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Secondary Metabolites from the leaves and stems of *Leonotis nepetifolia* (Lamiaceae)

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ABSTRACT

Leonotis nepetifolia (L.) R. Br. (Lamiaceae), commonly known as 'Shandilay', is well-known and widely used in Trinidadian ethnomedicine as well as in different countries for the treatment of various ailments. Biological activities for the plant and many phytoconstituents have been reported; however, the plant species located in Trinidad is yet to be investigated for its chemical constituents. This study was conducted to investigate the secondary metabolites that may be responsible for the observed traditional medicinal uses of the plant. A phytochemical investigation of the chloroform and 1-butanol fractions of the crude methanol extract of the leaves and stems from *L. nepetifolia* resulted in the isolation of 7 compounds which were identified as Stigmasterol-3-*O*-β-D-glucoside **(1)**, *trans*-caffeic acid **(2)**, *trans-p*-coumaric acid **(3)**, Apigenin-7-*O*-β-D-glucoside **(4)**, Uridine **(5)**, Luteolin-7-*O*-β-D-glucoside **(6)** and Adenosine **(7)**. Isolation of the seven compounds was performed by a combination of silica gel column chromatography and size exclusion column chromatography on Sephadex® LH-20. The chemical structures of these compounds were determined by comprehensive analyses of their ^{1}H and ^{13}C NMR, COSY, HSQC, and HMBC spectroscopic data, and HREIMS mass spectrometric data, as well as comparison of spectral data with that reported in the literature. To the best of the author's knowledge, this is the first report on the isolation of compounds **1–7** from *L. nepetifolia*, compounds **1-3** and **5** from the genus *Leonotis* and compound **7** from the Lamiaceae family. These reported bioactive constituents might be responsible for some of the observed biological activities of the plant *Leonotis nepetifolia*.

Keywords: *Leonotis nepetifolia*, Shandilay, Lamiaceae, Isolation, Structure elucidation, Trinidad

Introduction

Leonotis nepetifolia (L.) R. Br. (family: Lamiaceae, syn. Labiatae), also known as Lion's Ear and Klip Dagga, is an important medicinal plant with a long history of numerous traditional medicinal uses in various countries, which include the treatment of bronchial asthma, diarrhoea, fever, rheumatism, influenza and malaria.[1-3] This plant exhibits a diverse range of salutary pharmacological activities and phytochemical examination of its plant parts indicated the presence of a number of bioactive phytoconstituents. Characterized by orange-scarlet, coroneted, verticillaster inflorescences and a distinct plant odour, this species is widespread throughout tropical Africa, India, tropical America and the West Indies.[3-5] The genus *Leonotis* has 12 species and is represented by one species, *L. nepetifolia* in Trinidad, where it is commonly referred to as Shandilay.[2] An ethnobotanical survey of medicinal plants used in Trinidad indicated *L. nepetifolia* as the most frequently used plant species in traditional medicine on the island.[6] The ailments reportedly treated with *L. nepetifolia* in Trinidad are common cold, cough, fever, asthma, diabetes, womb prolapse, malaria, menstrual pain and as a cooling/cleanser.[2,6-10]
Previous phytochemical studies of *L. nepetifolia* indicated Labdane diterpenoids, such as nepetaefuran, leonotinin, and leonotin,[11] as the predominant class of chemical constituents isolated from *Leonotis nepetifolia*. The seed oil contains laballenic acid, an allenic acid.[12]
Other secondary metabolites such as flavonoids (apigenin and cirsiliol),[11] iridoid glycosides (10-*O*-(trans-3,4-dimethoxycinnamoyl) geniposidic acid and 10-*O*-(p-hydroxybenzoyl)geniposidic acid),[5] coumarins (4,6,7-Trimethoxy-5-methylchromen-2-one)[13] and phenylethanoid glycosides (acteoside, martynoside)[5] have been reported. In addition, a range of biological activities including antifungal, antibacterial, antidiabetic, antioxidant, anticancer, anti-inflammatory, anticonvulsant, anxiolytic, wound healing and hepatoprotective activities have been reported for the crude extracts or pure compounds from this plant, making it a potential source for the isolation of more bioactive chemical constituents.[1-5, 14-18]
In the search for phytochemicals from medicinal plants of Trinidad and Tobago, the chemical constituents of the leaves and stems of *L. nepetifolia* were investigated. Herein, we report the isolation and identification of seven compounds from the plant for the first time viz. Stigmasterol-3-*O*-β-D-glucoside **(1)**, *trans*-caffeic acid **(2)**, *trans-p*-coumaric acid **(3)**, Apigenin-7-*O*-glucoside **(4)**, Uridine **(5)**, Luteolin-7-*O*-β-D-glucoside **(6)** and Adenosine **(7)**.

Materials and Methods

General experimental procedures
NMR spectra were recorded on a Bruker AVANCE DRX-600 spectrophotometer at 600 MHz (^{1}H) and 150 MHz (^{13}C). Samples were prepared in CD_3OD and DMSO-d_6 with tetramethylsilane (TMS) as an internal standard. The chemical shift (δ) values were measured relative to the internal standard in ppm. High Resolution Electron Spray Ionization Mass Spectrometry (HRESIMS) data was recorded on a micrOTOF-Q (Bruker Daltonics) Mass Spectrometer. TLCs were performed on aluminium-backed plates pre-coated with 0.20 mm thick silica gel 60 F_{254} (Sigma-Aldrich). The developed plates were

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visualized by UV light (254/366 nm). Column chromatography (CC) was performed by gravity using glass columns of appropriate sizes with Silica gel 60 Å, 70-230 mesh (Sigma-Aldrich) or Sephadex® LH-20 (Sigma –Aldrich) as the adsorbent. The solvents used for extractions and chromatographic separations were freshly distilled prior to use according to standard procedures.

Collection and identification of the plant material

The leaves and stems of *L. nepetifolia* were collected from La Horquette Road, Glencoe, Trinidad in August 2013. The sample was identified and authenticated by Ms. Keisha Manuare at the National Herbarium of Trinidad and Tobago, W.I., where a voucher specimen (TRIN. 50158) has been deposited.

Preparation of the extracts

The combined leaves and stems of *L. nepetifolia* were oven-dried for two days at 40 ºC to obtain 932.8 g of dried sample (leaves and stems). This sample was pulverized in a 4 L waring blender and exhaustively extracted with methanol, filtered and evaporated at 40ºC in vacuo to yield 97.4 g of an oily dark green crude extract. The crude methanol extract was suspended in methanol:water (9:1), defatted with petroleum ether and sequentially partitioned with chloroform (3 × 500 mL) and 1-Butanol (3 × 500 mL), affording 16.5 g of chloroform fraction and 14.5 g of 1-Butanol fraction.

Isolation and structural elucidation

The chloroform fraction was subjected to silica gel column chromatography and eluted with gradient mixtures of Pet. ether:$CHCl_3$ (20:80, 10:90, 0:100) to yield 8 combined fractions (F1-F8). F6 (8.8 g) was further subjected to silica gel column chromatography and eluted with gradient mixtures of $CHCl_3$:MeOH (98:2, 95:5, 90:10, 85:15) to afford 5 sub-fractions (SF1-SF5). SF2 was obtained as compound **1** (63 mg). SF4 (569 mg) was further purified by silica gel column chromatography with gradient mixtures of Pet. ether:EtOAc (6:94, 0:100) to afford compounds **2** (6.9 mg), **3** (34.8 mg) and **4** (79.3 mg). The 1-butanol fraction was chromatographed over a silica gel column and eluted with gradient mixtures of $CHCl_3$:MeOH (88:12, 80:20) to afford 7 combined fractions (B1-B7). Fraction B4 (1.1 g) was further purified by silica gel column chromatography eluted with $CHCl_3$:MeOH (85:15) to yield ten sub-fractions (SB4-1 - SB4-10). SB4-6 (184 mg) was subjected to size exclusion column chromatography on Sephadex® LH-20 with $CHCl_3$:MeOH (95:5) to yield compound **5** (5.7 mg). Fraction B5 (498 mg) was subjected to silica gel column chromatography eluting with EtOAc:MeOH:H_2O (90:6:4) to afford 12 sub-fractions (SB5-1 – SB5-12), of which SB5-4 was compound **6** (7.2 mg). SB5-8 (21.9 mg) was purified using size exclusion column chromatography on Sephadex® LH-20 with $CHCl_3$:MeOH (95:5) to give compound **7** (7.3 mg).

The structures of the isolated compounds were elucidated unequivocally by means of spectroscopic experiments, mainly ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC and mass spectrometry (MS). All spectroscopic data were also compared with their respective literature data and the isolated compounds were identified as stigmasterol-3-*O*-*β*-D-glucoside (**1**),[19] trans-caffeic acid (**2**),[20] trans-p-coumaric acid (**3**),[21] Apigenin-7-*O*-glucoside (**4**),[22] uridine (**5**),[23] Luteolin-7-*O*-*β*-D-glucoside (**6**),[24] and adenosine (**7**)[25] (Figure 1).

Results and Discussion

Seven compounds were isolated from the titled plant for the first time. To the best of the author's knowledge, this is the first report on the isolation of compounds **1–7** from *L. nepetifolia*, compounds **1-3** and **5** from the genus *Leonotis* and compound **7** from the Lamiaceae family. This study revealed a qualitative difference in the production of secondary metabolites in *L. nepetifolia* from Trinidad, W.I. compared to the same species from other regions. The ecological function of secondary metabolites is primarily survival of the plant as defense (against microbes, herbivores, viruses or competing plants) and signal compounds (to attract pollinating or seed dispersing animals).[26] These secondary metabolites may have been produced in response to some factor in the external environment of the collected species *L. nepetifolia*. Factors such as physiological variations, geographic variations and environmental conditions influence the production and accumulation of secondary metabolites and account for the significant differences.[27] The seven isolates are classified as one plant sterol (Stigmasterol-3-*O*-β-D-glucoside), two phenylpropanoids of the hydroxycinnamic acids subcategory (*trans*-caffeic acid and *trans*-*p*-coumaric acid), two flavone glycosides (Apigenin-7-*O*-β-D-glucoside and Luteolin-7-*O*-β-D-glucoside) and two nucleosides (Uridine and Adenosine). Stigmasterol-3-*O*-β-D-glucoside and Apigenin-7-*O*-glucoside were the major compounds isolated from *L. nepetifolia* in the present study. Stigmasterol-3-*O*-β-D-glucoside has been shown to possess antifungal activity against *Aspergillus niger*[19] and apigenin-7-*O*-glucoside exhibited anti-HIV activity.[28] *Trans*-caffeic acid has shown neuroprotective and anti-oxidant effects[20] and *trans*-*p*-coumaric acid has displayed significant antioxidant and anti-inflammatory properties and is also reported to have potential in the primary prevention of vascular diseases.[29] Luteolin-7-*O*-glucoside has been reported to exhibit antibacterial activity against *Salmonella typhimurium* and antifungal activity against *Alternaria alternata.*[24] Uridine and Adenosine has demonstrated a range of therapeutic activities, such as, antidepressant and anti-inflammatory.[30]

These findings suggest *L. nepetifolia* collected in Trinidad as a potential source of interesting phytochemicals. The presence of these seven constituents may provide some scientific evidence in favour of the traditional medicinal uses of *L. nepetifolia*.

Stigmasterol-3-*O*-*β*-D-glucoside (1): White amorphous powder (63 mg); yielded a positive indication of steroid base on the Liebermann-Burchard test; HRESIMS (positive mode) *m/z* 597.3884 $[M+Na]^+$ (calcd. for $C_{35}H_{58}O_6Na$, 597.4126); ^{1}H-NMR (600 MHz, DMSO-d_6): δ 5.32 (1H, br d, J = 4.6 Hz, H-6), 5.14 (1H, dd, J = 15.2, 8.4 Hz, H-22), 5.02 (1H, dd, J = 15.2, 8.4 Hz, H-23), 4.90 (1H, br s, OH-3′), 4.88 (1H, br *s*, OH-2′), 4.45 (1H, br *s*, OH-4′), 4.37 (1H, t, J = 6.0 Hz, OH-6′), 4.21 (1H, d, J = 7.8 Hz, H-1′), 3.65-2.85 (6 H, m, H-2′, 3′, 4′, 5′ and 6′), 3.42 (1H, m, H-3), 2.36 (1H, m, H-1a), 2.11 (1H, m, H-1b), 2.00-1.00 (16 H, m, H-2, 4, 7, 11, 12, 15, 16 and 28), 1.60 (1H, m, H-25), 1.49 (1H, *m*, H-24), 1.48 (1H, m, H-8), 1.32 (1H, m, H-20), 1.11 (1H, m, H-17), 1.06 (1H, m, H-14), 0.99 (1H, br s, H-9), 0.97 (3H, s, H-19), 0.89 (3H, d, J = 6.4 Hz, H-21), 0.85 (3H, d, J = 6.8 Hz, H-26), 0.82 (3H, d, J = 6.8 Hz, H-27), 0.78 (3H, t, J = 7.1 Hz, H-29), 0.65 (3H, s, H-18); ^{13}C-NMR (150 MHz, DMSO-d_6): δ 140.5 (C-5), 138.1 (C-22), 128.9 (C-23), 121.3 (C-6), 100.8 (C-1′), 77.0 (C-3), 76.8 (C-3′), 76.7 (C-5′), 73.5 (C-2′), 70.2 (C-4′), 61.2 (C-6′), 56.2 (C-14), 55.4 (C-17), 50.6 (C-24), 49.7 (C-9), 41.9 (C-13), 41.8 (C-12), 38.4 (C-1), 36.9 (C-4), 36.3 (C-10), 35.5 (C-20), 33.4 (C-2), 31.9 (C-8), 31.5 (C-7), 31.4 (C-25), 29.3 (C-16), 24.9 (C-15), 23.9 (C-28), 22.7 (C-11), 19.1 (C-26), 19.0 (C-19), 18.9 (C-27), 18.7 (C-21), 11.72 (C-18), 11.71 (C-29).

***trans*-caffeic acid (2)**: White crystals (6.9 mg); HRESIMS (negative mode) *m/z* 179.0305 $[M-H]^+$ (calcd. for $C_9H_7O_4$, 179.0339); ^{1}H-NMR (600 MHz, CD_3OD): δ 7.53 (1H, d, J = 15.9 Hz, H-7), 7.04 (1H, d, J = 2.0 Hz, H-2), 6.93 (1H, dd, J = 8.1, 2.0 Hz, H-6), 6.78 (1H, d, J = 8.1, H-5), 6.22 (1H, d, J = 15.9 Hz, H-8); ^{13}C-NMR (150 MHz, CD_3OD): δ 171.1 (C-9), 149.4 (C-4), 147.1 (C-7), 146.8 (C-3), 127.8 (C-1), 122.8 (C-6), 116.5 (C-5),115.6 (C-8), 115.1 (C-2).

trans-p-coumaric acid (3): White amorphous powder (34.8 mg); ^{1}H-NMR (600 MHz, DMSO-d_6): δ 7.49 (1H, d, J = 15.9, H-7), 7.36 (2H, d, J = 8.3 Hz, H-2,6), 6.67 (2H, d, J = 8.3 Hz, H-3,5), 6.33 (1H, d, J = 15.9 Hz, H-8); ^{13}C- NMR (150 MHz, DMSO-d_6): δ 167.4 (C-9), 160.5 (C-4), 145.3 (C-7), 130.7 (C-2,6), 126.4 (C-1), 116.1 (C-3,5), 114.2 (C-8).

Apigenin-7-*O*-*β*-D-glucoside (4): Yellow amorphous powder (79.3 mg); yielded a positive response to the Shinoda test; ^{1}H-NMR (600 MHz, DMSO-d_6): δ 12.99 (1H, br s, OH-5), 7.93 (2H, d, J = 8.1 Hz, H-2′,6′), 6.90 (2H, d, J = 8.1 Hz, H-3′,5′), 6.81 (1H, s, H-3), 6.79 (1H, d, J = 2.1 Hz, H-8), 6.48 (1H, d, J = 2.1 Hz, H-6), 5.18 (1H, d, J = 7.2 Hz, H-1″), 4.50-3.20 (6 H, m, H-2″, 3″, 4″, 5″ and 6″); ^{13}C-NMR (150 MHz, DMSO- d_6): δ 182.9 (C-4), 164.8 (C-2), 163.2 (C-7), 162.3 (C-4′), 161.8 (C-5), 157.7 (C-9), 129.5 (C-2′,6′), 122.1 (C-1′), 116.6 (C-3′,5′), 105.4 (C-10), 103.4 (C-3), 100.0 (C-1″), 99.9 (C-6), 95.2 (C-8), 75.9 (C-5″), 73.6 (C-3″), 72.5 (C-2″), 69.5 (C-4″), 62.9 (C-6″).

Uridine (**5**): Yellow gum (5.7 mg); HRESIMS (positive mode) *m/z* 267.0627 $[M+Na]^+$ (calcd. for $C_9H_{12}N_2O_6Na$, 267.0588); ^{1}H-NMR (600 MHz, CD_3OD): δ 7.99 (1H, d, J = 8.1 Hz, H-6), 5.89 (1H, d, J = 4.6 Hz, H-1'), 5.68 (1H, d, J = 8.1 Hz, H-5), 4.17 (1H, t, J = 5.0 Hz, H-2'),

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4.14 (1H, t, *J* = 5.0 Hz, H-3'), 3.89 (1H, m, H-4'), 3.82 (1H, dd, *J* = 12.2, 2.7 Hz, H-5'a), 3.72 (1H, dd, *J* = 12.2, 3.1 Hz, H-5'b); ^{13}C-NMR (150 MHz, CD_3OD): *δ* 166.1 (C-4), 152.4 (C-2), 142.7 (C-6), 102.6 (C-5), 90.7 (C-1'), 86.3 (C-4'), 75.7 (C-2'), 71.3 (C-3'), 62.3 (C-5').

Luteolin-7-*O*-*β*-D-glucoside (**6**, Cynaroside): Yellow amorphous powder (7.2 mg); yielded a positive response to the Shinoda test; HRESIMS (positive mode) *m/z* 471.0878 $[M+Na]^+$ (calcd. for $C_{21}H_{20}O_{11}Na$, 471.0898); ^{1}H-NMR (600 MHz, DMSO-d_6): *δ* 13.03 (1H, br s, OH-5), 7.44 (1H, dd, *J* = 8.1, 2.1 Hz, H-6′), 7.40 (1H, d, *J* = 2.1 Hz, H-2′), 6.86 (1H, d, *J* = 8.1 Hz, H-5′), 6.78 (1H, d, *J* = 2.0 Hz, H-8), 6.73 (1H, s, H-3), 6.43 (1H, d, *J* = 2.0 Hz, H-6), 5.08 (1H, d, *J* = 7.6 Hz, H-1″), 3.70-3.17 (6 H, m, H-2″, 3″, 4″, 5″ and 6″); ^{13}C-NMR (150 MHz, DMSO-d_6): δ 181.5 (C-4), 164.1 (C-2), 162.7 (C-7), 161.2 (C-5), 156.6 (C-9), 149.7 (C-4′), 145.9 (C-3′), 121.1 (C-1′), 118.9 (C-6′), 115.7 (C-5′), 113.6 (C-2′), 105.2 (C-10), 103.2 (C-3), 99.6 (C-1″), 99.4 (C-6), 94.5 (C-8), 77.1 (C-5″), 76.2 (C-3″), 73.1 (C-2″), 69.5 (C-4″), 60.4 (C-6″).

Adenosine (**7**): White crystals (7.3 mg); HRESIMS (positive mode) *m/z* 268.1006 $[M+H]^+$ (calcd. for $C_{10}H_{14}N_5O_4$, 268.1040); ^{1}H-NMR (600 MHz, DMSO-d_6): *δ* 8.35 (1H, s, H-8), 8.13 (1H, s, H-2), 7.34 (2H, br s, H-NH_2), 5.87 (1H, d, *J* = 6.2 Hz, H-1'), 5.53 (1H, m, OH-2'), 5.43 (1H, m, OH-5'), 5.27 (1H, br s , OH-3'), 4.61 (1H, t, *J* = 5.6 Hz, H-2'), 4.14 (1H, m, H-3'), 3.96 (1H, m, H-4'), 3.66 (1H, m, H-5'a), 3.55 (1H, m, H-5'b); ^{13}C-NMR (150 MHz, DMSO-d_6): *δ* 156.2 (C-6), 152.3 (C-2), 149.2 (C-4), 139.1 (C-8), 119.5 (C-5), 87.8 (C-1'), 85.9 (C-4'), 73.7 (C-2'), 70.8 (C-3'), 61.7 (C-5').

Conclusion

The study revealed that *L. nepetifolia* is rich in phytochemicals, all of which have been reported to exhibit different physiological activities. This study, to the best of the author's knowledge, is the first report on the isolation of compounds **1**–**7** from *L. nepetifolia*, compounds **1**-**3** and **5** from the genus *Leonotis* and compound **7** from the Lamiaceae family. These reported bioactive constituents might be responsible for some of the observed biological activities of the plant *Leonotis nepetifolia*. Further validation of the ethnomedicinal usage of different parts of the plants in the management of diseases is warranted.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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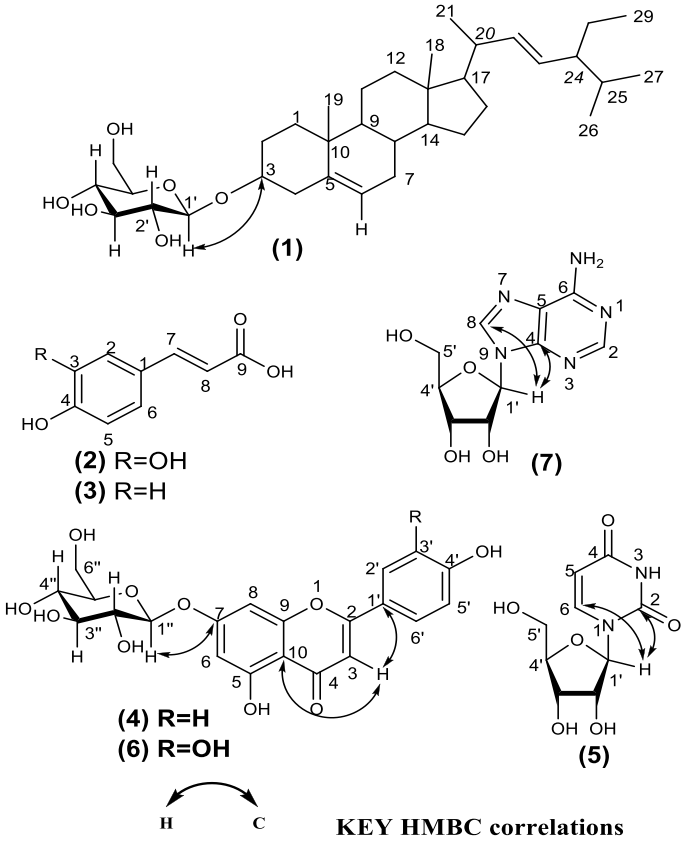

Figure 1: Chemical structures of compounds **1-7** isolated from *Leonotis nepetifolia*.

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