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Isolation of a unique dipyridodiazepinone metabolite nevirapine during large scale extraction of Cliv-92 from the seeds of *Cleome viscosa*

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ABSTRACT

Natural nevirapine (1), a dipyridodiazepinone metabolite has been isolated as a minor constituent with a yield of 0.00397% from the ethyl acetate extract during large scale extraction of Cliv-92 at a level of 100 kg/batch from the seeds of *Cleome viscosa*. Column chromatographic purification of the ethyl acetate extract afforded the molecule which was characterized as nevirapine through spectral analysis and confirmed by single crystal X-ray crystallography. The molecule also has been re-isolated from the same batch of seeds to confirm its presence in the seeds of *C. viscosa*. The molecule nevirapine has been fully characterized by co-TLC, prep-TLC-MS/MS and chiral HPLC analysis. Isotope Ratio Mass Spectrometric (IRMS) analysis of natural nevirapine and the synthetic drug revealed it to be different. It is an interesting discovery to find out that the natural nevirapine exists as an endogenous metabolite of the plant. This paper also reports the isolation of salicylic acid from the same batch of seeds which afforded nevirapine which may have been due to some plant–pathogenic interaction.

1. Introduction

Cleome viscosa (Syn. *Cleome icosandra*) (Capparidaceae) is an annual wasteland weed with yellow flowers and strong penetrating odour. This weed is very common in India. The leaves of the plant are rubefacient, vesicant and sudorific. The seeds are small, dark brown or black and granular. They are reported to have rubefacient, vesicant and anthelminthic properties. The seeds are used occasionally as condiment in curries. (Anon, 1950)

Chemical investigation of the plant has disclosed the presence of several interesting classes of chemical compounds, macrocyclic diterpene cleomaldic acid (Anon, 2001) glycoflavanones (Chauhan et al., 1979), lipoflavanones (Srivastava and Srivastava, 1979), glucosinolates (Songsak and Lockwood, 2002) and cleomeolide (diterpene lactone) (Mahato et al., 1979) which have anticancer properties. Phytochemical investigation of the seeds of the plant also has resulted in the isolation of a new class of chemical entities known as coumarinolignoids in which a lignan (C_6–C_3) unit is linked with a coumarin moiety through a dioxane bridge. Four coumarinolignoids cleomiscosins A, B, C and D have been isolated and identified from the seeds of *C. viscosa* (Ray et al., 1985; Kumar et al., 1988). Cleomiscosins A, B and C possess liver protective activity (Chattopadhyay et al., 1999a,b). The mixture of the three coumarinolignoids cleomiscosins A, B and C is termed as Cliv-92 which has hepatoprotective activity comparable to that of silymarin the antihepatotoxic drug currently in use throughout the world. Recent phytochemical investigations also have shown the presence of terpenoids, saponins and alkaloids (Koche et al., 2010). The occurrence of nitrogenous compounds has been exemplified by the first isolation of lactam nonanic acid from the root exudates of the plant (Jana and Biswas, 2011).

For one of our ongoing projects, we needed cleomiscoins A, B and C in large quantities. In order to get the compounds, we started extracting 100 kg seeds of *C. viscosa* to get the molecules. In that endeavor we isolated a molecule with a yield of 0.00397% which was characterized as nevirapine. In order to confirm the structure unequivocally, the crystal structure of the molecule also was studied.

After its structure was fully characterized, we published our findings in *Tetrahedron* (Chattopadhyay et al., 2011a). After publication of the paper in *Tetrahedron*, some criticisms have appeared on the natural occurrence of nevirapine as well as on its chiral property. Therefore, we had retracted the paper to verify the findings beyond doubt (Chattopadhyay et al., 2011b).

We have re-isolated the molecule from the same batch of seeds and confirmed that the natural nevirapine isolated from the seeds of *C. viscosa* was different from synthetic nevirapine through isotope ratio mass spectrometric (IRMS) analysis. In addition to nevirapine,

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we also have isolated salicylic acid during isolation of nevirapine. In this paper we are reporting the occurrence of nevirapine in the seeds of *C. viscosa* and confirmation of its structure by detailed spectral analysis including X-ray crystallography.

2. Materials and methods

2.1. General

Melting points were uncorrected and were recorded on a Buchi – Melting point Apparatus. UV spectra were recorded on a Spectronic® GENESYS™ with a 10 mm quartz cell and IR spectra were recorded on a Perkin-Elmer Spectrum BX FT-IR spectrometer. Sample pellets were prepared in KBr using hydraulic pellet press of Kimaya manufacturers. NMR spectra were recorded on a Bruker - Avance 300 MHz FT-NMR using $CDCl_3$, a deuterated solvent, the chemical shift of which was used as an internal standard. DART-HRMS data were obtained with a JEOL ACCU TOF DART JMS-T100LC mass spectrometer. IRMS analysis for $\partial^{13}C$ and $\partial^{15}N$ was performed with Thermo Finnigan Flash1112 elemental analyzer linked with a Thermo Finnigan Delta V Plus isotope ratio mass spectrometer. Sucrose with a $\partial^{13}C$ certified value of −10.8 and ammonium sulphate with a $\partial^{15}N$ certified value of +20, procured from IAEA (International Atomic Energy Agency) were used as standard for $\partial^{13}C$ and $\partial^{15}N$. The overall analytical precision for replicates of standard was 0.06‰ for $\partial^{13}C$ and 0.08 ‰ for $\partial^{15}N$.

2.2. Plant material

The seeds of *C. viscosa* were collected from the outskirts of Lucknow during the month of October. The seed samples have been deposited in the "National Gene Bank of Medicinal and Aromatic Plants" CIMAP, Lucknow (accession number CIMAP 3426). The harvested plants were dried in diffused sunlight and seeds were removed by thrashing.

2.3. Extraction and isolation of nevirapine from synthetic drug nevimune

One tablet containing 200 mg API nevirapine was powdered in a mortar and pestle. The powdered tablet was then allowed to stir with 1N aqueous HCl for about 1 h and was filtered. The aqueous filtrate was extracted subsequently with hexane and chloroform. Nevirapine (120 mg) was isolated from the chloroform fraction. Then the acidic layer was basified with ammonia (liquor) and extracted with chloroform. The chloroform layer gave an extra quantity (50 mg) of nevirapine.

2.4. Chiral HPLC method development for the analysis of nevirapine

Several methods were used for chiral resolution of nevirapine and its acetate. It was found to be well resolved, with good reproducibility, in a NP-isocratic elution mode consisting of solvent A (n-hexane) and solvent B (ethyl acetate containing 0.1% diethylamine) with 80:20 ratio and 0.5 mL/min flow in CHIRALPAK-IA (250 mm× 4.6 mm i.d.) column procured from DAICEL Chemical Industries, Tokyo, Japan. The solvents were procured from MERCK, India and were of analytical grade. Detections were performed at λ_{max} values of 283 nm for nevirapine and 340 nm for acetylated nevirapine in a Waters HPLC system (Waters Corporation, Milford, MA, USA) consisting of a Waters 600E multi solvent delivery system and equipped with Waters Empower software for data acquisition.

2.5. Extraction and isolation of nevirapine from the seeds

The process was aimed primarily at the extraction of coumarinolignoids which involved the fixed oil removal from 100 kg of dried seeds by subjecting them to an oil expeller. The miscella (89 kg) was charged into pilot scale extractors of 100 kg/batch of a multiutility solid–liquid extraction plant where the initial defatting of the crude material was done in hexane (200 L × 6). The marc left was extracted with methanol (210 L × 6). The solvent free dark green colored crude methanolic extract (12 kg) was then adsorbed onto celite (6.4 kg) which served as a base for the solid-matrix partition process. A nutsche type filtration unit of 50 kg capacity was packed with celite (6.0 kg) in toluene and the adsorbed extract was partitioned sequentially by toluene (25 L × 4), ethyl acetate (25 L × 4) and methanol (25 L × 4), respectively. The vacuum concentrated toluene, ethyl acetate and methanolic extracts were of 1.98%, 5.69%, 0.55% yields, respectively. The semi solid concentrated ethyl acetate extract was adsorbed with 11.0 kg silica gel and was loaded onto pilot scale stainless steel columns of 50 kg gel holding capacity. Silica gel (60–120 mesh) (23 kg) was packed with hexane along with the adsorbed slurry and the column was eluted with a mixture of hexane and ethyl acetate in the ratio of 1:1, 1:3 and finally with ethyl acetate. TLC analysis of the fractions of hexane: ethyl acetate (1:1) eluates in choloroform:acetone (93:7) showed almost a major spot in UV at a R_f of 0.46 which was stained with Dragendorff's reagent. The fractions containing the above spot were pooled and concentrated. Recrystallization of the concentrate from ethyl acetate yielded colorless needles of nevirapine (**1**) (3.54 g, 0.00397%); m.p. 241–243 °C; R_f (7% $CH_3COCH_3/CHCl_3$) 0.46; IR ν_{max} (KBr) 3047, 2921, 2868, 1744, 1656, 1586, 1463, 1417 cm^{-1}; UV ($CHCl_3$) λ_{max} (log ε) 325 nm (3.5), 275 nm (sh, 3.5) 240.4 nm (4.0); ^1H NMR and ^{13}C NMR spectroscopic data see Table 1. DART-HRMS *m/z* 267.1231 $[M+H]^+$ (calculated 267.1246).

2.6. Preparation of N-acetylated derivative ***2*** of ***1***

A mixture of **1** (50 mg, 0.019 mmol), acetic anhydride (4.0 mL, 42.3 mmol) and triethylamine (2.0 mL, 14.35 mmol) was stirred on an oil bath at 80 °C for 4 h. Standard work up of the reaction mixture and subsequent silica gel column purification in chloroform gave a fraction which on crystallization from cold methanol yielded the acetate derivative **2** (30 mg,Yield = 53%): m.p. 174–176 °C (from MeOH); UV ($CHCl_3$) λ_{max} 343 nm, 262 nm (sh), 244 nm; IR (KBr) ν_{max} 1684, 1585, 1559, 1455, 1413,1297 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data see Table 1; ESI-MS *m/z* 309.2 $[M+H]^+$; 331.3 $[M+Na]^+$.

2.7. Single crystal X-ray structure determination of nevirapine (***1***) at 293 (***2***) K

X-ray data were collected at 293 K with a Bruker Smart Apex CCD diffractometer with graphite monochromator and Mo Kα radiation (λ = 0.71073 Å), using SMART32 (Bruker) and SAINT (Bruker) softwares. The structure was solved by direct methods and refinements by full-matrix least-squares methods on F^2 using SHELXTL-NT [Bruker AXS Inc.: Madison, Wisconsin, USA 1997]. Crystal data: $C_{15}H_{14}N_4O$, Empirical formula $C_{17}H_{14}N_4O_2$, M_r = 306.32, triclinic, space group P(−1), *a* = 7.767(3) Å, *b* = 8.420(4) Å, *c* = 12.466(5) Å, α (deg) = 84.70, β (deg) = 89.37, γ (deg) = 68.39, *V* (Å^3) = 754.5(5), *Z* = 2, $\rho_{calc.}$ = 1.348 Mg/m^3, λ (Mo Kα) = 0.71073 Å, μ = 0.092 (mm^{-1}). Data collection and reduction: crystal size, 0.225 mm × 0.20 mm × 0.275 mm, θ range = 2.61–28.31, 4942 reflections collected, 3553 independent reflections (R_{int} = 0.0229), *R* indices (all data) = R_1 = 0.1219 and wR_2 = 0.3301 final *R* indices [$I > 2\sigma(I)$] R_1 = 0.0931 and wR_2 = 0.2450 for 233 variable parameters, GOF = 1.12. The X-ray crystallographic file of the synthetic