A new anti-feedant clerodane diterpenoid from *Tinospora cordifolia*

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Abstract: Phytochemical investigation of the chloroform extract of *Tinospora cordifolia* yielded a new clerodane diterpenoid tincordin (**1**) along with tinosporide (**2**), 8-hydroxytinosporide (**3**), columbin (**4**), 8-hydroxycolumbin (**5**) and 10-hydroxycolumbin (**6**). The structure of the new compound was elucidated comprehensively using 1D and 2D NMR methods. All major clerodane diterpenoids isolated were tested for their efficacy as insect antifeedants against *Earias vitella*, *Plutella xylostella* and *Spodoptera litura*.

Keywords: clerodane diterpenoids, *Tinospora cordifolia*, antifeedant activity.

1- Introduction

Tinospora genus (Menispermaceae) consists of deciduous woody climbers, distributed throughout the tropics of Asia, Africa and Australia. Members of this genus are known in Ayurveda for their adaptogenic and immuno-modulatory activity in fighting infections. (Dahanukar, Thatte, & Rege, 1999; Panchabhai, Kulkarni, & Rege, 2008; Prince, & Menon, 1999; Nayampalli, Ainapore, & Nadkarni, 1982).

The bitterness of the stem of *Tinospora cordifolia* is largely due to the clerodane glycosides (Swaminathan, Sinha, & Ramkumar, 1989; Gupta et al., 1967). Many compounds isolated from the plant are configurational isomers of clerodanes isolated from other Menispermaceae plants (Bhatt, & Sabata, 1990). As part of our pursuit to isolate clerodanes (Krishna Kumari et al.,

2003a; Krishna Kumari et al., 2003b; Aravind et al., 2010] and also to explore their antifeedant activity, the phytochemical examination of *Tinospora cordifolia* was taken up.

2-Results and Discussion

Six compounds were isolated from the chloroform extract (figure 1). Compound **2, 3** and **4** were previously reported from *T. cordifolia* (Bhatt, & Sabata, 1990; Hanuman, Bhatt, & Sabata, 1988; Swaminathan, Sinha, & Ramkumar, 1989). Compounds **5** and **6** are found for the first time from *T. cordifolia*. The spectral and physical data of **5** were in complete concurrence with the literature (Rasoanaivo et al., 1991; Oguakwa et al., 1986). Compound **6** was previously reported from *Tinospora malabaricum* (Atta-ur-Rahman, & Ahmad, 1988).

Compound **1** was obtained as an amorphous solid and had a molecular formula $C_{19}H_{22}O_6$ (HR-FAB-MS). Its IR spectrum showed absorptions corresponding to hydroxyl (3427 cm^{-1}), and furan (2923 cm^{-1}, 1442 cm^{-1}, 889 cm^{-1}) moieties. Apart from these, signals at 1705 and 1672 cm^{-1} indicated the presence of lactone carbonyl and unsaturated carbonyl groups, which were confirmed by the resonances at 164.6 and 201.6 ppm in the ^{13}C NMR spectrum.

^1H-NMR of **1** displayed signals at δ 7.71 (1H, *br s*), δ 7.67 (1H, *br s*) and δ6.55 (1H, *br s*) assignable to the protons of the β-substituted furan moiety common in clerodanes isolated from many *Tinospora* species (Huang et al., 2010; Bhatt, & Sabata, 1990; Hanuman, Bhatt, & Sabata, 1988; Atta-ur-Rahman, & Ahmad, 1988). Two angular methyls were observed as singlets at δ 1.52 and δ 1.06. Two D_2O exchangeable signals at δ 5.51 and δ 2.49 suggested the presence of two hydroxyl groups. The signals at δ 5.77 (1H, *dd*, J = 5.1 Hz), was assigned to the C-12 proton, bearing the β-substituted furan moiety. The signals at the aliphatic region δ 2.25 (1H, *dd*, *J* =14.3, 5.1 Hz), δ 2.08 (1H, *dd*, *J* = 14.3, 12 Hz) were attributed to the C-11 methylene protons.

On perusal of other signals, it was clearly evident that the NMR signals of **1** closely resembled the chemical structure of tinocallone C (decarboxycolumbin) (Song, & Xu, 1992) and fibaruretin E (Su et al., 2008). The olefinic proton resonances at δ 6.75 (1H, *dd*, *J* =10.1, 4.8 Hz) and δ 6.10 (1H, *d*, *J* =10.1 Hz) indicated the presence of α,β-unsaturated carbonyl system at C-4. The HMBC correlations observed from H-1 (δ 4.49) to C-3 (δ 128.7); H-2 (δ 6.75) to C-4 (δ 201.6) and CH_3-19 (δ 1.52) methyl protons to C-4 and C-6 (δ 73.0) augmented the above argument. Of the two hydroxyl resonances, the one at δ 2.49 was allocated to the carbon at position 1. The other deshielded hydroxyl peak at δ 5.51 was assigned to the C-6 based on

HMBC correlations. The relative configuration of **1** was deduced based on NOESY experiment. A cross peak was found from H-10 (δ 2.24) to CH_3-19 (δ 1.52), OH-6 (δ 5.51) and H-12 (δ 5.77) indicated the *cis* configuration for the A/B ring (Manabe, & Nishino, 1986) and also the *equatorial* orientations of C-6 OH and H-12. Furthermore, the NOE signal between H-6 (δ 3.78) and CH_3-20 (δ 1.06) substantiated the fact that they both have the same relative stereochemistry. Thus **1** was named tincordin and was assigned the following structure. (Figure 1)

All the clerodane diterpenoids isolated from *Tinospora cordifolia* were tested for their efficacy as insect antifeedants against *E. vitella*, *P. xylostella* and *S. litura*. The most potent insect antifeedant, azadirachtin-A, was used as a positive control for comparison. Initially the activities of these compounds were tested at 1 μg/cm^2 for *S. litura* and 1 μg/cm^3 for *E. vitella*, for which the results were not encouraging. Hence the dosage of the compounds for our study was fixed at 5 and 10 μg/cm^3 (approx.15 and 30 μg/ g of diet) for *E. vitella* and 5 and 10 μg/cm^2 of leaf area for *S. litura* respectively. Among the diterpenoids tested (Table 1), the most effective was 8-hydroxy tinosporide **3**. The differences in susceptibility of the insects to the diterpenoids were only marginal, *S. litura* being slightly more susceptible than *E. vitella*. The antifeedant activity of compound **3** at 5 and 10μg concentrations was equal to that of azadirachtin-A at 0.5 μg concentration.

3. **Experimental**

3.1 General procedures

Melting points were determined with a Concord melting point apparatus and are uncorrected. Optical rotations were determined on an Autopol apparatus. IR spectra were recorded on a IFS BRUKER FT-IR instrument. Routine ^{1}H (200 MHz) and ^{13}C (50 MHz) NMR spectra were recorded on a Bruker DPX200 spectrometer with TMS as internal standard. HMBC and NOE experiments of compound **1** were done on Varian unity plus spectrometer apparatus at ^{1}H (300 MHz) and ^{13}C (75 MHz). HRMS was done on a Micromass ESI-TOF instrument.

3.2 Plant material

The plant *Tinospora cordifolia* was collected from TAMPCOL farm, Chennai, India and was identified by Dr M.V.Rao, School of Biological Sciences, Bharathidasan University, Tiruchirapalli, India. A voucher specimen (RHT 18364) is deposited in the Herbarium of the Bharathidasan University.

3.3 Extraction and isolation of Clerodane diterpenoids

Finely powdered stems (9.5kg) were percolated with chloroform (10 L, three times) for 24 hrs. The resulting extract was then concentrated under vacuum to obtain a residue (100 g). Part of the crude extract (55g) was chromatographed over a silica column (70-325 mesh, ~750g, and column size - 90 cm x 15 cm) and eluted with $CHCl_3$/MeOH combinations [1-100%].

Initial fractions eluted in chloroform alone yielded sitosterol, stigmasterol and cycloartanol. Fractions 8-10 (eluted with 2% MeOH in $CHCl_3$) afforded **2** (75 mg) and Fraction 11 (eluted with 2% MeOH in $CHCl_3$) afforded **3** (36mg). Fractions 12-16 yielded a complex mixture of other clerodanes. Further chromatography on a silica column (230-400 mesh) and elution with 2% MeOH in $CHCl_3$ yielded **1** (7mg), **4** (42mg), **5** (13mg) and **6** (17mg).

Tincordin (1)
Molecular formula: $C_{19}H_{22}O_6$ **M.p:** 191°C
FAB-MS- Calculated *m/z* (M+ H^+) **-** Observed**-** 346.1413 Expected- 346.1416
IR (ν_{max}^{KBr} cm^{-1}): 3427, 2923, 1705, 1672, 1442, 889
$[\alpha]_D$ = -63^O ($CHCl_3$, c= 0.15)
UV(λ_{max}(log ε)): 232 (4.02), 208(5.29) nm
^{1}H-NMR (300MHz, $CDCl_3$): δ 4.49 (1H, *dd, J* = 5.2 and 4.8 Hz, H-1); 2.49 (1H, *d*, *J* = 5.2 Hz, OH-1); 6.75 (1H, *dd, J* = 10.1 and 4.8 Hz, H-2); 6.1(1H, *d, J* = 10.1 Hz, H-3); 3.78 (1H, *m*, H-6); 5.51 (1H, *d, J* = 4.3 Hz, OH-6); 1.56 (1H, *m*, H-7a); 1.86 (1H, *m*, H-7b); 2.95 (1H, *dd,* J = 9.2 and 8.9 Hz, H-8); 2.24 (1H, *br s*, H-10); 2.08 (1H, *dd, J* = 14.3 and 5.1 Hz, H-11a); 2.25 (1H, *dd*, *J* = 14.3 and 12 Hz, H-11b); 5.77 (1H, *dd, J* = 12 and 5.1 Hz, H-12); 6.55 (1H, *br s*, H-14); 7.67 (1H, *br s*, H-15); 7.71 (1H, *br s*, H-16); 1.52 (1H, *s*, CH_3-19); 1.06 (1H, *s*, CH_3-20).
^{13}C-NMR (75MHz, $CDCl_3$): δ (C 1) 63.9; (C-2) 143.8; (C-3) 128.7; (C-4) 201.6; (C-5) 42.2; (C-6) 73.0; (C-7) 26.5; (C-8) 49.7; (C-9) 35.7; (C-10) 47.2; (C-11) 36.5; (C-12) 72.4; (C-13) 127.3; (C-14) 110.9; (C-15) 141.9; (C-16) 145.8; (C-17) 164.6; (C-19) 32.4; (C-20) 22.1.

3.4 Insect rearing and antifeedant bioassay

Earias vitella: for rearing and bioassay, modified Klein medium was used replacing alfalfa meal with bhendi [*Abelmoschus esculentus*] seed powder (Krishna Kumari et al, 2003a).

Spodoptera litura: The test insect *S. litura* was reared in the laboratory on *Ricinus communis* (castor) leaf disks (180cm^2) were cut and kept in Petri-dishes (Krishna Kumari et al., 2003a) The individual compounds were dissolved in acetone and painted on the abaxial side using a pipette. Controls were treated with acetone alone and air-dried. Freshly moulted third-instar larvae were taken from the culture and starved for 4 h prior to testing. Five larvae were placed in each Petri-dish containing a leaf disc with a small piece of wet cotton to prevent dessication. Five replicates were maintained. The set-up was undisturbed for 24 h after which time the leaf disks were

removed for analysis. The disks were placed on a ΔT leaf area meter to determine leaf area consumed in treated versus control.

Plutella xylostella: Bioassay was conducted by no choice method (Krishna Kumari et al., 2003b). The test insect *P. xylostella* were reared in the laboratory on *Brassica oleracea var. capitata* (Cabbage) leaves at 25±2^{o}C respectively. Bioassay was conducted by no choice method. Fresh leaf discs (22 cm^2) were cut and kept in petri dishes. 110 µg of individual compound was dissolved in 500 µl of methanol to give 5 µg/cm^2 and 200 µg was dissolved in 500 µl to give 10 µg/cm^2 concentrations. The test solutions were painted on both sides of leaf using a pipette. Controls were treated with methanol alone and air-dried. Freshly moulted third-instar larvae were taken from the culture and starved for 1 h prior to testing. Five larvae were placed in each petri dish containing a leaf disc with a small piece of wet cotton to prevent desiccation. Five replicates were maintained for all treatments. The set up was undisturbed for 24 h, after which the leaf discs were removed for analysis. The discs were placed on ΔT leaf area meter to determine leaf area consumed in treated versus control.

The antifeedant activity percentage was calculated by the following method

$$\%AF = 100 - (Treated/control) * 100$$

Azadirachtin-A was tested at 0.5 µg/cm^2 concentration for all the insects.

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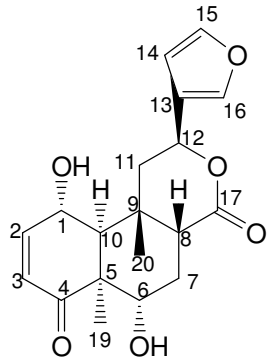
Table 1: Antifeedant activity of Diterpenoids

Compounds	Antifeedant activity (%)					
	Spodoptera litura¶ (μg/cm^2)		*Earias vitella*¶ (μg/cm^3)		*Plutella xylostella*¶ (μg/cm^2)	
	10	**5**	**10**	**5**	**10**	**5**
1	74.9 ±3.6	69.1 ±2.6	73.8±0.01	69.9±0.01	70.9 ±3.6	65.2±2.7
2	72.0 ±2.8	68.1±3.6	71.6 ±2.8	68.2±0.02	73.0 ±3.0	66.3±2.3
3	80.6 ±4.2	74.3±2.7	79.9 ±4.2	73.7±0.01	71.6 ±3.2	67.7 ±3.5
4	68.0±3.7	63.4±2.5	68.7±3.7	65.4±0.01	65.0±2.7	63.1 ±2.3
5	69.9±2.9	65.6±3.5	69.6±2.9	66.8±0.02	66.9±3.1	65.4 ±3.3
6	76.0±7.3	60.1±6.3	75.2±7.3	61.5±0.02	63.2±6.9	60.2±6.3
Azadirachtin A ‡	**79.2±4.7**		**78.2±4.6**		**71.02±2.4**	

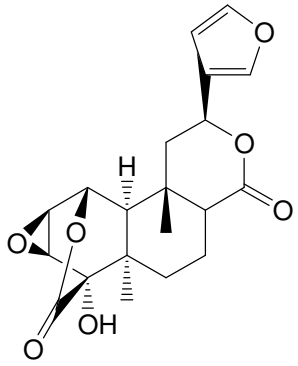
¶ Values are mean ± S.D.

‡ Treatment at 0.5 μg for all insects.

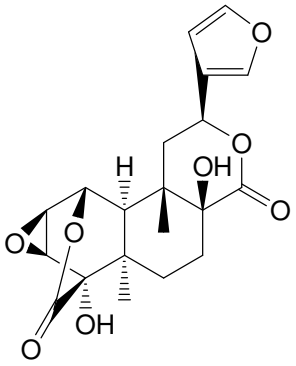
Figure 1: clerodanes isolated from *Tinospora cordifolia*



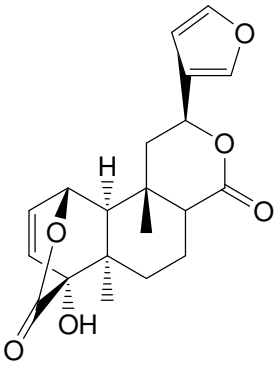
tincordin **1**



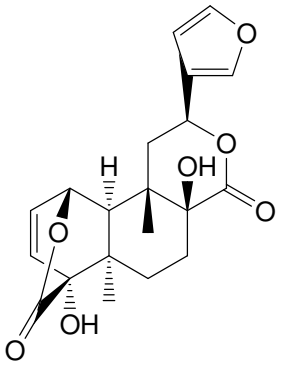
tinosporide (**2**)



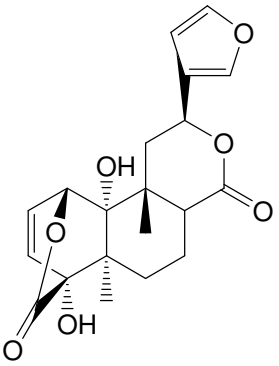
8-hydroxytinosporide (**3**)



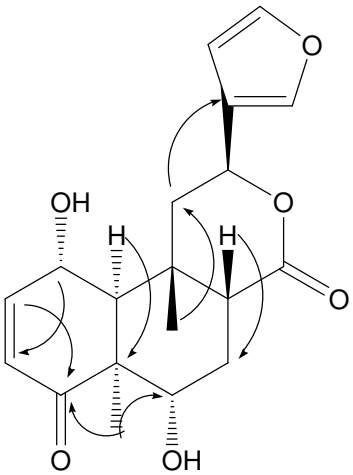
columbin (**4**)



8-hydroxycolumbin(**5**)



10-hydroxycolumbin(**6**)



HMBC interactions in **1**