SUPPLEMENTARY MATERIAL

New Chemical constituent from the stem of Cuscuta reflexa Roxb. and its biological activities

Zulfa Nooreen,¹ Sudeep Tandon,¹ Narayan Prasad Yadav,² and Ateeque Ahmad¹*  

¹Process Chemistry and Technology Department, Central Institute of Medicinal and Aromatic Plants, Lucknow-226015, India  
²Herbal Medicinal Product Department, Central Institute of Medicinal and Aromatic Plants Lucknow-226015, India.  

*Corresponding author. E-mail address: ateeque97@gmail.com; a.ahmad@cimap.res.in (A. Ahmad)  

ABSTRACT  

Chemical investigations on the stem of Cuscuta reflexa Roxb. (Convolvulaceae) led to the isolation of one new compound characterised as 3', 4'-dimethoxy-1-phenyl-1α, 2-ethanediol (1), along with eight known compounds as tridecanyl palmitate, palmitic acid, n-pentatriacontane, n-triacont-21, 27-dien-1-ol, kaempferol, chlorogenic acid, 5, 7-dimethoxyapigenin and quercitin. The chemical structures were established with the help of physical, chemical and spectroscopic methods. The antimicrobial potential of the new compound (1) was evaluated against three bacterial and three fungal pathogenic strain and showed significant activities.  

KEYWORDS: Cuscuta reflexa; Convolvulaceae; new compound; spectroscopic analysis; antimicrobial activity.
3. Experimental

3.1 General

The whole plant of *C. reflexa* was purchased from local market of Lucknow, State of Uttar Pradesh, India in the month of October, 2017 and separate the seed and stem part. The voucher specimen was deposited in the herbarium of our institute, Head of Botany Division, identified material *Cuscuta reflexa* accession number 774. The material after extraction was subjected to column chromatography over silica gel normal chromatography to give a new chemical compound.

All the chemicals, reagents and the solvents used in the assay protocols were of analytical grade. n-Hexane, EtOAc, MeOH, C₂H₅OH, H₂SO₄ of analytical grade and other chemicals were procured from Merck, India. Column chromatography was conducted by using silica gel (70–230 mesh) and LiChroprep RP-18 (40–63 µm; ODS silica gel) from Merck. Thin layer chromatography was achieved on glass backed precoated silica gel 60 F254 plates (Merck). Resolution of the spots in TLC plates was checked by dipping the plate in a solution of 5% vanillin-H₂SO₄ in C₂H₅OH. Standards were procured from Sigma-Aldrich, USA. Instrumentation details such as optical rotation, FT-IR, ESI/FT mass and NMR spectra were given in the literature (Nooreen et al., 2019).

**Extraction of fruits**

Dried stem of *C. reflexa* (2 Kg) in powdered form were extracted with methanol (4 L) by cold percolation for three days and concentrated in vacuo to obtained a semisolid brown liquid to yield (250 gm) of an extract was obtained.

**Isolation of the compounds from methanolic extract**

The entire methanolic extract (250 g) was subjected to silica gel column (4 kg; 60-120 mesh size) and wash out with solvenr of n-hexane, n-hexane-EtOAc (9:1-1:9, v/v), EtOAc, and MeOH to give 11 fractions (frs.; each of 15 L). Fraction were checked by TLC and showing complex mixtures, fraction 2 (19 g, n-hexane-EtOAc 9.5:0.5) were chromatographed over silica gel column (200 g; 60-120 mesh size, each fraction of 50 ml) yields (n-pentatriacone(42 mg), tridecanyl palmitate (15 mg), n-Tritriacon-21, 27-dien-1-ol (25 mg) and palmitic acid (19 mg), fraction 6 (38 g, obtained in n-hexane-EtOAc 3:7) were re-
chromatographed over silica gel column (350 g; 60-120 mesh size; each fraction of 100 ml). The elution was sequentially performed with CHCl₃, CHCl₃-MeOH (9.5:0.5, 9:1, 8:2, 7:3 v/v), MeOH to yield 104 fractions. Frs. 47-58 (obtained in CHCl₃-MeOH; 9.5:0.5) after rechromatography over Lichroprep RP-18 ODS (100 g, each fractions of 50 ml). The elution was sequentially performed with methanol 80%, 60%, 40%, 20%, 10% and 0% of water to yield mixture of two and further purification over silica gel column with Chloroform and yield one new compound 1 (32 mg) and 5,7-dimethoxyapigenin (45 mg) and quercetin (34 mg) over LiChroprep RP-18(ODS silica gel; 40-63 µm). Fraction 9 (42 g, n-hexane-EtOAc 7:3) were chromatographed over silica gel column (420 g; 60-120 mesh size, each fraction of 100 ml) yields 50 fractions. Fraction 15 The elution was sequentially performed over silica gel column (5.4 g; 200-400 mesh size, each fraction of 20 ml) with CHCl₃, CHCl₃-MeOH (9.5:0.5, 9:1, 8.5:1.5, 8:2 7.5:2.5, 7:3, 6.5:3.5, 6:4, 1:1, 6:4, 7:3, 8:2, 9:1 v/v), MeOH to yield 54 fractions yields kaemperol (29 mg) and chlorogenic acid (32 mg).

Antimicrobial activity

Antifungal assays

The antifungal activity of the compound 1 was tested against Candida albicans (ATCC 14053), Candida albicans clinical isolates (AI) and Candida kefyr (ATCC 204093) Cultures of fungi were grown on Sabouraud Dextrose Broth (Hi Media Pvt, Ltd., India) for 24 h at 37 °C and then turbidity was adjusted to 0.5 McFarland standards (approximately 1.2 × 106 CFU/mL). Inoculum (0.5 McFarland) of the fungal culture (100 µL) was withdrawn with caution and uniformly spread over the surface of Sabouraud Dextrose agar plate to get even lawn. The ZA/11 was impregnated on the sterile paper disc (5 mm diameter, Whatman No. 3 filter paper) and placed on the fungal lawns. The plates were then incubated for 24 h (37 °C) following which the diameter of the inhibition zone was measured. The net zone of growth inhibition was determined by subtracting the disc diameter (that is 5 mm) from the total zone of growth inhibition shown by the test disc in terms of clear halo fungal lawn around the disc. MIC (minimum inhibitory concentration) was estimated using micro dilution broth assays. For this purpose, serial dilution of two fold series was employed to determine the MIC of the compound. In each assay, 10 µL of fungal culture (0.5 McFarland) prepared as before was added to the 1.0 mL medium and incubated at 37 ± 1 °C and the killing or inhibition was examined by visible turbidity. MFC (minimal fungicidal concentration) was determined by plating 100 µL from each tube used for determining MIC
and observed for any growth after 2 days of incubation. Ketoconazole was used as standard in antifungal activity evaluation (Saikia et. al., 2001).

**Antibacterial assays**

Antibacterial activity of compound **1** was determined using filter paper disc diffusion assay [23]. Inoculums of the test bacteria [Gram-positive: Staphylococcus aureus (MTCC 96), *Escherichia coli* (DH5α) and *Pseudomonas aeruginosa* (MTCC 741)] were prepared equivalent to McFarland Standard 0.5. Bacterial lawns were made uniformly using 100-μL inoculums on a Mueller–Hinton agar plate. Filter paper (Whatman) discs (5.0 mm) soaked with test compound were placed over seeded plates. The plates were incubated at 37 °C for 24 h. Activity was measured in terms of zone of growth inhibition (mm) determined by subtracting the disc diameter (that is 5.0 mm) from the total zone of inhibition shown by the test disc in terms of clear zone around the disc. The tests were performed in triplicate. The bacterial strains were obtained from the Microbial Type Culture Collection Centre (MTCC), CSIR-Institute of Microbial Technology (IMT) Chandigarh, India. Antibacterial efficacy of the compound **1** was also determined by microdilution broth assay using 96 ‘U’ bottom micro-titer plates as per CLSI guidelines (Wayne et al., 2012). Samples were serially diluted two folds (in the range of 1000–1.95 μg/mL) in Mueller–Hinton Broth (MHB). The broth was inoculated with 10.0 μL of diluted 24-h grown culture of test organisms with a titre equivalent to 0.5 McFarland standards. The inoculated plates were incubated at 37 °C for 16–24 h and the growth was recorded spectrophotometrically at 600 nm using Spectramax 190-microplate reader (Molecular Devices, CA, and USA). The MIC value was determined from the turbid metric data as the lowest concentration showing growth inhibition as compared to control. An antibiotic norfloxacin was taken as positive control, while DMSO served as negative control. Experimental observations were performed in triplicate to reduce error during the procedure.

**Spectral data of known compounds 2–9.**

**n-Pentatriacontane (2).** Colourless crystalline mass; m.p. 74–76°; Rf 0.73 (n-hexane-EtOAc; (7:3); UV λ\text{max} (MeOH): UV λ\text{max} (MeOH0: 211 nm; IR\text{v}_\text{max} (KBr): 2927, 2841, 1620, 1377, 1128, 889, 720 cm\textsuperscript{-1}; \textsuperscript{1}H NMR (CDCl\textsubscript{3}; 500 MHz): δ 1.55 (2H, m, CH\textsubscript{2}), 1.28 (4H, m, 2 x CH\textsubscript{2}), 1.25 (60 H, br s, 30 x CH\textsubscript{2}), 0.89 (3H, t, J = 7.0 Hz, Me-1); 0.86 (3H, t, J = 7.0 Hz, Me-35); \textsuperscript{13}C NMR (CDCl\textsubscript{3}; δ 125 MHz): δ 37.12 (CH\textsubscript{2}), 31.94 (CH\textsubscript{2}), 30.06 (CH\textsubscript{2}), 29.72 (27 x
CH$_2$), 29.68 (CH$_2$), 29.38 (CH$_2$), 29.18 (CH$_2$), 27.11 (CH$_2$), 22.71 (CH$_2$), 14.12 (Me-1. Me-35); ESIMS m/z (re. int.): 493 [M+H]$^+$ (C$_{38}$H$_{73}$) (88.6).

**Tridecanyl palmitate (3).** Yellow crystals; mp 63–65$^0$; R$_f$ 0.55 (n-hexane:EtOAc; 9:1); UV $\lambda_{\text{max}}$ (MeOH): 208 nm; IR$_{\text{max}}$ (KBr): 3141, 2918, 2849, 1720, 1617, 1466, 1431, 1410, 1294, 1227, 1206, 1186, 939, 723 cm$^{-1}$; $^1$H NMR (CDCl$_3$; 500 MHz): $\delta$ 4.12 (2H, t, $J = 7.0$ Hz, H-2'), 2.33 (2H, t, $J = 7.5$ Hz, H-2'), 2.03 (4H, m, 2 x CH$_2$), 1.62 (2H, m, CH$_2$), 1.28 (4H, m, 2 x CH$_2$), 1.26 (34 H, br s, 17 x CH$_2$), 1.23 (4H, m, 2 x CH$_2$), 0.88 (3H, t, $J = 7.0$ Hz, Me-13'), 0.85 (3H, t, $J = 7.0$ Hz, Me-16'); $^{13}$C NMR (CDCl$_3$; 125 MHz): $\delta$ 171.20 (C-1), 60.42 (C-1), 33.78 (CH$_2$), 31.93 (CH$_2$), 29.70 (CH$_2$), 29.59 (CH$_2$), 29.44 (CH$_2$), 29.36 (CH$_2$), 29.25 (CH$_2$), 29.08 (CH$_2$), 24.72 (CH$_2$), 22.70 (CH$_2$), 21.05 (CH$_2$), 14.20 (Me-16), 14.12 (Me-13'); ESIMS m/z (rel. int.): 439 [M+H]$^+$ (C$_{29}$H$_{59}$O$_2$) (6.3), 255 (93.5), 239 (5.6), 199 (4.8), 183 (14.2).

**n-Tritriacon-21, 27-dien-1-ol (4).** Semi-solid brown; R$_f$ 0.63 (Chloroform-methanol; 9:5:0.5); UV $\lambda_{\text{max}}$ (MeOH): 210, 363 nm IR$_{\text{max}}$ (KBr): 3414, 2917, 2849, 1615, 1462, 1382, 1303, 1254, 1174, 720 cm$^{-1}$; $^1$H NMR (CDCl$_3$; 500 MHz): $\delta$ 5.34 (1H, m, H-21), 5.31 (1H, m, H-22), 5.25 (1H, m, H-27), 5.22 (1H, m, H-28), 3.31 (2H, d, $J = 6.4$ Hz, H-21), 2.28 (2H, m, H-22), 2.23 (2H, m, H-22), 1.63 (2H, m, H-26), 1.60 (2H, m, H-29), 1.39 (10H, s, 5 x CH$_2$), 1.28 (36H, br s, 18 x CH$_2$), 0.86 (3H, t, $J = 6.7$ Hz, Me-33); $^{13}$C NMR (CDCl$_3$; 125 MHz) $\delta$ 133.33 (C-21), 131.23 (C-22), 117.44 (C-27), 116.87 (C-28), 61.24 (C-1), 33.53 (C-20), 31.22 (C-23), 30.91 (46 H, br s, 23 x CH$_2$), 26.24 (CH$_2$), 22.68 (CH$_2$), 16.95 (Me-33); ESIMS m/z (re. int.): 477 [M+H]$^+$ (C$_{33}$H$_{64}$O) (78.3), 405 (44.8), 379 (40.2), 323 (22.6), 297 (10.5), 179 (2.6), 153 (9.8), 97 (36.1).

**Palmitic acid (5).** Colourless mass; m.p. 61–63$^0$; R$_f$ 0.40 (n-hexane-EtOAc; 9:1); UV $\lambda_{\text{max}}$ (MeOH): 208 nm; IR$_{\text{max}}$ (KBr): 3414, 2918, 2849, 1703, 1466, 1433, 1350, 1295, 1099, 939, 723 cm$^{-1}$; $^1$H NMR (CDCl$_3$; 500 MHz): $\delta$ 2.34 (2H, t, $J = 7.5$ Hz, H-2'), 1.62 (2H, m, H-3), 1.32 (2H, m, CH$_2$), 1.29 (2H, m, CH$_2$), 1.25 (20 H, br s 10 x CH$_2$), 0.87 (3H, t, $J = 6.5$ Hz, Me-16); $^{13}$C NMR (CDCl$_3$; 125 MHz): $\delta$ 180.45 (C-1), 33.81 (C-2), 31.92 (C-3), 29.67 (CH$_2$), 29.66 (CH$_2$), 29.60 (CH$_2$), 29.53 (CH$_2$), 29.44 (CH$_2$), 29.37 (CH$_2$), 29.33 (CH$_2$), 29.25 (CH$_2$), 29.14 (CH$_2$), 29.70 (CH$_2$), 24.53 (CH$_2$), 22.69 (C-15), 14.11 (C-16); ESIMS m/z (re. int.): 257 [M+H]$^+$ (C$_{16}$H$_{33}$O$_2$) (100).

**Kaempferol (6).** Pale yellow powder; R$_f$ : 0.60 (chloroform:methanol; 9:1); m.p. 276-78$^0$; UV $\lambda_{\text{max}}$ (MeOH): 265, 306nm; IR$_{\text{max}}$ (KBr): 3418, 3310, 1660, 1613, 1568, 1507, 1445,
1381, 1310, 1251, 1226, 1089, 1009, 818 cm\(^{-1}\); \(^1\)H NMR (CDCl\(_3\); 500 MHz): \(\delta\) 8.04 (2H, d, \(J = 8.4\) Hz, H-3', H-5'), 6.93 (2H, d, \(J = 8.5\) Hz, H-2', H-6'), 6.44 (1H, d, \(J = 2.0\) Hz, H-6), 6.19 (1H, d, \(J = 2.0\)Hz, H-8); \(^{13}\)C NMR (CDCl\(_3\); 125 MHz) \(\delta\) 146.85 (C-2), 135.70 (C-3), 175.95 (C-4), 156.21 (C-5), 98.23 (C-6), 163.92 (C-7), 93.52 (C-8), 160.74 (C-9), 103.07 (C-10), 121.70 (C-1'), 129.55 (C-2'), 115.48 (C-3'), 159.23 (C-4'), 115.16 (C-5'), 131.56 (C-6'); ESIMS \(m/z\) (rel. int.): 287 [M+H\(^+\)] (C\(_{15}\)H\(_{10}\)O\(_6\)) (88.1).

5, 7-Dimethoxyapigenin (7). Yellow solid; \(R_t\): 0.60 (chloroform:methanol; 9:1); UV \(\lambda_{\text{max}}\) (MeOH): 253, 289, 369 nm; IR\(_{\text{max}}\) (KBr): 3410, 2923, 2832, 1663, 1613, 1517, 1381, 1301; 1214, 1166, 1095, 1010, 940 cm\(^{-1}\); \(^1\)H NMR (CDCl\(_3\); 500 MHz): \(\delta\) 7.64 (1H, d, \(J = 8.0\) Hz, H-2'), 7.43 (1H, d, \(J = 8.0\) Hz, H-3'), 6.89 (1H, d, \(J = 8.0\) Hz, H-3'), 6.80 (1H, d, \(J = 8.0\) Hz, H-5'); 6.39 (1H, s H-3), 6.19 (1H, d, \(J=1.5\) Hz, H-6), 6.18 (1H, d, \(J=1.5\) Hz, H-8), 3.30, 3.32 \((2 \times\) OMe, s each); \(^{13}\)C NMR (CDCl\(_3\); 125 MHz) \(\delta\) 158.37 (C-2), 104.66 (C-3), 170.45 (C-4), 151.65 (C-5), 99.38 (C-6), 165.71 (C-7), 94.55 (C-8), 146.36 (C-9), 115.89 (C-10), 124.29 (C-1'), 121.82 (C-2'), 117.86 (C-3'), 146.20 (C-4'), 116.13 (C-5'), 132.32 (C-6'); 51.82, 51.99 (OMe); ESIMS \(m/z\) (rel. int.): 299 [M+H\(^+\)] (C\(_{17}\)H\(_{14}\)O\(_5\)) (20.4), 180 (4.3), 152 (12.6), 146 (11.2).

Quercitin (8): \(^1\)H NMR (CDCl\(_3\); 500 MHz): \(\delta\) 7.67 (1H, d, \(J = 2.0\) Hz, H-2'), 7.54 (1H, dd, \(J = 2.5, 8.5\) Hz, H-6'), 6.88 (1H, d, \(J = 8.5\) Hz, H-5), 6.40 (1H, d, \(J = 2.0\) Hz, H-8), 6.18 (1H, d, \(J = 2.0\) Hz, H-6); \(^{13}\)C NMR (CDCl\(_3\); 125 MHz) \(\delta\): 147.74 (C-2), 135.77 (C-3), 175.88 (C-4), 160.76 (C-5), 98.22 (C-6), 163.92 (C-7), 93.40 (C-8), 156..17 (C-9), 103.05 (C-10), 122.00 (C-1'), 115.04 (C-2'), 145.10 (C-3'), 146.84 (C-4'), 115.64 (C-5'), 119.98 (C-6'); ESIMS \(m/z\) (rel. int.): J. Y. Lallemand and M. Duteil. \(^{13}\)C NMR spectra of quercetin and rutin. Organic Magnetic Resonance, 9(3), 179—180 (1977).

Chlorogenic acid (9). \(^1\)H NMR (CDCl\(_3\); 500 MHz): \(\delta\) 7.53 (s, 1H, H-1), 7.00 (1H, d, \(J = 4.2,\) H-4), 6.77 (1H, d, \(J = 8.5\) Hz, H-5), 7.64 (1H, d, \(J = 1.6,\) H-6), 6.27 (1H, d, \(J = 1.8,\) H-7), 5.32 (1H, m H-8), 4.16 (1H, m H-9), 3.70 (1H, m H-10), 2.24 (2H, m, H-11), 2.22 (2H, m, H-12), 4.16 (2H, S, H-13, 14, -OH), 5.31 (1H, s, H-15, -OH), 11.20 (1H, COOH, H-16); \(^{13}\)C NMR (CDCl\(_3\); 125 MHz): \(\delta\) 115.20 (C-1), 146.60 (C-2), 149.40 (C-3), 116.50 (C-4), 123.00 (C-5), 127.80 (C-5'), 147.10 (C-6), 115.20 (C-7), 177.50 (-CO), 71.40 (C-8), 73.60 (C-9), 72.00 (C-10), 38.20 (C-11), 38.90 (C-12), 76.30 (C-COOH), 168.90 (COOH); ESIMS \(m/z\) (rel. int.): 255 [M+H\(^+\)] (C\(_{18}\)H\(_{18}\)O\(_9\)).

**Figures S1:** Chemical structures of 2-9 isolated from *Cuscuta reflexa* stem.
Spectra

$^1$HNMR
ANALYTICAL TEST REPORT

Sample Information for Direct Mass Analysis of Isolates/synthetic molecule
Sample Code : AA_ZN11
Solubility : MeOH
Name of the Scientist : Dr. A. Ahmad
Project Code: GAP-383
Mass Range: 50-500

Mass chromatogram
UV-Spectra

ESI+

ESI−