

Crispene A, B, C and D, Four New Clerodane Type Furanoid Diterpenes from *Tinospora crispa* (L.)

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ABSTRACT

Background: *Tinospora crispa* (L.) is used to alleviate the symptoms of diabetes mellitus in folk medicine. It is also used for hypertension and to treat malaria, remedy for diarrhea, and as vermifuge.

Materials and Methods: Stems of *T. crispa* were collected, sun dried for several days followed by oven dried for 24 h at a considerably low temperature and then ground into coarse powder. The powdered stems were soaked in methanol at room temperature for 14 days with occasional shaking. The extract was collected by filtration, and the solvent was evaporated under reduced pressure in a rotary evaporator to obtain a solid residue which was then subjected to fractionation using the modified Kupchan partitioning method into *n*-hexane, CCl₄, CHCl₃ and aqueous soluble fractions. The *n*-hexane soluble fraction was chromatographed over sephadex (LH-20) and the column was eluted with *n*-hexane: CH₂Cl₂:MeOH (2:5:1) followed by CH₂Cl₂:MeOH (9:1) and MeOH (100%) in order to increase the polarities. The column fractions were then concentrated and subjected to thin layer chromatography screening and the fractions with a satisfactory resolution of compounds were rechromatographed over silica gel to isolate the pure compounds.

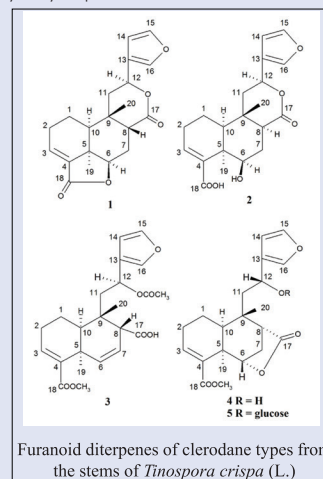
Results: Four new furanoid diterpenes of clerodane types, Crispene A, B, C, and D (1–4), including one known furanoid diterpene glucoside, borapetoside E (5), were isolated from the stems of *T. crispa*. The structures of these compounds were elucidated by means of extensive spectroscopic analysis and by comparison of their spectral data with closely related compounds. **Conclusion:** We have reported four new furanoid diterpenes of clerodane types, including one known furanoid diterpene glucoside. This is the first report of any clerodane diterpene having olefinic bond between C-6 and C-7.

Key words: Clerodane, Crispene, furanoid diterpene, *Menispermaceae*, *Tinospora crispa*

SUMMARY

- Crispene A, B, C, and D, four new furanoid diterpenes of clerodane types from *Tinospora crispa*
- Crispene C, an unusual furanoid diterpene with olefinic bond between C-6 and C-7

- First report of Crispene D as a free aglycone, though it was earlier reported as an enzymatic hydrolysis product.



Abbreviation used: TLC: Thin layer chromatography, NMR: Nuclear magnetic resonance, COSY: Correlation spectroscopy, NOE: Nuclear overhauser effect, HPLC: High-performance liquid chromatography, ESI-MS: Electrospray ionization mass spectroscopy.

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INTRODUCTION

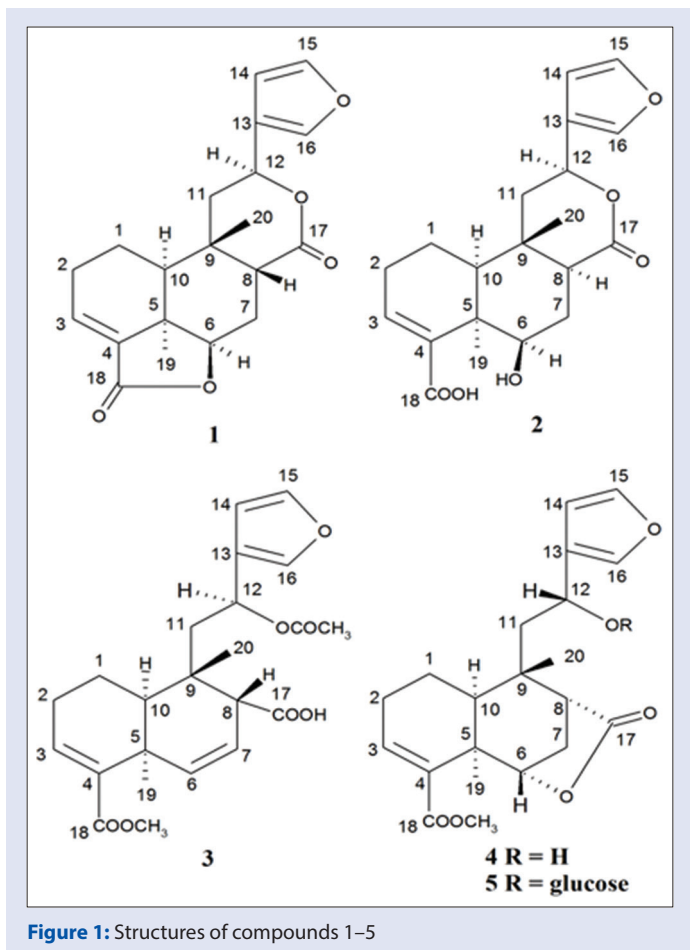
Tinospora crispa (L.) (Synonym: *Tinospora rumphii* Boerl., *Menispermum crispum* L.; Local name: Gulancha; family - *Menispermaceae*) is a woody climber with numerous protrusions on the stem and is native to Malesia, Indochina, Indian subcontinent, and China.^[1,2] In traditional medicine, it is used for hypertension, diabetes mellitus, to treat malaria, remedy for diarrhea and as vermifuge.^[1] Pharmacological studies on this plant also demonstrated its anti-inflammatory, antioxidant, antimalarial, antiprotozoal, and hypoglycemic activities.^[1] Previous chemical investigation on *T. crispa* have been reported the isolation of borapetol A and B, borapetoside A, B, C, E, and F, tinocrisposide, *N*-formylanondine, *N*-formylnormuciferine, *N*-acetyl normuciferine, *g*-sitosterol, picrotein, tinotubride, jatrorhizine, magnofl rine, palmatine, protoberberine, tembolarine, diosmetin, cycloeucalenol, cycloeucalene and other clerodane type furanoid diterpenes and their glycosides.^[3-8]

This paper describes the phytochemical investigation of the stems of *T. crispa*, which resulted in the isolation and structural elucidation of four new furanoid diterpenes of clerodane types, Crispene A, B, C, and D (1–4), along with a previously isolated and reported furanoid diterpene glucoside, borapetoside E (5) [Figure 1].^[9,10]

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MATERIALS AND METHODS

General experimental procedure

Column chromatography was performed on sephadex (LH-20). Optical rotations were measured on an ADP 220 polarimeter (Bellingham Stanley Ltd.) and concentrations (c) are given in g/100 ml. ^1H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were acquired at 300 K using Bruker Advance NMR spectrometer at 400 MHz and 100 MHz respectively. Chemical shifts are reported relative to TMS ($\delta = 0.0$ ppm). Chemical shifts (δ H) are quoted in ppm (parts per million) and referenced to CDCl_3 residual chloroform signal ^1H $\delta = 7.26$, ^{13}C $\delta = 77.2$. Electrospray ionization mass spectroscopy (ESI-MS) data were collected using a Waters Micromass ZQ instrument coupled to a Waters 2695 high-performance liquid chromatography with a Waters 2996 photodiode array. Waters Micromass ZQ parameters used were capillary (kV), 3.38; cone (V), 35; extractor (V), 3.0; source temperature ($^\circ\text{C}$), 100; desolvation temperature ($^\circ\text{C}$), 200; cone flow rate (L/h), 50; and desolvation flow rate (L/h), 250.

Plant material

Stems of *T. crispa* were collected from the Tangail district of Bangladesh, in the month of March 2009. The plant was identified by Mr. Sardar Nasir Uddin, Senior Scientific officer, Bangladesh National Herbarium, Dhaka, where a voucher specimen has been deposited (DACB accession number: 35291). The stems were sun dried for several days followed by oven dried for 24 h at a considerably low temperature. The dried stems were then ground into coarse powder

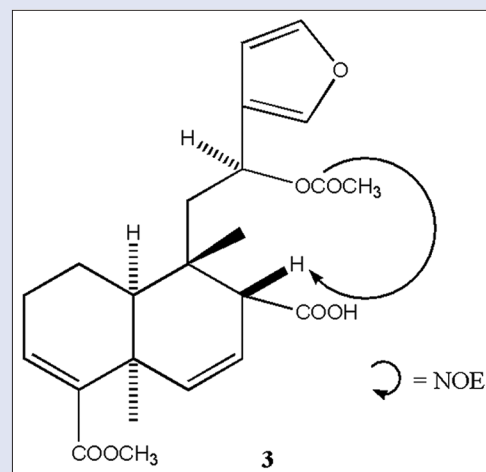


Figure 2: Structure of Crispene C showing nuclear overhauser effect

using high capacity grinding machine in the Phytochemical Research Laboratory, Faculty of Pharmacy, University of Dhaka.

Extraction and isolation

The powdered stems (600 mg) were soaked in methanol (3 L) at room temperature for 14 days with occasional shaking and the extract was collected by filtration. The solvent was evaporated under reduced pressure in a rotary evaporator to obtain a solid residue (5 g) which was then subjected to fractionation using the modified Kupchan partitioning method^[11] into *n*-hexane, CCl_4 , CHCl_3 , and aqueous soluble fractions. Evaporation of solvent afforded *n*-hexane (400 mg), CCl_4 (1.56 g), CHCl_3 (140 mg), and aqueous soluble fractions. The *n*-hexane soluble fraction was chromatographed over sephadex (LH-20), and the column was eluted with *n*-hexane: CH_2Cl_2 :MeOH (2:5:1) followed by CH_2Cl_2 :MeOH (9:1) and MeOH (100%) in order to increase the polarities. The column fractions were then concentrated and subjected to thin layer chromatography (TLC) screening. The fractions with a satisfactory resolution of compounds were rechromatographed over silica gel separately to obtain the pure compounds 1 (1.4 mg), 2 (1.3 mg), 3 (1.7 mg), 4 (1.6 mg), and 5 (3.8 mg).

RESULTS AND DISCUSSION

The methanol extract of the dried stems of *T. crispa* was successively partitioned with *n*-hexane, carbon tetrachloride, and chloroform. The *n*-hexane fraction was then subjected to column chromatography using sephadex (LH-20) followed by TLC screening and preparative TLC to isolate five pure compounds (1–5) [Figure 1]. The NMR spectra of these compounds suggested that four of them were furanoid diterpenes of clerodane series and another was furanoid diterpene glucoside of the same series.

The ^1H NMR spectra of 1–4, which are summarized in Tables 1 and 2, suggested some common structural features: The presence of an olefinic bond (C-3 and C-4), a furan ring (C-13–C-16), a carbonyl group (C-18), and two angular methyl groups (C-19 and C-20). The spectra also suggested the presence of two sp^3 -hybridized quaternary carbons (C-5 and C-9), two sp^3 -hybridized methines (C-8 and C-10) and an olefinic proton (C-3), which confirmed the absence of proton at C-4. The one proton broad signals at δ 6.72–7.01 and no cross connection between C-3 proton and any olefinic protons in correlation spectroscopy (COSY) experiment exclude the possibility of the double bond at C-1 and C-2.

Table 1: ¹H NMR spectral data of compounds 1-5 (400 MHz, CDCl₃)

Position	1	2	3	4	5
1a	2.01 m*	1.98-2.10 m*	1.98-2.03 m*	1.86-1.96 m*	1.90 m*
1b	2.01 m*	1.98-2.10 m*	1.98-2.03 m*	1.86-1.96 m*	1.90 m*
2a	2.10 m	2.34-2.46 m*	1.98-2.03 m*	2.39 m	2.36 m (15.2, 3.6)
2b	1.89 m	2.34-2.46 m*	1.98-2.03 m*	2.29 dq (16.4, 4.4)	2.29 dq (15.2, 5.0)
3	6.89 dd (3.6, 3.6)	6.88 dd (3.6, 3.6)	6.72	7.01 dd (4.0, 4.0)	6.99 dd (4.0, 3.6)
6	4.62 dd (10.6, 6.6)	4.46 dd (10.6, 7.4)	6.38 d (10.4)	5.46 d (6.0)	5.45 d (6.0)
7a	1.52 m	1.52 m	6.56 d (10.4)	1.98 bd (12.4)	1.95 bd (12.4)
7b	2.80 ddd (14.2, 7.0, 3.6)	2.58 ddd (14.2, 7.4, 2.4)	-	2.17 dd (12.0, 6.0)	2.13 dd (12.0, 6.0)
8	2.42 dd (4.0, 3.6)	2.48 dd (12.6, 2.2)	2.09 br s	2.63 d (5.6)	2.69 d (5.6)
10	2.00 bd (6.0)	1.94 bd (6.8)	2.54 dd (6.0, 2.0)	1.86-1.96 m*	1.90 m*
11a	2.33 dd (14.4, 4.4)	2.16 dd (14.4, 4.4)	1.92 dd (13.2, 8.4)	2.04 dd (15.2, 9.2)	2.19 ddd (15.2, 3.6, 3.6)
11b	1.82 dd (14.4, 11.6)	1.92 dd (14.4, 12.0)	2.28 dd (14.0, 8.6)	1.78 bd (14.4)	1.67 dd (15.2, 2.8)
12	5.51 dd (11.6, 4.8)	5.33 dd (12.0, 4.0)	5.94 dd (8.0, 7.6)	5.13 bd (8.4)	5.27 dd (9.6, 2.8)
14	6.41 br s	6.42 br s	6.41 br s	6.51 t (0.8)	6.46 d (1.2)
15	7.43 br s	7.42 br s	7.43 br s	7.36 t (1.6)	7.38 dd (1.2, 1.6)
16	7.46 br s	7.47 br s	7.46 br s	7.41 d (0.8)	7.48 br s
17	-	-	-	-	-
19	1.25 s	1.30 s	1.25 s	1.33 s	1.31 s
20	1.10 s	0.98 s	0.83 s	1.27 s	1.25 s
CH ₃ OCO-	-	-	3.73 s	3.73 s	3.72 s
AcO-	-	-	1.56 s	-	-
Glc 1'	-	-	-	-	4.29 d (8.0)

*Signals overlapped in each column. NMR: Nuclear magnetic resonance

Table 2: Comparison of ¹H NMR spectral data of compounds 4 and 5 with the published data

Position	4 (CDCl ₃ at 400 MHz)	Aglycone of borapetoside E (CDCl ₃ at 90 MHz) ^[9]	5 (CDCl ₃ at 400 MHz)	Borapetoside E (CDCl ₃ at 90 MHz) ^[9]
1a	1.86-1.96 m	-	1.90 m	-
1b	1.86-1.96 m	-	1.90 m	-
2a	2.39 m	-	2.36 m (15.2, 3.6)	-
2b	2.29 dq (16.4, 4.4)	-	2.29 dq (15.2, 5.0)	-
3	7.01 dd (4.0, 4.0)	7.02 dd (3.6, 4.1)	6.99 dd (4.0, 3.6)	7.01 dd (3.6, 4.1)
6	5.46 d (6.0)	5.46 br.d (5.1)	5.45 d (6.0)	5.46 br. d (5.1)
7a	1.98 bd (12.4)	-	1.95 bd (12.4)	-
7b	2.17 dd (12.0, 6.0)	-	2.13 dd (12.0, 6.0)	-
8	2.63 d (5.6)	2.63 br.d (5.1)	2.69 d (5.6)	2.71 br. d (5.1)
10	1.86-1.96 m	2.03 m	1.90 m	2.00 m
11a	2.04 dd (15.2, 9.2)	-	2.19 ddd (15.2, 3.6, 3.6)	-
11b	1.78 bd (14.4)	-	1.67 dd (15.2, 2.8)	-
12	5.13 bd (8.4)	5.08 dd (3.1, 8.2)	5.27 dd (9.6, 2.8)	5.24 dd (3.6, 8.2)
14	6.51 t (0.8)	6.51 m	6.46 d (1.2)	6.54 m
15	7.36 t (1.6)	7.44-7.32 (2H, m)	7.38 dd (1.2, 1.6)	7.36 m
16	7.41 d (0.8)	-	7.48 br s	7.52 m
19	1.33 s	1.33 s	1.31 s	1.32 s
20	1.27 s	1.28 s	1.25 s	1.27 s
CH ₃ OCO-	3.73 s	3.72 s	3.72 s	3.73 s
Glc 1'	-	-	4.29 d (8.0)	4.25 d (7.2)

NMR: Nuclear magnetic resonance

The spectra of 1, 2, and 4 showed signals attributed to four sp³-hybridized methylenes (C-1, C-2, C-7, and C-11) and two oxygenated methine groups (C-6 and C-12). Whereas, the spectrum of 3 indicated three sp³-hybridized methylenes (C-1, C-2, and C-11) and one oxygenated methine group (C-12).

The ESI-MS of compound 1 showed a pseudo molecular ion [M + Na]⁺ peak at m/z 365.0 corresponding to the molecular formula C₂₀H₂₂O₅ and compound 2 showed a pseudo molecular ion [M + H]⁺ peak at m/z 361.10 relevant to C₂₀H₂₄O₆. The ¹H NMR spectra of 1 and 2 [Table 1] displayed a one proton double doublet at δ 5.51 (*J* = 11.6, 4.8 Hz) and δ 5.33 (*J* = 12.0, 4.0 Hz), which were assigned to C-12. The difference in chemical shifts of C-12 (δ 5.51 and δ 5.33) is may be due to the dissimilarity in the fusion of B/C rings (*cis*-fused in 1 and *trans*-fused in 2).^[8] The downfield chemical

shifts of the protons at C-12 were also indicative of the presence of a lactone ring at C-12 and C-17. Strong cross peaks were observed between C-12 proton with the C-11β and C-11α protons in the COSY spectra. The one proton doublet of doublets (dd) at δ 2.42 (*J* 4.0, 3.6 Hz) in 1 and δ 2.48 (*J* = 12.6, 2.2 Hz) in 2 at C-8 were indicative of the presence of neighboring carbonyl group at C-17. The proton at C-6 of compound 1 showed a downfield dd at δ 4.62 (1H, *J* = 10.6, 6.6 Hz) which revealed the presence of another lactone ring between C-6 and C-18. The large coupling (10.6 Hz) is indicative of axial orientation of H-6. On the other, the proton at C-6 of compound 2 showed the corresponding downfield dd at δ 4.46 (1H, *J* = 10.6, 7.4 Hz), which indicated the presence of a free hydroxyl group (C-6). Again, the large coupling (10.6 Hz) confirms that the oxymethine proton at C-6 is axial. Based on these results and from a

comparison with the reported data of closely related compounds,^[9-13] the structure of 1 and 2 were characterized as shown. Compound 1 and 2 are found to be new diterpenes and given the trivial names Crispene A and Crispene B, respectively.

The relative configuration at various centers in 1 and 2 were derived from extensive dreiding model study and comparing the published ¹H NMR data of similar compounds. In the literatures, most of the bicyclic furanoid diterpenes of clerodane types isolated from *Tinospora* genus, the H-10 and C-19 methyl are in α orientation, i.e. A/B rings are *cis*-fused. Again, C-20 methyl and H-12 are in β and α configuration, respectively. In compound 1 and 2, the H-10 appears as broad doublet (6.0 Hz and 6.8 Hz) indicating that this proton is in axial (α) position (with respect to B ring) and the C-19 methyl is equatorial (α). As the H-10 is axial and α , from the biogenetic point the methyl group at C-9 must be in axial and β orientation. The H-8 proton in compound 1 appears as dd at δ 2.42 ($J = 4.0, 3.6$ Hz) showing its equatorial (β) orientation. Since both H-8 and C-20 methyl are in β orientation, B/C rings are *cis*-fused. The oxymethine proton at C-12 appears as dd at δ 5.51 ($J = 11.6, 4.8$ Hz) and the large coupling (11.6 Hz) confirms it to be axial (α). In compound 2, the H-8 proton appears as dd at δ 2.48 ($J = 12.6, 2.2$ Hz) and the large coupling of 12.6 Hz showed it to be in axial (α) position. Hence, the B/C rings are *trans*-fused. C-12 proton appears as dd at δ 5.33 ($J = 12.0, 4.0$ Hz), the large coupling of 12.0 Hz indicative of axial orientation and α as the C ring in half-chair conformation.^[14] In both 1 and 2, the relative deshielding of C-7 equatorial proton at δ 2.80 and 2.58, respectively, support earlier findings^[14] that C (8)-C (17)-O-C (12) are on the same plane.

ESI-MS of compound 3 gave a mol. wt. 417.20 [M + H]⁺ suggestive of C₂₃H₂₈O₇. The ¹H NMR spectrum of 3 [Table 1] displayed a one proton double doublet at δ 5.94 ($J = 8.0, 7.6$ Hz), which was assigned at C-12. The downfield shift (δ 5.94) of this proton was also indicative of the presence of an acetoxy group (OAc) at C-12,^[15] which was confirmed by the methyl signal at δ 1.56 (3H, s). In the mass spectrum, a presence of a strong fragment at m/z 358 produced by the loss of 59 also supports the presence of OAc group. The relative shielding of the acetoxymethyl is quite rare but not uncommon.^[15-17] The location of the OAc group at C-12 was also augmented by the cross-linking observed in the COSY between the oxymethine proton (δ 5.94) with C-11 protons at δ 2.28 and 1.92. Two downfield doublets at δ 6.38 (1H, $J = 10.4$ Hz) and δ 6.56 (1H, $J = 10.4$ Hz) revealed the presence of two olefinic protons and COSY showed that these protons only couple with each other, hence this double bond must be at C-6 and C-7. It was also substantiated by the presence of a ¹H singlet of C-8 (β and equatorial) proton at δ 2.09. It is interesting to note that in the related diterpenes, the H _{β} -8 proton appears as broad singlet, even when there are two protons at C-7.^[18,19] The placement of oxymethine proton at C-12 is determined on the basis of its close similarity in the chemical shift value (δ 5.94) with those reported for the same proton (H-12) in amritoside A and B.^[15] In addition, the nuclear overhauser effect experiment showed cross peaks between H _{β} -8 and CH₃OCO⁻ indicated that the orientation of H-12 is α [Figure 2]. The location of another CH₃OCO⁻ (δ 3.73, 3H, s) was placed at C-18 on common biogenetic ground. From these spectral data, the structure of 3 was elucidated as shown. To our knowledge, there is no record of any clerodane diterpene having olefinic bond between C-6 and C-7. This new compound is given the trivial name Crispene C.

Compound 4 has the molecular formula C₂₁H₂₆O₆ as determined by ESI-MS at m/z 375.20 [M + H]⁺. The ¹H NMR spectrum of 4 [Tables 1 and 2] suggested the presence of all the common structural features of 5 except the data for glucose moiety at C-12. Compound 5 exhibited a one proton dd at δ 5.27 ($J = 9.6, 2.8$ Hz) which was assigned to the C-12 proton and revealed the presence of glycosidic linkage. On

the other, compound 4 exhibited the corresponding signal at δ 5.13 (1H, *bd*, $J = 8.4$ Hz) which indicated the presence of free hydroxyl group at C-12. The signal at δ 3.73 (3H, s) was due to the ester methyl group at C-18. On the basis of these results and from a comparison with the published data,^[9,10] compound 4 was identified as the aglycone of 5. Though it was earlier reported as an enzymatic hydrolysis product of borapetoside E (5),^[9,10] this is the first record of 4 as free aglycone and the trivial name Crispene D has been proposed.

Compound 5 was characterized as borapetoside E by comparing the spectral data [Tables 1 and 2] with the published data of this compound.^[9,10] This diterpene glucoside was first reported from *Tinospora tuberculata*^[9] and later on isolated and the structure was revised as 5.^[10]

Crispene A (1)

Amorphous powder. ¹H-NMR (CDCl₃, 400 MHz): Table 1. ESI-MS (positive-ion mode) m/z: 365.0 [M + Na]⁺ (Calculated for C₂₀H₂₂O₅Na: 365.40), 350, 329, 288, 255, 155, 151, 102, 100, 91, 89.

Crispene B (2)

Amorphous powder. ¹H-NMR (CDCl₃, 400 MHz): Table 1. ESI-MS (positive-ion mode) m/z: 361.10 [M + H]⁺ (Calculated for C₂₀H₂₅O₆: 361.41), 289, 238, 214, 158, 141, 116, 101, 100, 90, 89.

Crispene C (3)

Amorphous powder. ¹H-NMR (CDCl₃, 400 MHz): Table 1. ESI-MS (positive-ion mode) m/z: 417.20 [M + H]⁺ (Calculated for C₂₃H₂₉O₇: 417.48), 379, 358, 347, 288, 255, 198, 142, 123, 100, 91, 89, 84.

Crispene D (4)

Colorless gum. ¹H-NMR (CDCl₃, 400 MHz): Tables 1 and 2. ESI-MS (positive-ion mode) m/z: 375.20 [M + H]⁺ (Calculated for C₂₁H₂₇O₆: 375.44), 357, 339, 289, 214, 158, 141, 130, 119, 102, 89.

Borapetoside E (5)

Amorphous powder: 47.1 (c 0.34, CDCl₃). ¹H-NMR (CDCl₃, 400 MHz): Tables 1 and 2. ¹³C-NMR (CDCl₃, 100 MHz) δ : 16.46 (C-1, CH₃), 24.17 (C-2, CH₃), 142.37 (C-3, CH), 134.30 (C-4, C), 39.30 (C-5, C), 82.87 (C-6, CH), 29.45 (C-7, CH₂), 46.55 (C-8, CH), 39.26 (C-9, C), 45.57 (C-10, CH), 46.62 (C-11, CH₂), 69.0 (C-12, CH), 125.86 (C-13, C), 108.93 (C-14, CH), 143.66 (C-15, CH), 140.20 (C-16, CH), 178.39 (C-17, C), 166.8 (C-18, C), 27.15 (C-19, CH₃), 21.69 (C-20, CH₃), 51.69 (CH₃COO-), 98.99 (C-1', CH), 73.86 (C-2', CH), 76.40 (C-3', CH), 70.73 (C-4', CH), 75.08 (C-5', CH), 62.55 (C-6', CH₂). ESI-MS (positive-ion mode) m/z: 559.14 [M + Na]⁺ (Calculated for C₂₇H₃₆O₁₁Na: 559.58), 357, 356, 204, 152, 119, 80.

CONCLUSION

We have isolated and characterized four new furanoid diterpenes of clerodane types, Crispene A, B, C and D (1-4), including one known furanoid diterpene glucoside, borapetoside E (5), from the stems of *T. crispa* (L.). To the best of our knowledge, there is no record of any clerodane diterpene, like Crispene C (3), having olefinic bond between C-6 and C-7.

Acknowledgments

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Nil.

Conflicts of interest

There are no conflicts of interest.

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