Rarely Occurring Natural Products Isolated from *Vincetoxicum stocksii*

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Summary: Silica gel column chromatography of the ethyl acetate fraction of methanol extract of *Vincetoxicum stocksii* resulted in the separation of three new rarely occurring natural products; [4-(4-(methoxycarbonyl)benzyl)phenyl] carbamic acid (**1**), bis[di-*p*-phenylmethane]ethyl carbamate (**2**), methyl 2-hydroxy-3-(2-hydroxy-5-(3-methylbut-2-enyl)phenyl)-2-(4-hydroxyphenyl) propanoate, stocksiloate(**3**), along with five known compounds; 1-(4-hydroxy-3-methoxyphenyl)-1,2,3,-propanetriol (**4**), feruloyl-6-O-*β*-D-glucopyranoside (**5**), 4-hydroxy-3,5-dimethoxybenzoic acid (**6**), apocynin (**7**) and vincetomine (**8**). The structures of compounds **1** and **2** were established with help of 1D, 2D-NMR techniques and high resolution mass spectrometry, whereas, compound **3** could only be characterized tentatively by 1D, 2D-NMR techniques. Compounds **1** is new compound while **2** is synthetically known but never been reported from natural source. The known compounds were identified due to 1D NMR analysis and in comparison with the literature values. Compounds **1**-**3** were found inactive in an anti-urease assay.

Key words: *Vincetoxicum stocksii*; Secondary metabolites; Carbamic acid derivatives; Isolation; Structure elucidation.

Introduction

The Genus *Vincetoxicum* of plant the family Asclepiadaceae (milkweed) is widely distributed in Europe and Asia. Six species including *V. sakesarense*, *V. canescens*, *V. cardiostephanum*, *V. arnottianum V.hirundinaria* and *V. stocksii* are growing in different regions of Pakistan [1]. *Vincetoxicum stocksii* is one among the medicinally important but poisonous plants in this genus. This perennial leafy vine is not well studied for its pharmacological activities, however, previous phytochemical investigation of this plant revealed the presence of some glycosides and alkaloids [2]. Traditionally, *V. stocksii* is used for healing of wounds and for cancer treatment [3].

Its crude extract shows high cytotoxic, antibacterial, antifungal, antidiarrheal and antispasmodic activities [2]. Previously, we investigated the extract of this plant for its secondary metabolites and isolated four new and six known compounds [4]. Due to low amount some of the compounds isolated from EtOAc fraction remained un-identified therefore, the re-investigation of the extract of this plant resulted in the discovery of (4-(4-(methoxycarbonyl) benzyl) phenyl) carbamic acid (**1**) and bis[di-*p*-phenylmethane]ethyl carbamate (**2**), as synthetically known but new natural products, methyl 2-hydroxy-3-(2-hydroxy-5-(3-methylbut-2-enyl)phenyl)-2-(4-hydroxyphenyl) propanoate, stocksiloate (**3**), as new discovery, along with five previously reported natural products; 1-(4-hydroxy-3-methoxyphenyl)-1,2,3,-propanetriol (**4**), feruloyl-6-O-*β*-D-glucopyranoside (**5**), 4-hydroxy-3,5-dimethoxybenzoic acid (**6**), apocynin (**7**) and vincetomine (**8**) [4] (Figure 1).

Experimental

Equipment

Column chromatography was carried out using Silica gel (Kieselgel 230-400 mesh, E. Merck), packed in glass columns of different sizes. The fractions obtained or purification of the compounds were monitored by thin layer chromatography on TLC plates, silica gel pre-coated on aluminum sheets (Kieselgel 60 F_{254}, thickness 0.25 mm 70-230 mesh, E. Merck, Darmstadt, Germany). The TLC

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chromatograms were visualized under UV lamp operating at 254 and 366 nm, followed by heating with ceric sulfate to locate UV inactive components. IR spectra were recorded on JASCO-320-A (Duisburg, Germany) spectrophotometer, whereas, UV data was scanned in methanol on a Hitachi UV-3200 Spectrometer. The ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) spectra were recorded on Bruker (Zurich, Switzerland) spectrometer AMX-600, using TMS as an internal reference. The 2-D NMR (COSY, HMQC, and HMBC) spectra were also recorded on the same instrument operating at 600 MHz. FAB-MS and HRFAB-MS were calculated on Finnigan (Varian MAT, Waldbronn, Germany) JMS H×110 with a data system and JMSA 500 mass spectrometers.

Plant material

Vincetoxicum stocksii Ali & Khatoon was collected from Ziarat valley in September 2011, Baluchistan, and was identified by Prof. Dr. Rasool Bakhsh Tareen, Department of Botany, University of Baluchistan, Quetta, Pakistan, where a voucher specimen (RBT-VS-11) has been deposited in the herbarium.

General procedure

The plant was dried under shade for 15 days, ground into semi-powder (20 kg) and was extracted with methanol (18 L) for 5 days (twice). The solvent was evaporated under vacuum to get a dark brown gummy mass (217 g). The crude methanolic extract was suspended in water (2 L) and was extracted with *n*-hexane and ethyl acetate to get 105 g and 81 g fractions respectively. The water-soluble part was weighed after drying as 30 g.

The ethyl acetate fraction (81 g) was subjected to column chromatography over silica gel eluting with *n*-hexane, *n*-hexane-ethyl acetate, ethyl acetate, ethyl acetate-methanol in increasing order of polarity to get ten fractions (V1-V10).The Fraction V5 (6 g) obtained with *n*-hexane:ethyl acetate (4:6) was further chromatographed on silica gel column eluting with *n*-hexane: ethyl acetate to get 3 sub-fractions VS1-VS3.

The sub-fraction VS2 (2.5 g) which was subjected to repeated silica gel column chromatography eluting with *n*-hexane: ethyl acetate (2.5:7.5) to get compound **1** (21 mg). The sub-fraction VS3 (1.5 g), which was also purified on silica gel column eluting with *n*-hexane: ethyl acetate (2:8), provided compound **7** (12 mg).

The fraction V3 (2.8 g) obtained from the main column with *n*-hexane: ethyl acetate (6:4) was further subjected to silica gel column to give compound **2** (19 mg) and **8** (17 mg) when eluted with *n*-hexane:ethyl acetate (5:5). Fraction V6 (1.5 g) which was eluted with *n*-hexane: ethyl acetate (3:7) on further purification with silica gel and isocratic elution with *n*-hexane:ethyl acetate (3:7) yielded compound **3** (6 mg) and **6** (14 mg). The main fraction V9 (5.0 g) from the first column eluted with pure ethyl acetate give two sub-fractions VY1-VY2 on further silica gel column chromatography. The sub-fraction VY2 when further purified on silica gel column eluting with ethyl acetate:methanol (9:1) yielded compound **4** (11 mg) and **5** (~15 mg).

*[4-(4-(methoxycarbonyl)benzyl)phenyl]carbamic acid (**1**):* White amorphous powder (21 mg); IR (KBr): λ_{max} 3385-2470, , 1730, 1705, 1605, 1545 and 1490 cm^{-1}; UV (MeOH): ν_{max} 360 (3.12), 282 (3.15), 245 (3.2); ^{1}H- &^{13}C- NMR (Table-1); EIMS: *m/z* 282 (100%) $[M\text{-}H_2O]^+$, 267, 256, 240, 223 and 208; +ve-HR-FABMS: *m/z* 301.1189 $[M+H]^+$(calcd. 301.1188) for $C_{16}H_{17}N_2O_4$ corresponding to the formula as $C_{16}H_{16}N_2O_4$.

*Bis[di-p-phenylmethane]ethyl carbamate (**2**)*: White amorphous powder (19 mg); IR (KBr): λ_{max} 3480, 3090, 2930, 1730, 1635, 1600, 1545 and 1485 cm^{-1}; UV (MeOH): ν_{max} 282 (4.01), 255 (4.12); ^{1}H- &^{13}C- NMR (Table-1); EIMS: *m/z* 342 (100%) $[M]^+$, 313, 296, 270, 241, 223, 197 and 106; HR-EIMS: *m/z* 342.1579 $[M]^+$(calcd. 342.1579 for $C_{19}H_{22}N_2O_4$).

*Stocksiloate (**3**):* White amorphous powder (6 mg); IR (KBr): λ_{max} 3505, 3050, 1737, 1655, 1605 and 1545 cm^{-1}; ^{1}H- &^{13}C- NMR (Table-1)

Results and Discussion

White amorphous solid of compound **1** displayed diagnostic IR absorption bands at 3385-2470, 1730, 1705, 1605, 1545 and 1490 cm^{-1} due to carboxylic, amide functions and aromatic system. The EIMS spectrum displayed heaviest ion at *m/z* 282 $[M\text{-}H_2O]^+$, however, the molecular formula ($C_{16}H_{16}N_2O_4$) with 10 DBE could be determined due to HR-FABMS analysis in positive mode, thus indicating the molecular mass of **1** as 300 amu.

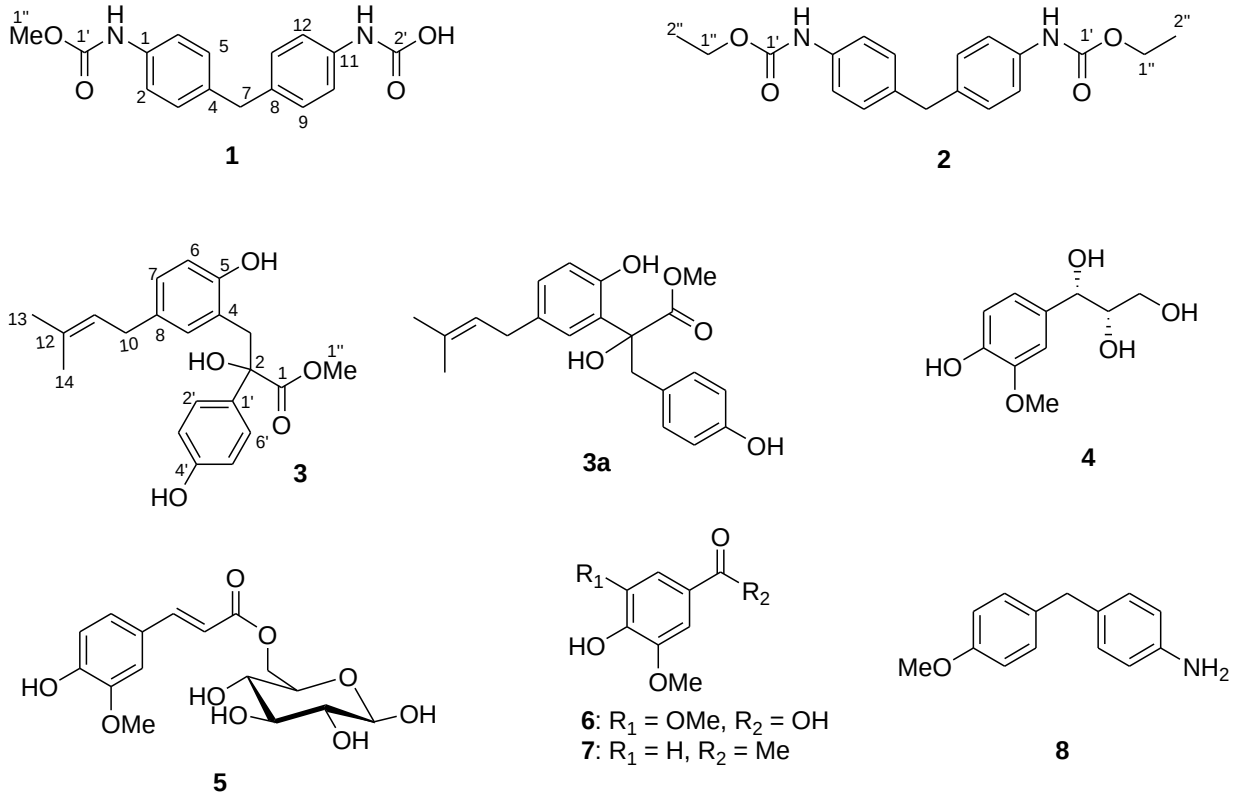

Fig. 1: Secondary metabolites **1**-**8** (except **3a**) isolated from *Vincitoxicum stockii*.

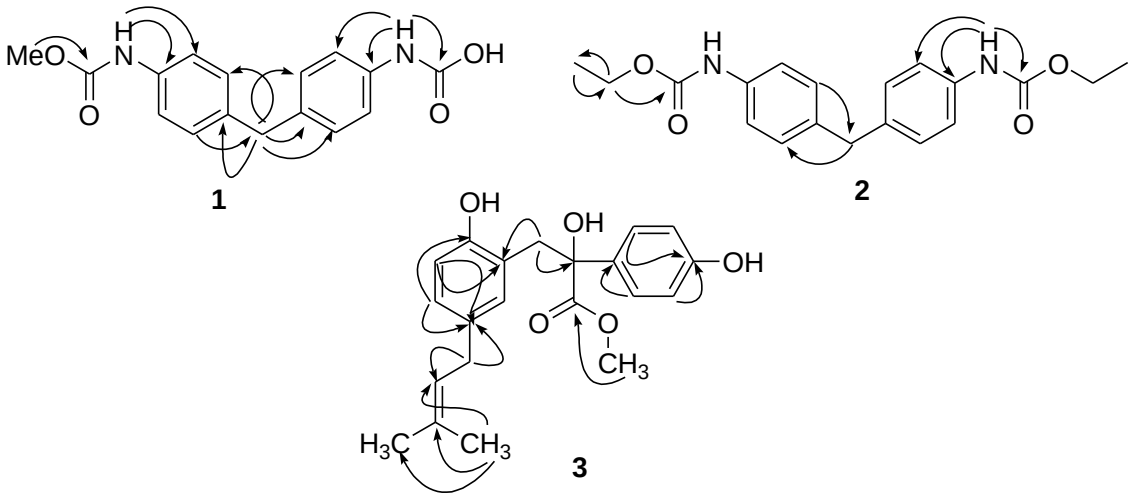

Fig. 2: HMBC correlations observed in the spectra of compounds **1-3.**

The ^{1}H-NMR spectrum (Table-1) of **1** afforded four signals in the aromatic region at δ 7.33 (2H, d, J = 8.4 Hz), 7.32 (2H, d, J = 8.4 Hz), 7.10 (2H, d, J = 8.4 Hz) and 7.08 (2H, d, J = 8.4 Hz), which depicted two *p*-substituted benzene rings in the molecule. The most downfield resonances observed at δ 9.50 (1H, s) and 8.49 (1H, s) were attributed to two secondary amines, besides the same spectrum displayed two more singlets at δ 3.78 (2H, -CH_2) and 3.62 (3H, -OMe). The correlation of the methylene proton in HSQC spectrum with the carbon at δ 39.9 revealed its presence between two aromatic systems.

The ^{13}C NMR spectrum (Table-1) of **1** displayed altogether 12 carbon resonances, which were identified as δ 153.9 (C), 152.5 (C), 137.6 (C), 137.0 (C), 135.5 (C), 134.8 (C), 128.8 (2CH), 118.2 (2CH), 51.4 (CH_3) and 39.9 (CH_2). Information collected from the IR data and resonances of two quaternary carbons at δ 153.9, 152.5 suggested carbamic acid moieties in **1** [5]. Fragmentation pattern (*m/z* 282, 267, 256, 240, 223 and 208) in EIMS spectrum depicted an isocyanato nature of the ion *m/z* 282 (100 %) that not only supported the idea of carbamic acid function but also justified low stability of this function and higher stability of the isocyanato ion, which could have been generated on dehydration from the carbamic acid group of molecular ion. Therefore, it is concluded that the fragment ions in EIMS spectrum at *m/z* 282, 267, 256, 240, 223 and 208 came from dehydrated isocyanato derived ion of **1** [5].

The two aromatic systems accommodated 8 DBE, whereas, the remaining two DBE could be attributed to two carbamic acid moieties, which were further substantiated due to HMBC correlation (Figure 2) of the two amine protons (δ 9.50 and 8.49) with the most downfield carbons at δ 153.9 and 152.5 respectively. The methoxyl proton (δ 3.62) also exhibited HMBC interaction with the carbon at δ 153.9, indicating as one group must be methylcarbamate. Reading the other HMBC correlations (Figure 2), the two amine protons were found further interacted with the carbons at δ 137.6, 137.0 and 118.2 respectively, which substantiated an N-aryl linkage of the two carbamic acid groups. The HMBC interaction of the methylene proton (δ 3.78) with the aromatic carbons at δ 135.5, 134.8, 128.8 and that of aromatic protons (δ 7.10 and 7.08) with the methylene carbon (δ 39.9) confirmed the presence of methylene group between two benzene rings. All proton and carbon assignments were fully established due to the analysis of COSY, HSQC and HMBC spectra, which finally led to the structure of **1** as [4-(4-(methoxycarbonyl)benzyl)phenyl]carbamic acid, which is a new natural product.

IR spectrum of compound **2** missed the absorption band (3485-2470 cm^{-1}) for carboxylic acid function, while other bands were seen nearly at the same wave number when compared to the spectrum of **1**, indicating the similar nature of **2**. The EIMS spectrum of **2** showed molecular ion at *m/z* 342 (100%) $[M]^+$, with other fragment ions were seen at *m/z* 313, 296, 270, 241, 223, 197 and 106. The HR-EIMS displayed molecular ion at *m/z* 342.1579 $[M]^+$ that depicted the molecular formula $C_{19}H_{22}N_2O_4$.

Aromatic region of the ^1H-NMR spectrum (Table-1) of **2** showed two doublets at δ 7.30 (4H, *J* = 8.4 Hz) and 7.08 (4H, *J* = 8.4 Hz), which could be attested for two *p*-substituted benzene rings that revealed a symmetrical dimeric nature of **2**. One N-H signal was observed at 8.6 Hz. A singlet methylene proton displayed its position at δ 3.84, which was correlated in HSQC spectrum with a ^{13}C resonance at δ 41.4. Besides, the same spectrum afforded signals for ethoxy group at δ 4.16 (4H, q, *J* = 7.2 Hz) and 1.29 (6H, t, *J* = 7.2 Hz). The downfield chemical shift (δ 4.16) of the methylene proton was attributed to its attachment with carboxylate function.

The ^{13}C NMR spectrum (Table-1) of **2** fully supported the ^1H-NMR data as it displayed eight signals, which were identified as one methyl (δ 14.9), two methylene (δ 41.1 and 61.8), two methine (δ 130.1 and δ 120.1) and three quaternary carbons (δ 156.2, 138.1 and 137.5), on the basis of DEPT experiments. Since the molecular formula afforded 19 carbon atoms, the resonances of only eight carbon nuclei in the ^{13}C NMR spectrum substantiated symmetrical dimeric nature of **2** with two *p*-substituted benzene rings. Thus the combination of the above spectroscopic data with HMBC (Figure 2) information, compound **2** was finally characterized as bis[di-*p*-phenylmethane]ethyl carbamate, which is also a new natural carbamate derivative. The ^1H-NMR data was identical to the reported data of synthetic bis[di-*p*-phenylmethane]ethyl carbamate [6], thus compound **2** was found to be the same, which has been reported for the first time from natural source.

Compounds **1** and **2** are derivatives of methylene diphenyl diisocyanate (MDI), which is a synthetic organic compound used to synthesize polyurethane (PU) and have showed excellent cytotoxic activity against Chinese hamster and human cancer cell line. Further, compound **2** is commonly known as MDU, which is a derivative of important elastic, tensile and widely used PU (Because of importance of PU in medical devices, its derivatives are also getting attention for medical use. In a previous study, MDU was tested for its anti-cancer activity in comparison with taxol, and vincristine, and has been reported as better anticancer drug as compared to taxol, and vincristine, which possess complex structures [**7**]. *Vincetoxicum stocksii* is the first natural source of MDU. *Vincetoxicum stocksii* has anti cancer potential, it may be because of presence of MDU and other related analogues. Since all the solvents were used after distillation for extraction and chromatography thus the possibility of artifacts is excluded, further, this is not the first report on the isolation of carbamic acid derivatives as phytochemicals. The first report describes the isolation

of this class of compounds from a Taiwanian plant *Magnolia kachirachirai* [5].

The IR spectrum of **3** displayed characteristic absorption bands at 3505 (O-H), 3050 (C-H), 1737 (C=O), 1655 (C=C), 1605 and 1545 cm^{-1}. The splitting pattern of the signals in aromatic region of the ^{1}H-NMR spectrum of **3** witnessed *p*-substituted [δ 7.60(2H, d, J = 9.2 Hz), 6.85 (2H, d, J = 8.8 Hz)] and 1,2-4-trisubstituted benzene ring [δ 6.54 (1H, dd, J = 8.4, 2.0 Hz), 6.48 (1H, d, J = 8.4 Hz), 6.42 (1H, d, J = 2.0 Hz) systems in its structure, which were fully supported by the ^{13}C NMR spectrum of **3** (Table-1). A prenyl moiety in **3** was predicted due to the resonances of two allelic methyl protons at δ 1.65 (d, J = 0.8 Hz), 1.56 (br s), an olefinic methine at δ 5.07 (m) and a methylene at δ 3.07 (d, J = 7.2). The ^{1}H-NMR spectrum further displayed signal for methoxyl proton at δ 3.75 (s) and two overlapped doublets due to geminal protons of another methylene group at δ 3.39 (1H, d, J = 12.4 Hz) and 3.42 (1H, d, J = 12.4 Hz).

The ^{13}C NMR spectrum (Table-1) of **3** displayed 19 carbon resonances, attested for 21 carbon atoms. Due to COSY and HSQC spectral analysis, the resonances of prenyl group carbon nuclei were identified at δ 17.7 (CH_3), 25.9 (CH_3), 28.7 (CH_2), 123.6 (CH) and 128.4 (C), whereas, the most downfield quaternary carbon signal at δ 172.1 was attributed to carboxylate system in **3**. In addition to the signals due to two benzene rings (Table-1), the ^{13}C NMR spectrum also displayed resonances at δ 39.6 (-CH_2), 53.6 (-OMe) and 86.7 (C).

The attachment of the prenyl group at C-8 of tri-substituted benzene ring was determined due to HMBC analysis

(Figure 2), in which the methylene proton (δ 3.07) exhibited correlation with the aromatic carbons at δ 132.4 (C-9) and 128.4 (C-8), which could be substantiated due to the HMBC interaction of H-9 (δ 6.42) with C-10 (δ 28.7). H-3 (δ 3.39 and 3.42) exhibited weak HMBC correlations with C-2 (δ 86.7) and C-4 (δ 125.3), whereas, methoxyl proton was correlated with C-1 (δ 172.1). Other HMBC correlations are shown in figure 2. All the assignments were made with the combination of COSY, HSQC and HMBC spectral information. Due to certain limitations, we could not get the molecular formula of this compound, further, the absence of HMBC interactions between H-3 and C-1′ and vice versa and that of any of the aromatic proton with C-2 and C-3 only allowed us to establish a tentative structure as **3**, which was substantiated by theoretical calculations of NMR values using ACD lab software. The calculated ACD lab NMR values best fit to the structure **3**, instead of **3a**, thus we finalized the structure on comparison basis. This compound is named as stocksiloate (**3**).

Using previously described method [8] compounds **1-3** were screened in anti-urease bioassay, where at a concentration of 0.5mM, all the test compounds were found inactive.

Table-1: ^{1}H and ^{13}C NMR data of 1 (DMSO-d_6, 600 and 150 MHz, respectively), 2 (CD_3OD, 600 and 150 MHz, respectively) and 2 (CD_3OD, 400 and 100 MHz, respectively)

Position	1		2		3	
	δ_H (J = Hz)	δ_C	δ_H (J = Hz)	δ_C	δ_H (J = Hz)	δ_C
1	-	137.6	-	138.1	-	172.1
2	7.33 (d, 8.4)	118.2	7.30 (d, 8.4)	120.1	-	86.7
3	7.08 (d, 8.4)	128.8	7.08 (d, 8.4)	130.1	3.42 (d, 12.4) 3.39 (d, 12.40)	39.6
4	-	135.5	-	137.5	-	125.3
5	7.08 (d, 8.4)	128.8	7.08 (d, 8.4)	130.1	-	155.0
6	7.33 (d, 8.4)	118.2	7.30 (d, 8.4)	120.1	6.48 (d, 8.4)	115.0
7	3.78 (s)	39.9	3.84 (s)	41.1	6.54 (dd, 8.4, 2.0)	129.7
8	-	134.8	-	137.5	-	128.4
9	7.10 (d, 8.4)	128.8	7.08 (d, 8.4)	130.1	6.42 (d, 2.0)	132.4
10	7.32(d, 8.4)	118.2	7.30 (d, 8.4)	120.1	3.07 (d, 7.2)	28.7
11	-	137.0	-	138.1	5.07 (m)	123.6
12	7.32 (d, 8.4)	118.2	7.30 (d, 8.4)	130.1	-	132.9
13	7.10 (d, 8.4)	128.8	7.08 (d, 8.4)	120.1	1.56 (brs)	17.7
14	-	-	-	-	1.65 (d, 0.8)	25.9
15	-	-	-	-	-	-
16	-	-	-	-	-	-
17	-	-	-	-	-	-
18	-	-	-	-	-	-
1′	-	153.9	-	156.2	-	128.4
2′	-	152.5	-	-	7.60 (d, 9.2)	130.0
3′, 5′					6.85 (d, 8.8)	116.4
4′					-	158.7
6′					7.60 (d, 9.2)	130.0
1″	3.62 (s)	51.4	4.16 (q, 7.2)	61.8	3.75 (s)	53.6
2″	-	-	1.29 (t, 7.2)	14.9	-	-
NH	9.50 (s) and 8.49 (s)	-	8.6(s)	-	-	-

Conclusion

Vincetoxicum stocksii is one among the medicinally important poisonous plant growing in different regions of Pakistan. Phytochemical investigation on this plant leads to the isolation of rarely occurring natural products, they include [4-(4-(methoxycarbonyl)benzyl)phenyl] carbamic acid (**1**), a new compound while bis[di-*p*-phenylmethane]ethyl carbamate (**2**), as a new natural product but synthetically known compound, along with another new compound, methyl 2-hydroxy-3-(2-hydroxy-5-(3-methylbut-2-enyl)phenyl)-2-(4-hydroxyphenyl) propanoate, stocksiloate (**3**).

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