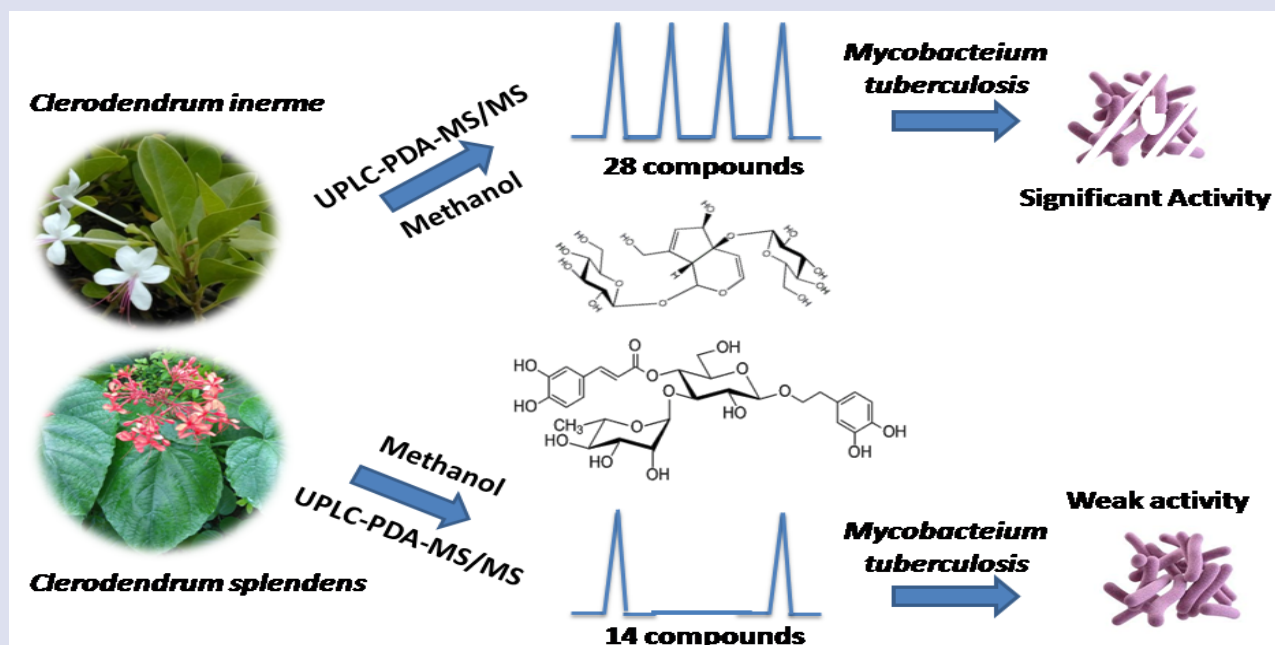
GRAPHICAL ABSTRACT



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