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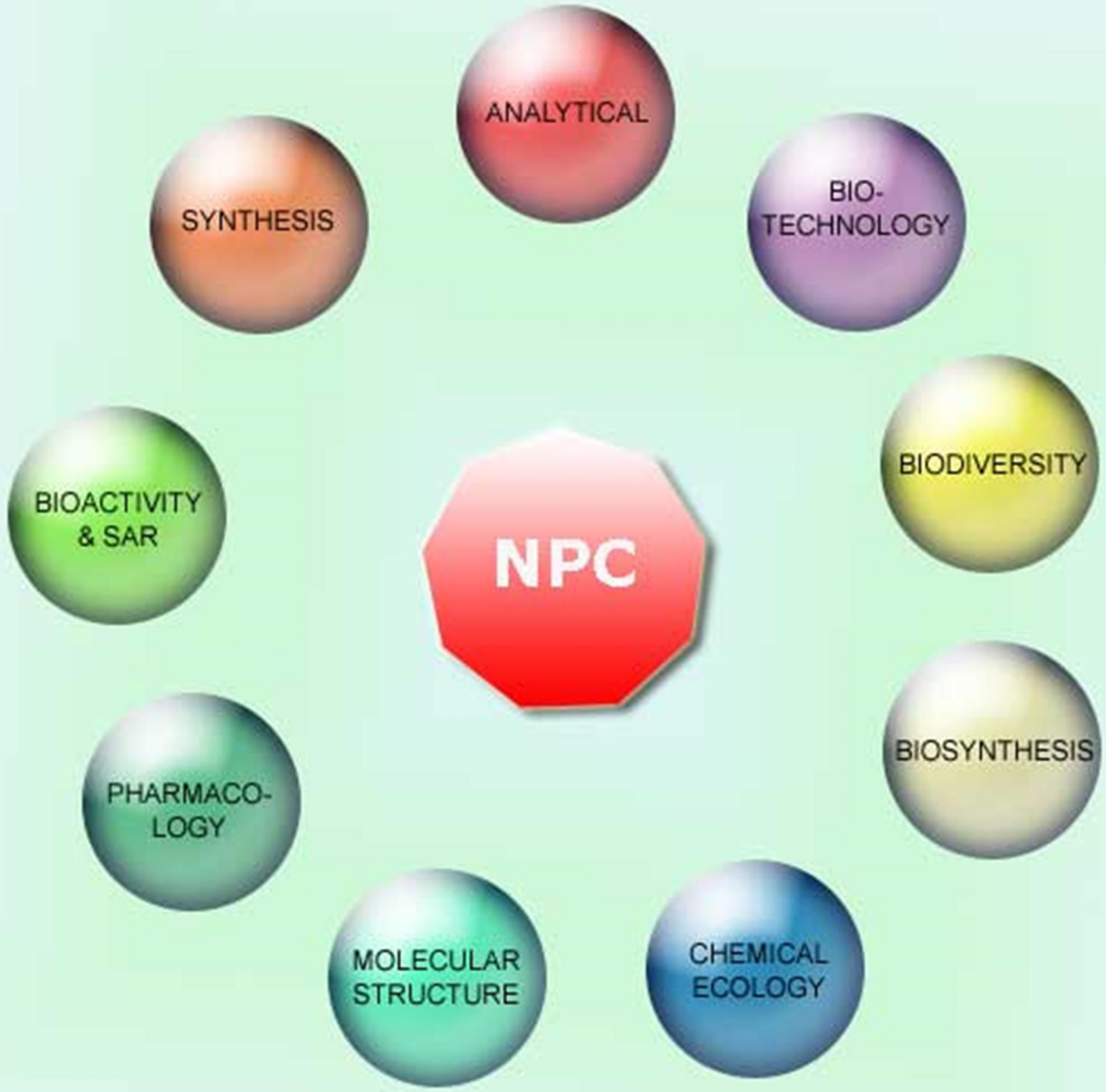

Volume 10. Issue 9. Pages 1495-1640. 2015
ISSN 1934-578X (printed); ISSN 1555-9475 (online)
www.naturalproduct.us

NPC Natural Product Communications

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NPC Natural Product Communications

2015
Vol. 10
No. 9
1525 - 1528

Triterpenoid Saponins from *Clematis graveolens* and Evaluation of their Insecticidal Activities

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Received: April 10th, 2015; Accepted: May 14th, 2015

A new hederagenin based triterpenoid saponin, clematograveolenoside A (**1**), along with three known saponins, tomentoside A (**2**), huzhangoside D (**3**) and clematoside S (**4**), were isolated from the roots and rhizomes of *Clematis graveolens*. The structure of new compound was elucidated on the basis of detailed analysis of chemical and spectroscopic data including 1D- and 2D NMR spectra. Compound **2** was found the most effective against aphid (*Aphis craccivora*) with an LC_{50} of 1.2 and 0.5 mg/mL after treatment for 72 and 96 h, respectively and was followed by compound **4** (LC_{50} = 2.3 and 1.9 mg/mL) and **1** (LC_{50} = 3.2 and 2.6 mg/mL). In case of termite (*Coptotermis homii*), compound **1** was found more toxic with an LC_{50} of 0.1 mg/L after 24 h of treatment followed by compound **2, 3** and **4** (LC_{50} = 0.1, 0.2 and 0.2 mg/mL, respectively).

Keywords: *Clematis graveolens*, Triterpenoid saponin, Insecticidal activity, *Aphis craccivora*, *Coptotermis homii*.

The genus Clematis L. (Ranunculaceae) consists of 295 species indigenous in north and south temperate, oceania and tropical African mountains [1]. In India, it is represented by thirty-two species including four sub species and five varieties [2]. The triterpenoids saponins, as dominant components of this genus possess extensive biological activities such as anti-inflammatory [3], antitumor [4], analgesic [5], cardio protective [6] and cytotoxic agents [7]. Apart from saponins, flavonoids have also been isolated from *Clematis hexapetala* [8]. *Clematis graveolens* occurs as climber, distributed between the altitudes 900-3000 m in the Himalayas [9]. The leaves cause blistering [10] and tincture prepared in spirit is used for the treatment of goiters and tumors of the neck [11]. This plant is mainly explored for biological activities and chemical investigation remains unexplored [12].

The termites are known to cause damage to the buildings, forestry crops, agriculture soils and stored products [13] in countries of America and Asia. The economic damage impact of termites was estimated 2-10 times greater to any other pest [14]. In recent past, the termite control was totally based upon inorganic chemicals and the synthetic insecticides. But the hazards on environment and carcinogenic caused by the synthetics underscored direct need for alternative insecticides, which would have environmental acceptability, farmer safety and termiticidal efficacy. The natural chemicals derived from the plants are possible alternatives. Many plant extracts [15] and essential oils [16] are reported to have potential termiticidal activity.

Herein, we report the isolation and characterization of a new hederagenin based triterpenoid saponin, clematograveolenoside (**1**), along with three known triterpenoid saponins (**2**-**4**) [Figure 1]. Insecticidal activities of compounds **1**-**4** were also evaluated.

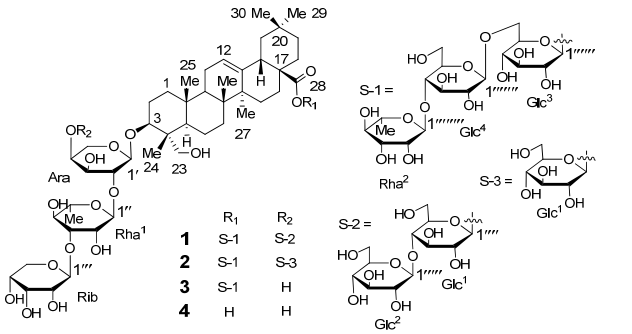

Figure 1: Structures of compounds (**1**-**4**).

Compound **1** was isolated as a brownish amorphous powder, and showed positive Molisch and Libermann-Burchard reactions, indicating **1** as triterpenoid or steroidal glycoside. The molecular formula was determined as $C_{76}H_{124}O_{40}$ based on pseudomolecular ion peak at m/z: 1711.7214 $[M+Cl]^-$ (calcd. for $C_{76}H_{124}O_{40}Cl$, 1711.7357) in negative HRESI-MS of **1**. The ^1H NMR spectrum (Table 1) of **1** displayed six tertiary methyl singlets in the upfield region at δ_H 0.83 (3H, s, Me-29), 0.91 (3H, s, Me-25), 0.96 (3H, s, Me-30), 1.06 (3H, s, Me-26), 1.15 (3H, s, Me-24), 1.23 (3H, s, Me-27), an olefinic proton at 5.36 (1H, br s, H-12), and an oxymethine at 4.20 (1H, overlapped, H-3). These signals indicate a pentacycle in compound **1**. Further, the downfield carbon signals at δ_C 81.3 (C-3) and 177.3 (C-28) in the ^{13}C-NMR spectrum of compound **1** indicated the structure to be bidesmoside of hederagenin [17]. Eight anomeric proton signals at δ_H 4.92 (1H, d, J = 7.2 Hz), 4.96 (1H, d, J = 7.8 Hz), 5.05 (1H, d, J = 7.2 Hz), 5.12 (1H, d, J = 8.0 Hz), 5.79 (br s), 5.91 (1H, d, J = 5.0 Hz), 6.19 (1H, d, J = 7.8 Hz) and 6.32 (1H, br s), along with two methyl at δ_H 1.53 (3H, d, J = 6.2 Hz),

Table 1: ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) spectroscopic data for glycone moiety of **1** (C_5D_5N, *J* in Hz).

Position	δ_C	δ_H (*J* in Hz)	Position	δ_C	δ_H (*J* in Hz)
	3-O-Ara			28-O-Glc	
1	104.9[a]	5.05 (d, *J* = 7.2)	1‴″	96.0	6.19 (d, *J* = 7.8)
2	74.9	4.52[a]	2‴″	74.1	4.11[a]
3	75.2	4.11[a]	3‴″	78.9	4.17[a]
4	81.0	4.08[a]	4‴″	70.5	4.29 (m)
5	66.1	4.28 (d, *J* = 9.5)	5‴″	78.2	3.99 (m)
		3.66 (d, *J* = 10.1)	6‴″	69.3	4.64 (d, *J* = 10.4)
					4.52[a]
	Rha			Glc	
1′	101.5	6.32 (br s)	1⁗″	105.1	4.96 (d, *J* = 7.8)
2′	72.1	4.86 (br s)	2⁗″	75.9	4.09[a]
3′	81.3	4.72[a]	3⁗″	76.7	3.90 (m)
4′	73.0	4.38[a]	4⁗″	78.8	4.32 (m)
5′	70.5	4.61 (m)	5⁗″	77.3	3.61 (m)
6′	18.7	1.53 (d, *J* = 6.2)	6⁗″	61.2	4.16[a]
					4.05 (m)
	Rib			Rha	
1″	104.9[a]	5.91 (d, *J* = 5.0)	1⁗‴″	103.0	5.79 (br s)
2″	72.5	4.23 (m)	2⁗‴″	72.9	4.65 (br s)
3″	70.0	4.52 (m)	3⁗‴″	72.8	4.52[a]
4″	71.6	4.12 (m)	4⁗‴″	74.9	4.29[a]
5″	65.5	4.40[a]	5⁗‴″	70.1	5.00 (m)
		4.17 (d, *J* = 10.9)	6⁗‴″	18.5	1.66 (d, *J* = 6.2)
	Glc				
1‴	107.0	5.12 (d, *J* = 8.0)			
2‴	76.4	4.00[a]			
3‴	78.9	4.17[a]			
4‴	80.1	4.14[a]			
5‴	78.8	3.82 (m)			
6‴	62.4	4.24 (m)			
		4.10[a]			
	Glc				
1⁗″	104.8	4.92 (d, *J* = 7.2)			
2⁗″	75.6	4.20[a]			
3⁗″	78.8	4.19 (m)			
4⁗″	70.5	4.25 (m)			
5⁗″	78.2	3.92 (m)			
6⁗″	62.8	4.52 (m)			
		4.45 (m)			

[a]overlapped signals.

1.66 (3H, d, *J* = 6.2) revealed the presence of eight monosaccharides including two deoxyhexose. From a comparison of ^{1}H and ^{13}C NMR data of the aglycone moiety of compound **1** with those reported in the literature [18], it is clear that the aglycon of compound **1** is (3*β*)-3,23-dihydroxyolean-12-en-28-oic acid. This is further confirmed by acidic hydrolysis of compound **1** and comparing the NMR data of aglycone moiety with reported values [19].

The ^{13}C and HMQC spectra of **1** suggested the presence of eight methyls at δ_C 14.4, 16.6, 17.9, 18.5, 18.7, 24.1, 26.5 and 34.4 for C-24, C-25, C-26, C-6⁗″″ of rhamnose-1, C-6′ of rhamnose-2, C-30, C-27 and C-29, respectively. Eight anomeric signals were observed at δ_C 96.0, 101.5, 103.0, 104.8, 104.9 (2C), 105.1 and 107.0 for glucose, rhamnose, rhamnose, arabinose, glucose ribose, glucose and glucose, respectively (Table 1).

The identity of the monosaccharide was determined on the basis of ^{1}H and ^{13}C NMR signals, which is further confirmed by acid hydrolysis of **1** and comparison with reference sugars by TLC and GC-MS analysis of the alditol acetate derivatives of the glycone portion [20, 21]. The sugars arabinose, ribose, rhamnose and glucose were found in a ratio of 1:1:2:4. The ^{1}H coupling constant (*J* >7Hz) were consistent with *β*-configuration of glucose [22, 23, 24]. An *α*-configuration of rhamnose was established on the basis of the C-5 signal of rhamnose at 70.1 and 70.5 [23-25].

Relatively large coupling constants (5.0-8.0 Hz) of anomeric signals suggested *α*-configuration of arabinose and *β*-configuration of ribose [26]. In HMBC spectrum of **1**, the signal of C-3 at δ_C 81.3 correlates with H-1′ (5.05, d, *J* = 7.2 Hz) of arbinopyranosyl indicating the linkage of arabinose sugar at C-3 of the aglycone. Similarly cross correlation between C-28 at δ_C 177.3 and H-1 (6.19, d, *J* = 7.8 Hz) of glucopyranosyl confirmed the linkage of glucose at C-28 through easter linkage. The ^{1}H NMR spectrum showed signals for other protons of the sugar residues at δ_H 3.90-5.00. A comparison of the ^{13}C NMR signals and the anomeric proton signals of the sugar moieties of compound **1** with reported values suggested the presence of a terminal *β*-D-ribose, *β*-D-glucose *α*-L-rhamnose, 2,4-substituted *α*-L-arabinose, 4-substituted *α*-L-rhamnose, two 4-substitued *β*-D-glucose and 6-substitued *β*-D-glucose. The linkages between the sugars were established on the basis of ^{13}C NMR values and HMBC correlations. Accordingly, the structure of compound **1** was established as 3-O-*β*-D-ribopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→2)-[*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→4)]-*α*-L-arabinopyranosyl hederagenin 28-*O*-*α*-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranoside.

Compounds **2**, **3** and **4** were identified as tomentoside A [27], huzhangoside D [28] and clematoside S [29], respectively by comparing the spectral data with reported values.

Insecticidal activity of compounds 1-4 against aphid, *Aphis craccivora* and termite, *Coptotermis homii:* The activity of pure compounds against aphid, *Aphis craccivora* in terms of lethal concentration (LC_{50}) to kill 50% of the population relative control values and other statistical parameters generated by linear regression analysis is summarized and presented in Table 2 and 3.

Table 2: Insecticidal activity of compounds **1-4** against aphid, *Aphis craccivora*.

Compounds	LC_{50} (mg/mL)	95% Cl (mg/mL)	Slope	Chi square	P
1(72 h)	3.2	2.0–13.7	1.4 ± 0.4	0.2	0.9
1 (96 h)	2.6	1.8–6.4	1.5 ± 0.4	0.1	1.0
2 (72 h)	1.2	0.8 –3.1	1.1 ± 0.3	0.3	1.0
2 (96 h)	0.5	3.1–6.9	1.3 ± 0.3	0.5	0.9
3 (72 & 96 h)	–	–	–	–	–
4 (72 h)	2.3	1.7–3.9	1.9 ± 0.5	1.4	0.5
4 (96 h)	1.9	1.5–2.7	2.2 ± 0.5	1.3	0.5
Dimethoate	0.002	0.002–0.003	3.3± 0.6	1.2	0.6

Cl: Confidence limits; h-hours after treatment; – LC_{50} values are not calculated for the treatment showing < 50% mortality

Table 3: Insecticidal activity of compounds **1-4** against termite, *Coptotermis homii* after 24 h.

Compounds	LC_{50} (mg/mL)	95% Cl (mg/mL)	Slope	Chi square	P
1	0.1	0.1 – 0.2	1.9 ± 0.5	1.2	0.5
2	0.1	0.1 – 0.3	1.7 ± 0.5	2.4	0.3
3	0.2	0.1 – 0.3	1.5 ± 0.5	3.8	0.2
4	0.2	0.1 – 0.4	1.8 ± 0.5	3.4	0.2
Imidacloprid	0.1	0.04 –0.2	3.1± 1.3	0.1	0.9

Cl: Confidence limits; h-hours after treatment

A. craccivora: Among the compounds tested, compound **2** was more effective against *A. craccivora* with an LC_{50} of 1.2 and 0.5 mg/mL after treatment for 72 and 96 h, respectively and was followed by compound **4** (LC_{50} = 2.3 and 1.9 mg/mL) and **1** (LC_{50} = 3.2 and 2.6 mg/mL). The compound **3** was not effective and its mortality was < 50%. Present results were also supported by Geyter et al. 2011 [30]. Similarly, saponins extracted from alfalfa cultivars against pea aphid (*Acrithosiphum pisum*) nymphs reared on an artificial diet containing 0.01% saponin showed 50% mortality after 4 days [31].

C. homii: Our effort to investigate insecticidal activity of these isolated compounds **1-4** against the *C. homi* showed encouraging results. Among the compounds tested, compound **1** was more

effective against *C. homi* with an LC_{50} of 0.1 mg/mL after 24 h of treatment and was followed by compound **2, 3** and **4** (LC_{50} = 0.1, 0.2 and 0.2 mg/mL respectively). All these compounds are comparable with positive control, imidacloprid (LC_{50} = 0.1 mg/mL) [Table 3] and can be used as effective biotermiticide and an efficient alternative to synthetic insecticides.

Experimental

General: Optical rotations were measured with a Horiba-SEPA-3 digital polarimeter. The elemental analysis was done on Elementor, Vacio EL apparatus. NMR spectra were recorded on BrukerAvance 111-600 spectrometer in *pyridine-d_5* with TMS as internal standard. HRESI-MS were obtained on Q-TOF-Waters Micromass and Maxis Bruker spectrometer. The melting points were measured on Barnstead Electrothermal 9100 instrument. Column chromatography (CC) was carried out on silica gel (60-120 mesh) Merck (8x80 cm). TLC was performed on percoated silica gel 60 F_{254} plates (Merck). The GC-MS was performed on a Shimadzu (QP 2010) series GC-MS (Tokyo, Japan) equipped with a AOC-20i auto-sampler coupled and a DB-5MS capillary column, (30 m 6 0.25 mm i.d., 0.25mm).

Plant material: The roots and rhizomes of *Clematis graveolens* Lindl. were collected in August 2013 from Kangra, Himachal Pradesh (India). The identification was performed from Punjab University Chandigarh (India) with PAN 6640.

Extraction and isolation: The dried roots and rhizomes (4 kg) of *C. graveolens* were extracted with 85% ethanol (10 L×2). After removal of the solvent under reduced pressure the residue (900 g) was dissolved in H_2O and partitioned between petroleum ether, CCl_4, EtOAc and finally with *n*-BuOH. The *n*-BuOH extract (120 g) was re-dissolved in MeOH (300 mL) and addition of Et_2O gave flocculent precipitates. Precipitates were washed three times with Et_2O to afford 50 g of crude saponin fraction. This saponin fraction was subjected to column chromatography over silica gel (60-120 mesh) in ($CHCl_3$-MeOH) and eluted with increasing polarity by MeOH. Compound **4** (30 mg), **3** (65 mg), **2** (30 mg) and **1** (70 mg) were obtained at polarity 15%, 18%, 20% and 25% MeOH in $CHCl_3$, respectively.

Acid hydrolysis of compounds 1-4: Each compound (5 mg) was refluxed with 2M HCl-MeOH (20 mL) (1:1) for 4 h at 95 ºC. The aglycones from hydrolyzed mixtures were extracted with EtOAc (3×10 mL) and dried. The aqueous layer was neutralized with 2M NaOH, extracted with pyridine and evaporated to dryness. Sugars were identified by TLC [*n*-BuOH: AcOH: EtOAc: H_2O (4: 2: 2: 1)] comparison with authentic samples. The hydrosylate part (2 mg) of each compound was acetylated with pyridine: acetic acid (1:1) mixture by continuous stirring for 24 h at room temperature. The mixture was diluted with water and extracted with EtOAc and dired. The EtOAc extract was subjected to GC-MS analysis. The absolute configurations of monosaccharide units were determined to be L-Arb (42.7 min), D-Rib (43.2 min), L-Rha (43.5 min), D-Glc (49.2 min), by comparison of retention time with standard acetates prepared in same manner from authentic samples and their respective mass fragments interpretation [20, 21]. The sugars L-arabinose, D-ribose, L-rhamnose, D-glucose were obtained in a ratio of (1: 1: 2: 4) for compound **1**, (1: 1: 2: 3) for compound **2**, (1: 1: 2: 2) for compound **3** and (1:1:1) for compound **4**.

Clematograveolenoside A
Brownish amorphous powder.
MP: 286-288°C.
$[\alpha]_D^{20}$: -28 (*c* 1.0, MeOH).
^1H and ^{13}C NMR (C_5D_5N, 600 MHz): Table 1.
HRESI-MS *m/z*: 1711.7214 $[M+Cl]^-$ (calcd. for $C_{76}H_{124}O_{40}Cl$, 1711.7357).

Insecticidal activity of compounds (1-4) against *A. craccivora* and *C. homii*: Toxicity of pure compounds was tested following Potters spray tower method against *A. craccivora*. 3000 mg of the test samples were dissolved in 10 mL of 0.05 percent Tritone (SD Fine Chemicals Limited, www.sdfine.com) in water and then ultra-sonicated for complete dissolution. Four to five concentrations (125 to 3000 ppm) of test solutions were prepared from stock solutions from higher concentration for dose response bioassay. Fresh bean discs (3 cm diameter) were prepared and pressed over the water-agar medium in Petri plates sprayed with 2 mL of the test solution at different concentrations under Potter's spray tower operated at 1.1 Kg/cm^2 pressure and the solvent was evaporated under a fume hood for 2 h. For control, leaf disks were sprayed with distilled water containing 0.05 percent Tritone. In each Petri dish, 10 numbers of wingless adult aphids were released then sealed with parafilm and kept in the laboratory conditions at 25±2°C temperature, 60±5% relative humidity and a photoperiod of 16:8 (L: D) for observations. All the treatments including control were replicated three times. Mortality was determined after 72 and 96 h of treatment. The synthetic insecticide dimethoate at recommended dose (1-25 ppm) was used as a positive control for comparison.

C. homii: Termiticidal activity of each compound was performed by the force-feeding method reported by Chang *et al.* 2007 [32]. The test solutions of compound 1-4, and the imidacloprid (chemical insecticide) were prepared at different concentrations (50, 100, 500, 1000 ppm/mL). The whatmann filter paper discs (6 No.) of the size of a petri dish (9 x 9 cm) were cut and dipped in each solution. After 10 minutes the discs were dried and placed in their respective Petri dish. The filter paper disc dipped in distilled water was used as control. In each dish 10 termites were released then covered with muslin cloth and incubated at 28°C for 24 h. Observations on number of termites in each treatment were counted.

Statistical analysis: Toxicity data from all bioassays was corrected for control mortality using Abbott formula [33]. The median lethal concentration (LC_{50}) and their corresponding 95 percent confidence limits were determined following probit analysis [34] and SPSS 10.00 statistical tool.

Acknowledgment - IHBT Communication No. for this MS is 3621. Authors are grateful to Director and HOD, NPC & PDD, CSIR-IHBT for providing the support. Mr. Rajeev Rattan thanks Dr. Satish Sharma (Principal) to provide necessary facilities and Dr. Ajay Ramdev for the help to record biological activities. The second and third author also thank CSIR, New Delhi for providing financial assistance (BSC-0110).

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Natural Product Communications
2015
Volume 10, Number 9

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