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Leaf surface *n*-alkanes of *Momordica cochinchinensis* Spreng as short-range attractants for its insect pest, *Aulacophora foveicollis* Lucas (Coleoptera: Chrysomelidae)

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ABSTRACT

Extraction, thin layer chromatography and gas chromatography-mass spectrophotometry analyses of surface wax *n*-alkanes of young, mature and senescent leaves of *Momordica cochinchinensis* Spreng (Cucurbitaceae) revealed the presence of 19, 20 and 18 alkanes between *n*-C_{15} and *n*-C_{35}, representing 85.62, 89.11 and 86.66 % of alkanes, respectively. The cuticular alkanes from young, mature and senescent leaves attracted the female insect, *Aulacophora foveicollis* Lucas (Coleoptera: Chrysomelidae) between 2-12, 1-12 and 6-12 µg concentrations, respectively; whereas the mixtures of synthetic alkanes mimicking cuticular alkanes of three types of leaves showed attraction between 6-12 µg concentrations in Y-shaped glass tube olfactometer bioassay. Individual synthetic nonadecane, nonacosane, hentriacontane, tritriacontane and pentatriacontane at the minimal amount of 65, 741, 729, 796 and 7144 ng, respectively, elicited attraction of insect. A synthetic blend of 476, 607, 508 and 6689 ng of nonacosane, hentriacontane, tritriacontane and pentatriacontane, respectively, showed highest attraction of insect and could be used as trapping tool for pest management.

Keywords: Attractant, *Aulacophora foveicollis*, Cucurbitaceae, insect pest**,** leaf surface alkanes, *Momordica cochinchinensis*, Y-shaped olfactometer bioassay

INTRODUCTION

In recent decades the red pumpkin beetle [*Aulacophora foveicollis* Lucas (Coleoptera: Chrysomelidae)] has become a serious pest of cucurbitaceous plants (1,30,31). Its both larvae and adults cause serious damage to host plants [*Cucurbita maxima* Duchesne*, C. moschata* Duchesne*, C. pepo* L.*., Cucumis sativus* L.*., Lagenaria vulgaris* Ser.*., Luffa cylindrica* L.*., Momordica cochinchinensis* Spreng*, Benincasa hispida* Thumb.*,* etc. (11,21,25,32,37)]. Neonate larvae feed on young and healthy roots of plants and the larvae continue to feed for 12-13 days on the roots before pupating in soil. After pupation (11-12 days), newly emerged adults consume leaf and flower tissue voraciously for 8-9 weeks (25). The feeding kills branches and shoots of hosts but not the entire plant (31). The insect can withstand wide ranges of humidity and temperature and switch from one crop to another within same growing season.

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Momordica cochinchinensis (Cucurbitaceae) is an important summer vegetable in Southeast Asian countries [India, Bangladesh and Vietnam (6,38)]. Its leaves are cooked as vegetables. The adults of *A. foveicollis* due to their gregarious feeding habit, rapidly consume leaves of this plant and thereby cause serious reduction in crop yields. Growers are often forced to apply insecticides [Carbofuran and Diazinon-60EC] for control of this insect (20,32,33). To reduce yield losses and environmental risks associated with insecticide application, it is necessary to develop new environment friendly products, for inclusion in integrated pest management (IPM). Hence, studies on the allelochemicals of *M. cochinchinensis* plant may provide clues such as baited traps, for pest control of adult and to inform the plant breeders to develop insect resistant crop varieties.

The major classes of cuticular waxes of diverse plant families are *n*-alkanes (*n*-C_{15} to *n*-C_{36}) (3,18) and the amount and composition of *n*-alkanes in epicuticular wax of plant, among the species and also within the species with different stages of leaf development (10,19,23,27). Alkanes attract (8,22,28,30) and/or stimulate the oviposition in coleopteran and lepidopteran insects (9,27). Long-chain alkanes [heneicosane, docosane, tricosane, tetracosane, pentacosane, hexacosane and heptacosane] from labellum extracts of orchid flower (*Ophrys sphegodes* Miller) attracts the solitary bee [*Andrena nigroaenea* (Kirby)] (28). Further, alkanes from leaf surface waxes of bitter gourd (*Momordica charantia* L.) showed attraction of *Epilachna dodecastigma* (Wied.) (26). We reported previously that free fatty acids from *M. cochinchinensis* leaf surface waxes elicited attraction of *A. foveicollis* (16). As part of our continuous work on bioassay guided isolation and characterization of secondary chemicals from leaves, here we report alkane profile throughout developmental stages of *M. cochinchinensis* leaves [i.e., young (1 ≤ week old), mature (2-4 weeks old) and senescent (5-7 weeks old)] and whether these differences in alkane concentrations can act as olfactory cues to attract the insect, *A. foveicollis* using a Y-maze olfactometer under laboratory conditions. If the alkanes present throughout the various ages of leaves are used by the insect in host location, then these compounds may provide clues to sustainable pest management strategies such as baited traps. Further, we studied the role of mixtures of synthetic alkanes mimicking cuticular alkanes of young, mature and senescent leaves followed by individual synthetic alkanes and a combination of synthetic alkanes that elicited response to the insect as an olfactory cue for *A. foveicollis*.

MATERIALS AND METHODS

I. Plant materials

Momordica cochinchinensis plants were cultivated in the field of our University Crop Research Farm, Burdwan (23°16′ N and 87°54′ E), West Bengal, India in first week of May 2013 (temperatures 30 - 37 °C). The plants were irrigated on alternate days and kept free from insecticide or herbicide use but weeds were removed weekly from the field by hand-picking method. The voucher specimen numbers are Mukherjee and Barik 1 and 2, one of which has been deposited in the Ecotaxonomy Laboratory, Department of Botany of our University. Different ages of leaves were classified based on developmental time following leaf emergence, leaf size, colour and texture of leaves at the time of collection, i.e., young (1≤ week old), mature (2-4 weeks old) and senescent (5-7 weeks old).

II. Extraction of leaf surface n-alkanes and identification

Fresh young (1≤ week old), mature (2-4 weeks old) and senescent (5-7 weeks old) leaves of *M. cochinchinensis* were randomly collected from the field during July - September 2013. Leaves were initially rinsed with distilled water and paper toweled for drying. One hundred g of each leaf type [i.e., young (number of leaves : 187.33), mature (number of leaves : 77.33) and senescent (number of leaves : 71.67); was separately dipped in 2 L *n*-hexane for 5 min at room temperature (27±1°C) for extraction of surface wax from the leaves, which yielded a light straw coloured extract without trace of chlorophyll (5,23,26). The crude extract was then passed through Whatman (Maidstone, UK) No. 41 filter paper and the solvent was removed under reduced pressure. The extract was further passed through a column of aluminium oxide (Alcoa, Frankfurt, Germany: F-20 grade) and eluted with petroleum ether. The eluent was fractioned by thin layer chromatography (TLC) on silica gel G (Sigma St. Louis, MO, USA) layers (thickness 0.5 mm), which had been prepared using a Unoplan (Shandon, London) coating apparatus, with carbon tetrachloride as the mobile phase. The single hydrocarbon band was eluted from the silica gel layer with chloroform, which showed no absorption of detectable functional groups by IR spectroscopy. The extraction process was repeated for thrice with one hundred grams of each type of leaf, separately (5, 15, 23, 26). The amounts of 2.4, 4.54 and 1.93 mg of alkane samples from one hundred grams of young, mature and senescent leaves, respectively were kept for quantification of the alkane compounds by gas chromatography (GC) and identification with coupled gas chromatography-mass spectrometry (GC-MS) and the 2 mg alkanes from each type of leaf were also used for the olfactory bioassay. All solvents used were of GR grade and purchased from E. Merck, India Pvt. Ltd.

Three separate extracts of each treatment (i.e., young, mature and senescent leaves) were analyzed by a Techcomp GC (Em Macau, Rua De Pequim, Nos. 202A-246, Centro Financeiro F7, Hong Kong) model 7900 fitted with an HP-1 capillary column (Agilent, USA; length: 30 m×0.25 mm×0.25-µm film thickness) and a flame ionization detector. The oven temperature programme was initially held at 170°C for 1 min, then raised at 4°C/min to 300°C and finally held at this temperature for 15 min (5, 23, 26). The carrier gas was nitrogen with a flow rate of 18.5 mL/min. The injector port temperature was maintained at 280°C. The volume of the sample injected was 1µL with a split ratio of 1:10. The limit of detection of the GC instrument is $\leq 5 \times 10^{-12}$ g/s (*n*-hexadecane). The peaks were tentatively identified by comparison of their retention times with those of the standard *n*-alkanes from *n*-C_{15} through *n*-C_{35} and the percentage composition of *n*-alkanes in respective leaves was computed from GC peak areas. Separate calibration curves were prepared for standard alkane from *n*-C_{15} to *n*-C_{35} to calculate R^2 values (i.e., 0.9990 - 0.9999), limit of detection (LOD) and limit of quantification (LOQ) of alkanes (Table 1). All *n*-alkanes (>99% purity) from *n*-C_{15} to *n*-C_{35} were purchased from Sigma Aldrich.

To confirm the identifications, the extracts were analyzed with an Agilent 6890 GC coupled to a 5973 Mass Selective Detector, which was run under same temperature conditions as mentioned in GC analysis and using an HP-1 column. The carrier gas was helium. The MS parameters were 280°C at the interface, ionization energy 70 eV, scan speed approximately 1 sec and scanned over the mass range 40-600 mass units. The

Table 1. Calibration curves, R^2 values, limit of detection (LOD) in µg and limit of quantification (LOQ) in µg for each alkane compound

Alkane	Calibration curves	R^2	LOD	LOQ
Pentadecane (*n*-C_{15})	Y = 409.21 x - 0.75	0.9998	0.0024692	0.008231
Hexadecane (*n*-C_{16})	Y = 445.98 x - 0.3333	0.9998	0.0029969	0.00999
Heptadecane (*n*-C_{17})	Y = 469.35 x - 0.7917	0.9999	0.0029212	0.009737
Octadecane (*n*-C_{18})	Y = 419.25 x + 0.3333	0.9996	0.0037753	0.012584
Nonadecane (*n*-C_{19})	Y = 377.07 x + 0.875	0.9998	0.0070866	0.023622
Eicosane (*n*-C_{20})	Y = 358.45 x + 0.4167	0.9998	0.004708	0.015693
Heneicosane (*n*-C_{21})	Y = 351.37 x + 0.5417	0.9995	0.0230225	0.076742
Docosane (*n*-C_{22})	Y = 345.67 x + 0.2083	0.9996	0.0050627	0.016876
Tricosane (*n*-C_{23})	Y = 350.26 x + 0.2083	0.9995	0.0041354	0.013785
Tetracosane (*n*-C_{24})	Y = 331.83 x + 0.125	0.9996	0.0041323	0.013774
Pentacosane (*n*-C_{25})	Y = 311.48 x + 0.2083	0.9998	0.0050489	0.01683
Hexacosane (*n*-C_{26})	Y = 305.96 x + 0.3333	0.9998	0.0194055	0.064685
Heptacosane (*n*-C_{27})	Y = 298.73 x + 0.5417	0.9992	0.0059221	0.01974
Octacosane (*n*-C_{28})	Y = 292.01 x + 0.375	0.9988	0.0064261	0.02142
Nonacosane (*n*-C_{29})	Y = 288.09 x + 0.3333	0.9994	0.0063218	0.021073
Triacontane (*n*-C_{30})	Y = 285.76 x + 0.5417	0.9990	0.0052198	0.017399
Hentriacontane (*n*-C_{31})	Y = 287.21 x + 0.25	0.9992	0.002623	0.008743
Dotriacontane (*n*-C_{32})	Y = 286.13 x + 0.5833	0.9993	0.0027281	0.009094
Tritriacontane (*n*-C_{33})	Y = 264.38 x + 0.8333	0.9999	0.0110399	0.0368
Tetratriacontane (*n*-C_{34})	Y = 249.7 x + 0.2083	0.9993	0.0047563	0.015854
Pentatriacontane (*n*-C_{35})	Y = 246.01 x + 0.375	0.9997	0.0434526	0.144842

identity of compounds was confirmed by injections of mixture of synthetic *n*-alkanes (i.e., *n*-C_{15} to *n*-C_{35}). Alkanes were verified by comparison of diagnostic ions and GC retention times with those of respective authentic standards.

III. Test insects

The insects used in this study were collected by light trap from the bottle gourd (*Lagenaria siceraria* (Molina) Standl.) plants growing in our Farm and maintained in 1 L glass jars, containing bottle gourd leaves (covered with fine-mesh nylon nets) at 27±1°C, 65±5% RH and 12 L: 12 D photoperiod in a biochemical oxygen demand (BOD) incubator (ADS instruments and Tech., Calcutta). *Aulacophora foveicollis* female insects of different ages were provided with water and starved for 10 h prior to use in olfactory bioassays. Age was not considered during olfactory bioassay as the adult females consume leaves of this plant voraciously for 8-9 weeks until death (31). Females were used in bioassay because they are guided by olfactory cues for both adult feeding and location of suitable larval hosts.

IV. Olfactory bioassay

Two mg cuticular alkanes from each type of leaf were dissolved in 20 mL petroleum ether to prepare 7-concentrations (1, 2, 4, 6, 8, 10 and 12 µg/mL) of alkanes for olfactory bioassay. These concentrations were chosen by preliminary olfactometer trials assuming that a single mature leaf was sufficient to attract the insect and gradually the doses of alkane were lowered to the point, where the insect did not indicate clear response

to the test odour. The highest dose of 12 µg/mL was used in this study, because this amount produced same significant ($P < 0.00001$) response to the test insect as a single mature leaf. To prepare the synthetic mixtures of alkanes mimicking cuticular alkanes of young, mature and senescent leaves, the identified mole percentage of each alkane was converted to weight percentage and a mixture of 2 mg synthetic alkanes was prepared mimicking each type of leaf; subsequently different concentrations were prepared by the above-mentioned procedure (Table 2A, 2B and 2C). The amounts of individual alkanes present in various concentrations (1, 2, 4, 6, 8, 10 and 12 µg/mL) of leaf surface wax alkanes were also prepared using synthetic alkanes for bioassay to observe role of individual alkanes in insect response. The Y-shaped glass tube olfactometer has two 0.6 cm radius × 5 cm long arms and a 0.6 cm radius × 5 cm long glass common arm (14,15,16). The right and left arm of Y-tube were connected to two micro kit adapters which were fitted into two long glass vials (1 cm radius × 3 cm long). One glass vial contained a piece (2 cm^2) of Whatman No. 41 filter paper moistened with 1 mL of a particular concentration of alkanes, whilst the other glass vial contained a same size of filter paper moistened with 1 mL of the control solvent (petroleum ether). Each membrane pump produced an air-flow of 450 mL min^{-1}, were first purified by passing through charcoal pellets and then led into left and right glass vials. All the connections between different parts of the set-up consisted of silicon tubing.

The effectiveness of alkanes as attractants was evaluated as under in laboratory [27±1ºC, 70±3 % RH and 150 lux light intensity]. One ml of alkanes concentration from each type of leaf and the pure solvent were applied to the filter paper pieces and allowed to evaporate the solvent in open space under given laboratory condition and these filter papers were introduced into the glass vials. One adult female, *A. foveicollis* was introduced into the porous glass vial (1 cm radius × 3 cm long) which was then attached with the common arm of the olfactometer and exposed to a particular odor, consisting of 1 mL of the control solvent (petroleum ether) in one glass vial and 1 mL of one of the different odors (alkanes) in another glass vial. This insect was not attracted by the control solvent in preliminary assays. The behaviour of insects, i.e., olfactory responses of insects toward alkane odor air flowing through one arm and control solvent air flowing through other arm was studied in a Y-tube olfactometer for 30 min in preliminary bioassays and subsequently, it was observed that olfactory responses of the insects either in the alkane odor-loaded arm or control solvent-loaded arm at 3 min and 30 min were same. Hence, the behavior of each female was observed for 3 min in the Y-tube in all further bioassays. A female was considered to have made a choice if it reached the end of the odor-loaded arm or solvent-loaded arm of the olfactometer, the insect was removed from the Y-tube and the choice of the insect was recorded either as a positive response or negative response by one unit, respectively. By contrast, a female was considered not having made a choice, i.e., "no response" if it remained in the common arm of the Y-tube by the end of the observation period, because only the scores for odor loaded and control air-flow can be compared with each other (2, 4, 12, 15, 16, 24, 26, 27). Each experiment with one alkane sample was conducted with a group of 90 (i.e., 30 insects ×3) naïve insects; and after testing 5 insects the olfactometer set-ups were cleaned with petroleum ether followed by acetone and the position of the two arms was systematically changed in order to avoid positional bias.

Table 2A. Proportions of different synthetic alkanes mixed to prepare concentrations mimicking young leaves of *M. cochinchinensis*

Synthetic alkanes	Concentration (ng)*						
	1000	2000	4000	6000	8000	10000	12000
n-C_{15}	1	2	5	7	10	12	15
n-C_{16}	2	5	10	14	19	24	29
n-C_{17}	1	1	3	4	5	7	8
n-C_{18}	5	9	18	28	37	46	55
n-C_{19}	5	11	22	33	44	55	65
n-C_{20}	3	6	12	18	24	30	35
n-C_{21}	3	6	12	18	24	30	36
n-C_{22}	3	5	11	16	22	27	32
n-C_{23}	2	4	8	12	16	20	24
n-C_{24}	2	4	8	12	16	19	23
n-C_{25}	2	4	9	13	17	21	26
n-C_{26}	2	3	6	10	13	16	19
n-C_{28}	3	6	13	19	25	31	38
n-C_{29}	71	142	284	426	567	709	851
n-C_{30}	4	7	15	22	30	37	45
n-C_{31}	49	99	198	297	396	495	594
n-C_{32}	13	26	52	79	105	131	157
n-C_{33}	41	82	163	245	326	408	489
n-C_{35}	644	1288	2577	3866	5155	6443	7732

* To prepare the synthetic mixtures of alkanes mimicking the combinations and proportions of alkanes in young leaves, the identified mole % of each alkane was converted to weight % (i.e., mole % of the alkane × molecular weight of the alkane = Fraction and then Fraction × 100 / Fraction total = Weight %). Adding of each alkane in each mixture of alkanes concentration will not be exactly equal because of absence of some branched chain alkanes.

Table 2B. Proportions of different synthetic alkanes mixed to prepare concentrations mimicking mature leaves of *M. cochinchinensis*

Synthetic alkanes	Concentration (ng)*						
	1000	2000	4000	6000	8000	10000	12000
n-C_{15}	1	2	5	7	9	11	13
n-C_{16}	1	3	5	8	11	14	16
n-C_{17}	2	4	8	13	17	21	25
n-C_{18}	3	7	13	20	26	32	39
n-C_{19}	2	4	9	13	18	22	27
n-C_{20}	3	6	13	19	25	32	38
n-C_{21}	1	2	4	6	8	10	12
n-C_{22}	3	5	10	15	20	25	30
n-C_{23}	1	2	4	7	9	11	13
n-C_{25}	2	5	10	14	19	24	29
n-C_{26}	4	7	15	22	29	36	43
n-C_{27}	4	7	14	21	28	35	42
n-C_{28}	2	3	6	9	13	16	19
n-C_{29}	48	95	190	286	381	476	571
n-C_{30}	4	8	17	25	34	42	51
n-C_{31}	61	121	243	364	486	607	729
n-C_{32}	23	46	92	138	184	230	276
n-C_{33}	51	102	203	305	407	508	610
n-C_{34}	7	14	27	41	55	68	82
n-C_{35}	668	1338	2676	4013	5351	6689	8027

*see under Table 2A

Table 2C. Proportions of different synthetic alkanes mixed to prepare concentrations mimicking senescent leaves of *M. cochinchinensis*

Synthetic Alkanes	Concentration (ng)*						
	1000	2000	4000	6000	8000	10000	12000
n-C_{16}	2	4	8	13	16	20	24
n-C_{17}	2	4	9	13	17	22	26
n-C_{18}	4	8	15	23	30	38	45
n-C_{19}	2	5	10	15	20	25	30
n-C_{20}	3	6	13	19	25	32	38
n-C_{21}	3	7	13	20	27	34	40
n-C_{22}	4	7	15	22	29	37	44
n-C_{24}	3	5	10	16	21	26	31
n-C_{26}	2	4	7	11	14	18	22
n-C_{27}	5	9	18	28	37	46	55
n-C_{28}	2	5	9	14	18	23	27
n-C_{29}	62	123	247	370	494	617	741
n-C_{30}	7	14	27	41	55	68	82
n-C_{31}	71	142	283	425	567	708	850
n-C_{32}	25	51	101	152	203	254	304
n-C_{33}	66	133	265	398	531	663	796
n-C_{34}	8	17	33	50	66	83	99
n-C_{35}	595	1191	2381	3572	4763	5954	7144

*see under Table 2A

Non-respondents were not included in the analysis. Experiments with leaf alkane samples, synthetic alkanes mixtures or individual synthetic alkanes were conducted in the same manner.

Statistical analyses

Two-way ANOVA following Tukey test were performed to find any differences between the leaf ages and concentrations of cuticular hydrocarbons as variables using responses of *A. foveicollis* to cuticular alkanes as dependent variable (SPSS 16.0; SPSS Inc., IL, USA). The data obtained on responses of *A. foveicollis* to different concentrations of cuticular alkanes, synthetic mixtures of alkanes and individual synthetic alkanes were analyzed based on the null hypothesis that the probability of scores for the test compound(s) or control solvent is equal to 50%, i.e., chi-square analysis (2, 4, 12, 15, 16, 24, 26, 27, 34). Insects that did not respond by selecting either arm of the olfactometer (i.e., insect replaced by new one when the insect did not respond) were not included in the analyses.

RESULTS AND DISCUSSION

The *n*-hexane extracts of 100 g of young, mature and senescent leaves yielded 4.4 ± 0.17, 6.54 ± 0.06 and 3.93 ± 0.09 mg of purified *n*-alkanes, respectively; whereas a single young, mature and senescent leaf contained 23.47 ± 0.53, 83.97 ± 1.32 and 54.87 ± 0.32 µg of *n*-alkanes (mean ± SE), respectively. The GC-FID and GC-MS analyses detected 19, 20 and 18 *n*-alkanes ranging from *n*-C_{15} to *n*-C_{35} alkanes in the surface waxes of young, mature and senescent leaves, respectively (Table 3). The identified hydrocarbons of young, mature and senescent leaves represented 85.62, 89.11 and 86.66% of the total

alkanes with the balance consisting of unidentified branched-chain alkanes, respectively (Table 3). Pentatriacontane (*n*-C_{35}) was the predominant *n*-alkane accounting for 60.93, 61.99 and 55.18% in young, mature and senescent leaves, respectively; whereas heptadecane (*n*-C_{17}), heneicosane (*n*-C_{21}) and hexacosane (*n*-C_{26}) were the least abundant alkanes in young, mature and senescent leaves, representing 0.13, 0.17 and 0.24%, respectively. Heptacosane (*n*-C_{27}) and tetratriacontane (*n*-C_{34}) were absent in young leaves, whereas *n*-C_{15}, tricosane (*n*-C_{23}) and pentacosane (*n*-C_{25}) were absent in senescent leaves and tetracosane (*n*-C_{24}) was absent in mature leaves. Nonacosane (*n*-C_{29}) is the second most abundant alkane in young leaves while hentriacontane (*n*-C_{31}) is the second most abundant alkane in mature and senescent leaves and rest of the alkanes displayed different patterns throughout different ages of leaves. In literature, changes in *n*-alkanes profiles throughout the developmental ages of leaves are different (19,24,26,35) and the *n*-alkane profile throughout the developmental ages of *M. cochinchinensis* leaves also exhibited important modifications. Li and Ishikawa (13), Cui *et al.* (7) and Sonibare *et al.* (35) demonstrated that *n*-C_{29}, *n*-C_{35} and heptriacontane (*n*-C_{37}) were the predominant *n*-alkanes in the epicuticular waxes of leaves of *Fallopia japonica* Houtt., Fiddle wood and *Antheocleista nobilis* G. Don, respectively. However in the present investigation, *n*-C_{35} was the predominant alkane throughout the developmental ages of *M. cochinchinensis* leaves.

Table 3. Alkanes in different ages leaves of *M. cochinchinensis*

Alkanes	Diagnostic ions (Molecular ion, M^+)	Mole % of alkanes (Mean ± SE, *N*=3)		
		Young	Mature	Senescent
Pentadecane (*n*-C_{15})	212	0.27 ± 0.03	0.27 ± 0.05	-
Hexadecane (*n*-C_{16})	226	0.50 ± 0.09	0.30 ± 0.03	0.43 ± 0.07
Heptadecane (*n*-C_{17})	240	0.13 ± 0.02	0.45 ± 0.02	0.43 ± 0.09
Octadecane (*n*-C_{18})	254	0.84 ± 0.14	0.65 ± 0.08	0.72 ± 0.15
Nonadecane (*n*-C_{19})	268	0.95 ± 0.06	0.42 ± 0.05	0.44 ± 0.13
Eicosane (*n*-C_{20})	282	0.49 ± 0.05	0.56 ± 0.06	0.54 ± 0.04
Heneicosane (*n*-C_{21})	296	0.47 ± 0.02	0.17 ± 0.02	0.55 ± 0.08
Docosane (*n*-C_{22})	310	0.41 ± 0.02	0.41 ± 0.04	0.57 ± 0.06
Tricosane (*n*-C_{23})	324	0.29 ± 0.02	0.18 ± 0.04	-
Tetracosane (*n*-C_{24})	338	0.27 ± 0.02	-	0.37 ± 0.06
Pentacosane (*n*-C_{25})	352	0.28 ± 0.03	0.34 ± 0.01	-
Hexacosane (*n*-C_{26})	366	0.20 ± 0.01	0.50 ± 0.02	0.24 ± 0.04
Heptacosane (*n*-C_{27})	380	-	0.46 ± 0.04	0.58 ± 0.05
Octacosane (*n*-C_{28})	394	0.37 ± 0.02	0.20 ± 0.03	0.28 ± 0.06
Nonacosane (*n*-C_{29})	408	8.09 ± 0.41	5.88 ± 0.46	7.29 ± 0.79
Triacontane (*n*-C_{30})	422	0.41 ± 0.08	0.50 ± 0.04	0.78 ± 0.22
Hentriacontane (*n*-C_{31})	436	5.28 ± 0.33	7.02 ± 0.28	7.83 ± 0.36
Dotriacontane (*n*-C_{32})	450	1.36 ± 0.19	2.57 ± 0.25	2.71 ± 0.39
Tritriacontane (*n*-C_{33})	464	4.09 ± 0.26	5.52 ± 0.14	6.89 ± 0.61
Tetratriacontane (*n*-C_{34})	478	-	0.72 ± 0.21	0.84 ± 0.06
Pentatriacontane (*n*-C_{35})	492	60.93 ± 0.49	61.99 ± 0.77	55.18 ± 1.24
Total		85.62 ± 1.99	89.11 ± 2.31	86.66 ± 4.01

The two-way ANOVA results revealed that *A. foveicollis* responded to cuticular alkanes significantly among the leaf types (F = 9.25, $P < 0.05$) and different concentrations of cuticular alkanes from different leaf types showed significant positive responses to *A. foveicollis* (F = 11.62, $P < 0.05$). Furthermore, among the leaf types there are significant differences between young and mature leaves (|q| = 2.19, $P < 0.05$), young and senescent leaves (|q|= 3.14, $P < 0.05$), mature and senescent leaves (|q|= 5.33, $P < 0.05$). Olfactory bioassay results with cuticular alkanes from three types of leaves are presented in Figure 1. *Aulacophora foveicollis* females were attracted by 1, 2, 4, 6, 8, 10 and 12 µg concentrations of alkanes from mature leaves with 62, 70, 74, 79, 83, 87 and 89 % insects, respectively. The alkanes from young leaves over the range of 2-12 µg concentrations showed an increase in insect attraction (62-86 %) in a dose dependent manner. Alkanes from senescent leaves were attractive to 61, 63, 67 and 73 % of the insects at 6, 8, 10 and 12 µg concentrations, respectively.

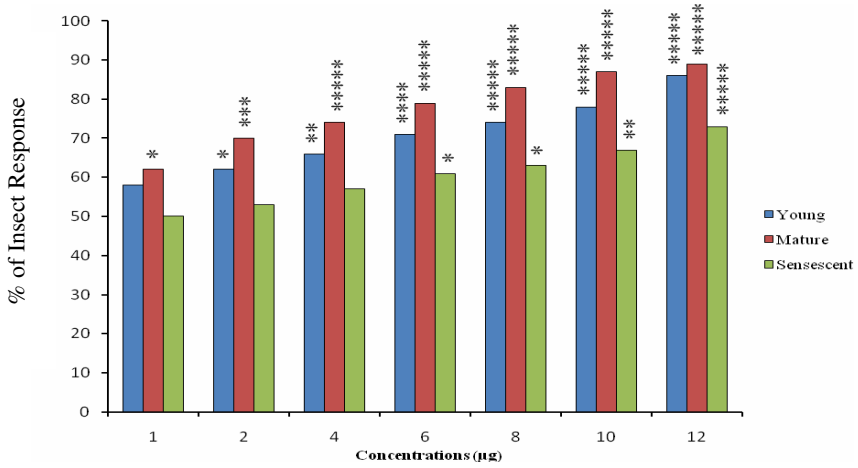

Figure 1. Attractiveness of alkanes isolated from three types of leaves of *M. cochinchinensis* to *A. foveicollis* choosing odor arm (%) in the Y-shaped glass tube olfactometer bioassay (N = 90 in each bioassay); *P = 0.05, **P = 0.01, ***P = 0.001, ****P = 0.0001, and *****P = 0.00001, significant responses.

Bioassay results of *A. foveicollis* with the mixtures of synthetic alkanes, mimicking cuticular alkanes of young, mature and senescent leaves are summarized in Figure 2. The alkane mimic of young and mature leaves attracted 66, 68, 72 and 73 % and 67, 70, 74 and 78 % of the insects at 6, 8, 10 and 12 µg concentrations, respectively. The alkane mimic of senescent leaves from 6-12 µg concentrations attracted 61-64 % of the insects.

Table 4 presents olfactory bioassay experiments of *A. foveicvollis* to individual synthetic alkanes mimicking the individual cuticular alkanes of young leaves. Nonadecane (n-C_{19}) at 65 ng was attractive to 61 % insects. Application of 709 and 851 ng of n-C_{29} elicited positive response in 61 and 65 % of the insects, respectively. A clear positive

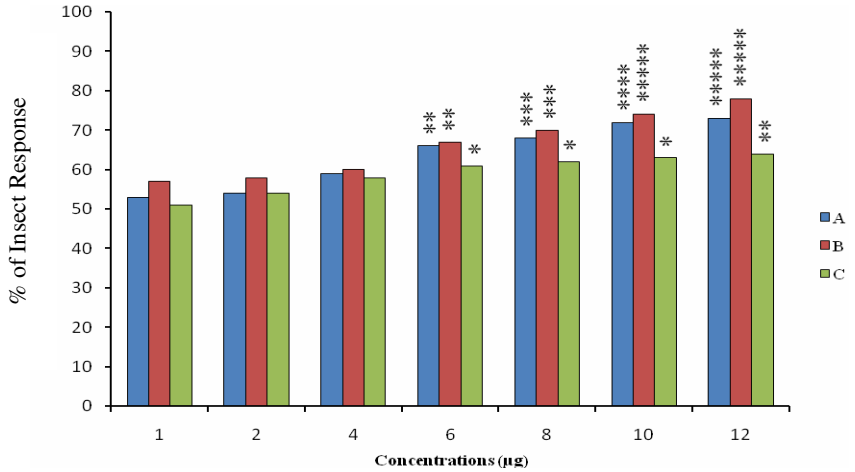

Figure 2. Attractiveness of synthetic alkanes mixtures mimicking the alkanes of three types of leaves (A: Young, B: Mature, C: Senescent) of *M. cochinchinensis* to *A. foveicollis* choosing odour arm (%) in the Y-shaped glass tube olfactometer bioassay (N = 90 in each bioassay); *P = 0.05, **P = 0.01, ***P = 0.001, ****P = 0.0001, and *****P = 0.00001, significant responses.

Table 4. Attractiveness of individual synthetic alkanes mimicking the proportions of individual cuticular alkanes and their combinations detected in young leaves of *M. cochinchinensis* to *A. foveicollis* choosing odor arm (%) in the Y-shaped glass tube olfactometer (N = 90 in each bioassay)

Alkanes	Concentration (µg)						
	1	2	4	6	8	10	12
Nonadecane (n-C_{19}) (a)	NR[a] (5)[c]	NR (11)	NR (22)	NR (33)	NR (44)	NR (55)	61*[b] (65)
Nonacosane (n-C_{29}) (b)	NR (71)	NR (142)	NR (284)	58 (426)	60 (567)	61* (709)	65** (851)
Tritriacontane (n-C_{33}) (c)	NR (40)	NR (82)	NR (163)	NR (245)	NR (326)	NR (408)	57 (489)
Pentatriacontane (n-C_{35}) (d)	NR (644)	NR (1288)	NR (2577)	NR (3866)	NR (5155)	58 (6443)	70*** (7732)
a + b + c + d	51	52	58	63*	66**	68***	72****

[a]: No response; [b]: *, **, *** and **** indicate significant responses at the level of 0.05, 0.01, 0.001 and 0.0001, respectively; [c]: The actual amount (ng) of each alkane represented in the bioassay.

reaction was observed with 7732 ng of n-C_{35} with 70 % of insects tested. Individual n-C_{33} at 489 ng indicated no clear preference to the insect. The rest of the 15 identified alkane compounds in the young leaves individually did not make any choice to the insect. The mixture of 4 alkanes attracted 63, 66, 68 and 72 % of this insect at the amount of equivalent leaf alkane of 6, 8, 10 and 12 µg, respectively.

Table 5 provides olfactory bioassay experiments of *A. foveicollis* using the individual synthetic alkanes mimicking individual cuticular alkanes of mature leaves. A clear positive reaction was observed with 729 ng of *n*-C_{31} with 61 % insects responding. Application of 8027 ng of *n*-C_{35} attracted 69 % insects. An amount of 571 ng of *n*-C_{29} or 610 ng of *n*-C_{33} showed a slightly positive reaction with 60 % insects tested. The rest of the 16 synthetic alkanes did not produce any response to the insect. The mixture of 4 alkanes attracted 66, 68, 73 and 76 % of this insect at the amount of equivalent leaf alkane of 6, 8, 10 and 12 µg, respectively.

Table 5. Attractiveness of individual synthetic alkanes mimicking the proportions of individual cuticular alkanes and their combinations detected in mature leaves of *M. cochinchinensis* to *A. foveicollis* choosing odor arm (%) in the Y-shaped glass tube olfactometer (*N* = 90 in each bioassay)

Alkanes	Concentration (µg)						
	1	2	4	6	8	10	12
Nonacosane (*n*-C_{29}) (a)	NR[a] (48)[c]	NR (95)	NR (190)	NR (286)	52 (381)	58 (476)	60 (571)
Hentriacontane (*n*-C_{31}) (b)	NR (61)	NR (121)	NR (243)	NR (364)	NR (486)	NR (607)	61*[b] (729)
Tritriacontane (*n*-C_{33}) (c)	NR (51)	NR (102)	NR (203)	NR (305)	NR (407)	59 (508)	60 (610)
Pentatriacontane (*n*-C_{35}) (d)	NR (668)	NR (1338)	NR (2676)	NR (4013)	NR (5351)	57 (6689)	69*** (8027)
a + b + c + d	53	56	59	66**	68***	73****	76****

[a]: No response; [b]: *, **, *** and **** indicate significant responses at the level of 0.05, 0.01, 0.001 and 0.00001, respectively; [c]: The actual amount (ng) of each alkane represented in the bioassay.

Table 6 shows olfactory bioassay experiments of *A. foveicollis* using individual synthetic alkanes mimicking individual cuticular alkanes of senescent leaves. An amount of 741 ng *n*-C_{29} attracted 63 % insects. Application of 850 ng of *n*-C_{31} or 7144 ng of *n*-C_{35} attracted 63 % insects. An amount of 796 ng of *n*-C_{33} elicited attractions of 62 % insects. Individual triacontane (*n*-C_{30}) at 84 ng indicated negative response, i.e., repellency to the insect. The rest of the 12 identified alkanes did not provoke any response to the insect. The mixture of 5 alkanes attracted 61 and 62 % of this insect at the amount of equivalent leaf alkane of 10 and 12 µg, respectively; whereas the subtraction of *n*-C_{30} from the mixture elicited attraction of 62 and 71 % of this insect at the amount of equivalent leaf alkane of 10 and 12 µg, respectively.

Alkanes are one of the commonest constituents of plant epicuticular waxes (3,10,17,18) and play an important role in plant-insect interactions study as attractant for feeding or oviposition (8,13,15,17,22,25,26,29,36). Our bioassay results revealed clear olfactory responses to long-chain alkanes, which are low volatile substances that might act as close range allelochemicals after arrival of the insect to the plant. The gravid *Cotesia plutellae* (Kurdjumov) females preferred the odor of *n*-C_{16} to *n*-C_{30} alkanes except *n*-C_{20}, *n*-C_{25} and *n*-C_{28} as 60 to 70 % insects oriented toward these saturated hydrocarbons in a dual choice bioassay with a wind tunnel (30). Dutton *et al.* (8) analyzed the leaf mines of

Table 6. Attractiveness of individual synthetic alkanes mimicking the proportions of individual cuticular alkanes and their combinations detected in senescent leaves of *M. cochinchinensis* to *A. foveicollis* choosing odor arm (%) in the Y-shaped glass tube olfactometer ($N = 90$ in each bioassay)

Alkanes	Concentration (µg)						
	1	2	4	6	8	10	12
	NR[a]	NR	NR	52	58	60	63*[b]
Nonacosane (n-C_{29}) (a)	(62)	(123)	(247)	(370)	(494)	(617)	(741)
	NR[c]	NR	NR	NR	NR	47	*33****[d]
Triacontane (n-C_{30}) (b)	(7)	(14)	(27)	(41)	(55)	(68)	(82)
	NR	NR	NR	NR	NR	57	63*
Hentriacontane (n-C_{31}) (c)	(71)	(142)	(283)	(425)	(567)	(708)	(850)
	NR	NR	NR	NR	NR	60	62*
Tritriacontane (n-C_{33}) (d)	(66)	(133)	(265)	(398)	(531)	(663)	(796)
	NR	NR	NR	NR	NR	54	63*
Pentatriacontane (n-C_{35}) (e)	(595)	(1191)	(2381)	(3572)	(4763)	(5954)	(7144)
a + b + c + d + e	50	53	56	58	58	61*	62*
a + c + d + e	50	53	56	58	58	62*	71****

[a]: No response; [b]: *, **, *** and **** indicate significant responses at the level of 0.05, 0.01, 0.001 and 0.0001, respectively; [c]: The actual amount (ng) of each alkane represented in the bioassay; [d]: Negative response or repellency, values in italics.

Phyllonorycter pomonella Zeller, i.e., damaged plant tissue and showed that a leaf miner parasitoid uses plant derived semiochemicals (alkanes from n-C_{27} to n-C_{33}) for host location. In the present study, the cuticular alkanes from young, mature and senescent leaves attracted the insect, *A. foveicollis* at 2-12, 1-12 and 6-12 µg concentrations, respectively, suggesting that *A. foveicollis* insects are attracted to different effective combinations of alkanes present in *M. cochinchinensis* leaves in the field. The cuticular alkanes from senescent leaves indicated lower amounts of n-C_{19} and n-C_{35} and higher amount of n-C_{30}, which failed to provoke positive responses like the responses of *A. foveicollis* to cuticular alkanes from young leaves. Further, absence of n-C_{27} and lower amount of n-C_{31} in young leaves failed to produce responses of *A. foveicollis* like mature leaves. However, a single young, mature, senescent leaf indicated presence of 23.47, 83.97 and 54.87 µg of cuticular alkanes, respectively, suggesting that each type of a single leaf is attractive to the insect *A. foveicollis*, because 12 µg cuticular alkanes from young, mature and senescent leaves produced 86% ($P < 0.00001$), 89% ($P < 0.00001$) and 73% ($P < 0.00001$) responses to the insect, respectively (Figure 1).

The cuticular alkanes of young, mature and senescent leaves attracted the insect between 2-12, 1-12 and 6-12 µg concentrations, respectively; whereas the mixtures of synthetic alkanes mimicking cuticular alkanes of young, mature and senescent leaves showed attraction between 6-12 µg concentrations; hence the differences in attraction between cuticular alkanes and alkane mimics are due to absence of unidentified branched-chain alkanes in the synthetic mixtures. However, 10 µg concentration of cuticular alkanes from mature leaves, a synthetic blend of alkanes mimicking this concentration of mature leaves, or a synthetic blend of 476, 607, 508 and 6689 ng of n-C_{29}, n-C_{31}, n-C_{33} and n-C_{35}, respectively, elicited the same responses ($P < 0.00001$) of *A. foveicollis*. Hence, the last combination of 4 synthetic alkanes might be used for insect pest management programme

such as baited traps. Further, this study indicates that the above mentioned combination of 4 synthetic alkanes might be an alternative of a synthetic blend of nonadecane, heptacosane and nonacosane mimicking the proportions as present in *M. cochinchinensis* flower surface waxes (15). However, bioassays in a greenhouse to evaluate responses of *A. foveicollis* to the combinations of 4 synthetic alkanes present at the amount of equivalent to 10 µg of the mature leaf alkanes are necessary to authenticate the attractiveness tested in the present study.

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REFERENCES

1. Abe, M. and Matsuda, K. (2005). Chemical factors influencing the feeding preference of three *Aulacophora* leaf beetle species (Coleoptera: Chrysomelidae). *Applied Entomology and Zoology* **40**: 161-168.
2. Adhikary, P., Mukherjee, A. and Barik, A. (2014). Role of surface wax alkanes from *Lathyrus sativus* L. seeds for attraction of *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae). *Journal of Stored Products Research* **59**: 113-119.
3. Baker, E.A. (1982). Chemistry and morphology of plant epicuticular waxes. In: *The Plant Cuticle* (Eds., D.F. Cutler, K.L. Alvin and C.E. Price) pp. 139-165. Academic Press, London.
4. Barik, A. and Banerjee, T.C. (2005). The role of triterpenes in the weed insect interaction. *Allelopathy Journal* **15**: 259-266.
5. Barik, A., Bhattacharya, B., Laskar, S. and Banerjee, T.C. (2004). The determination of *n*-alkanes in the cuticular wax of leaves of *Ludwigia adscendens* L. *Phytochemical Analysis* **15**: 109-111.
6. Burke, D.S., Smidt, C.R. and Vuong, L.T. (2005). *Momordica cochinchinensis*, *Rosa roxburghii*, Wolfberry and Sea buckthorn-highly nutritional fruits supported by tradition and science. *Current Topics in Nutraceutical Research* **3**: 259-266.
7. Cui, J.W., Huang, J.H. and Xie, S.C. (2008). Characteristics of seasonal variations of leaf *n*-alkanes and *n*-alkenes in modern higher plants in Qingjiang, Hubei Province, China. *Chinese Science Bulletin* **53**: 2659-2664.
8. Dutton, A., Mattiacci, L. and Dorn, S. (2000). Plant-derived semiochemicals as contact host location stimuli for a parasitoid of leafminers. *Journal of Chemical Ecology* **26**: 2259-2273.
9. Eigenbrode, S.D. and Espelie, K.E. (1995). Effects of plant epicuticular lipids on insect herbivores. *Annual Review of Entomology* **40**: 171-194.
10. Jetter, R., Schaffer, S. and Riederer, M. (2000). Leaf cuticular waxes are arranged in chemically and mechanically distinct layers: Evidence from *Prunus laurocerasus* L. *Plant, Cell and Environment* **23**: 619-628.
11. Khan, M.M.H., Alam, M.Z. and Rahaman, M.M. (2011). Host preference of red pumpkin beetle in a choice test under net case condition. *Bangladesh Journal of Zoology* **39**: 231-234.
12. Koschier, E.H., Kogel, W.J.D. and Visser, J.H. (2000). Assessing the attractiveness of volatile plant compounds to western flower thrips *Frankliniella occidentalis*. *Journal of Chemical Ecology* **26**: 2643-2655.
13. Li, G. and Ishiwaka, Y. (2006). Leaf epicuticular wax chemicals of the Japanese knotweed *Fallopia japonica* as oviposition stimulants for *Ostrinia latipennis*. *Journal of Chemical Ecology* **32**: 595-604.
14. Mukherjee, A. and Barik, A. (2014). Long-chain free fatty acids from *Momordica cochinchinensis* Spreng flowers as allelochemical influencing the attraction of *Aulacophora foveicollis* Lucas (Coleoptera: Chrysomelidae). *Allelopathy Journal* **33**: 255-266.

15. Mukherjee, A., Sarkar, N. and Barik, A. (2013). Alkanes in flower surface waxes of *Momordica cochinchinensis* influence attraction of *Aulacophora foveicollis* Lucas (Coleoptera: Chrysomelidae). *Neotropical Entomology* **42**: 366-371.
16. Mukherjee, A., Sarkar, N. and Barik, A. (2014). Long-chain free fatty acids from *Momordica cochinchinensis* leaves as attractants to its insect pest, *Aulacophora foveicollis* Lucas (Coleoptera: Chrysomelidae). *Journal of Asia-Pacific Entomology* **17**: 229-234.
17. Müller, C. (2006). Plant-insect interactions on cuticular surfaces. In: *Biology of the Plant Cuticle* (Eds., M. Riederer and C. Muller) pp. 398-422. Blackwell Publishing, Oxford.
18. Müller, C. and Riederer, M. (2005). Plant surface properties in chemical ecology. *Journal of Chemical Ecology* **31**: 2621-2651.
19. Piasentier, E., Bovolenta, S. and Malossini, F. (2000). The *n*-alkane concentrations in buds and leaves of browsed broadleaf trees. *The Journal of Agricultural Science* **135**: 311-320.
20. Rahaman, M.A. and Prodhan, M.D.H. (2007). Effects of net barrier and synthetic pesticides on Red pumpkin beetle and yield of cucumber. *International Journal of Sustainable Crop Production* **2**: 30-34.
21. Raman, K. and Annadurai, R.S. (1985). Host selection and food utilization of the red pumpkin beetle, *Raphidopalpa foveicollis* (Lucas) (Chrysomelidae: Coleoptera). *Proceedings, Indian Academy of Science (Animal Science)* **94**: 547-556.
22. Roy, N. and Barik, A. (2012). Alkanes used for host recognition by the arctiid moth, *Diacrisia casignetum* Kollar. *Journal of Entomological Research* **36**: 345-350.
23. Roy, N., Laskar, S. and Barik, A. (2012a). Determination of *n*-alkane profile through developmental state of sunflower leaves. *The South Pacific Journal of Natural and Applied Sciences* **30**: 72-76.
24. Roy, N., Laskar, S. and Barik, A. (2012b). The attractiveness of odorous esterified fatty acids to the potential biocontrol agent, *Altica cyanea*. *Journal of Asia-Pacific Entomology* **15**: 277-282.
25. Santhosh Kumar, K. and Nadarajan, L. (2008). Evidence of female-produced sex pheromone in red pumpkin beetle, *Aulacophora foveicollis* Lucas (Coleoptera: Chrysomelidae). *Current Science* **94**: 1369-1371.
26. Sarkar, N., Mukherjee, A. and Barik, A. (2013a). Long-chain alkanes: allelochemicals for host location by the insect pest, *Epilachna dodecastigma* (Coleoptera: Coccinellidae). *Applied Entomology and Zoology* **48**: 171-179.
27. Sarkar, N., Mukherjee, A. and Barik, A. (2013b). Olfactory responses of *Epilachna dodecastigma* (Coleoptera: Coccinellidae) to long-chain fatty acids from *Momordica charantia* leaves. *Arthropod-Plant Interactions* **7**: 339-348.
28. Schiestl, F.P., Ayasse, M., Paulus, H.F., Löfstedt, C., Hansson, B.S., Ibarra, F. and Francke, W. (1999). Orchid pollination by sexual swindle. *Nature* **399**: 421-422.
29. Schoonhoven, L.M., Van Loon, J.J.A. and Dicke, M. (2005). *Insect-Plant Biology*. Oxford University Press, Oxford. 421 pp.
30. Seenivasagan, T. and Paul, A.V.N. (2011). Gas-chromatography and electroantennogram analysis of saturated hydrocarbons of cruciferous host plants and host larval body extracts of *Plutella xylostella* for behavioural manipulation of *Cotesia plutellae*. *Indian Journal of Experimental Biology* **4**9: 375-386.
31. Singh, D. and Gill, C.K. (1979). Estimation of losses in growth and yield of muskmelon due to *Aulacophora foveicollis* (Lucas). *Indian Journal of Entomology* **44**: 294-295.
32. Sinha, A.K. and Krishna, S.S. (1969). Feeding of *Aulacophora foveicollis* (Lucas) on cucurbitacin. *Journal of Economic Entomology* **62**: 512-513.
33. Sinha, S.N. and Chakrabarti, A.K. (1983). Effect of seed treatment with carbofuran on the incidence of red pumpkin beetle, *Rhaphidopalpa foveicollis* (Lucas) on cucurbits. *Indian Journal of Entomology* **45**: 145-151.
34. Sokal, R.R. and Rohlf, F.J. (1995). *Biometry: The Principles and Practice of Statistics in Biological Research*, 3rd edition. WH Freeman, New York. 887 pp.
35. Sonibare, M.A., Soladoye, M.O. and Ekine-Ogunlana, Y. (2007). A chemotaxonomic approach to the alkane content of three species of *Anthocleista* Afzel. (Loganiaceae). *African Journal of Biotechnology* **6**: 1516-1520.
36. Srinivasan, R., Uthamasamy, S. and Talekar, N.S. (2006). Characterization of oviposition attractants of *Helicoverpa armigera* in two solanaceous plants, *Solanum viarum* and *Lycopersicon esculentum*. *Current Science* **90**: 846-850.
37. Tandon, P. and Sirohi, A. (2009). Laboratory assessment of repellent properties of ethanolic extracts of four plants against *Raphidopalpa foveicollis Lucas* (Coleoptera: Chrysomelidae). *International Journal of Sustainable Crop Production* **4**: 1-5.
38. Vuong, L.T. and King, J.C. (2003). A method of preserving and testing the acceptability of gac fruit oil, a good source of beta-carotene and essential fatty acids. *Food and Nutrition Bulletin* **24**: 224-230.