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RESEARCH ARTICLE

Isolation, Fractionation, Identification and Bioassay Studies of the Pheromone of the *Maconellicoccus hirsutus* (Green, 1908)

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Abstract

Maconellicoccus hirsutus (Green, 1908) is one of the invasive pests known to infest the agricultural crops of Vadodara, Gujarat. Large amount of pesticides are in use to control this pest which ultimately pose threat to both environment and mankind. So eco-friendly measures are required to limit the invasion of this pest. Hence the objective is to isolate the pheromone from *Maconellicoccus hirsutus* to uncover its different components through GC-MS along with the bioassay studies. Pheromone was isolated from the species through adsorbent method and the volatiles were then subjected to GC-MS for fractionation. Crude extract was further employed for behavioral bioassay to identify the nature of the pheromone. Combined result of GC and GC-MS indicated the presence of phenolic component in the extract. Additionally observations from bioassay studies confirmed it as sex pheromone where attractive index of males towards volatiles was higher (0.88) than the females. Hence, identification of different pheromonal components will be helpful in synthesizing chemical analogues and their use in controlling the concerned pest in an eco-friendly way.

Keywords: Attractive index, Bioassay Studies, GC-MS, *Maconellicoccus hirsutus*, Phenolic component

Introduction

Maconellicoccus hirsutus (Green, 1908), The pink *Hibiscus* mealybug (Phm) is a serious pest of a wide range of plant species including agricultural, horticultural and ornamental crops. Phm is originally known to belong to the Southern Asia from where it got migrated to the every possible habitats of the world (Roltsch *et al.*, 2006; Daane *et al.*, 2008). Its quick adaptability to survive on different plant species has supported its invasion to the old world as well as tropical and subtropical parts of the new world (Williams, 1996; Daane *et al.,* 2012). This invasive species was reported to extend its ranges in 24 different island of Carribean where it causes significant damage to the economically important crops (Sagarra and Peterkin, 1999). It was first recorded from Grenada, Carribean in 1994 after which it has been given the status of a serious pest in India and Egypt (Kairo *et al.,* 2000). In a recent survey conducted in Roraima has documented the existence of this pest for the first time in Brazil where it caused serious infestation to nine host plants (Marsaro Junior *et al.,* 2013). Phm continued to invade different parts of the country and reported to cause severe infestation to the cotton plants of all the nine cotton growing states of India (Nagrare *et al.,* 2009). Further studies revealed that Phm was also seen to infest the cotton and Hibiscus plants of Vadodara city of Gujarat (Singh, 2012).

This is a polyphagous pest which causes destruction by sucking the sap of the host plant with the characteristic manifestations like deformed leaves, stems and fruits, shortening of internodes, bunchy top etc. (Hodges and Hodges, 2006; Bhosle *et al.,* 2009).While feeding, Pink hibiscus mealybug injects toxic saliva in to the plant tissue which adds to the severity of damage (Moghaddam, 2006). "Honeydew secretion", major characteristic of mealybug infestation is a suitable media for fungal growth which prevents photosynthesis to occur leading to the total destruction of the host plant (OEPP/EPPO. 2005; Aristizabal *et al.,* 2012). This leads to the significant loss in country's economy as well as ruin the visual beauties of ornamental crops (Sharma *et al.,* 2008; Patil *et al.,* 2011).

Management studies of this pest mostly confined to the use of parasitoids to control their population (Sagarra *et al.,* 2000; 2001). Biological control was found to be more effective and eco-friendly (Aida *et al.,* 2010) but time taking and introduction and augmentation of parasitoids on fields are limiting factors. Hence, there is a need to develop a control strategy which can address all the limitations. Biorationals perfectly fits the category. Few studies were conducted on the isolation and characterization of pheromones which were efficient to control the pests (Zhang *et al.,* 2004 ; Zhang and Nie, 2005; Waghole and Naik, 2014).

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Lack of proper controlling mechanism has supported the pest to grow and infest new areas. So its management is of utmost importance. The present study was designed to manage Phm in an efficient but eco- friendly way.

Materials and Methods

Insects

Clusters of insect pest (*Maconellicoccus hirsutus*) found clenched on different parts of their host plants were collected from the fields of Vadodara, Gujarat. Collection was done in the morning hours i.e. from 7.30 am to 9.30 am and continued for a period of two years (January 2013 to March 2015) based on the thorough field survey which reports that mild temperature (max 33.56°C and min 12.32°C) and 50-60% RH; best suitable for their growth and survival (Deb and Kumar, 2016). The pests were then identified using standard references (Douglass, 1999; OEPP/EPPO 2005). The present work calls for the separation of virgin adult females from the clusters which were done with the help of the soft paint brush. The last instar nymphs (approx. 1.4 mm -1.9 mm) were separated from the bulk and maintained in plastic boxes. Hibiscus leaves were provided as a food for their survival and growth. After attaining the adult stage which is confirmed by the size i.e. 3 mm – 3.2 mm were used for pheromone isolation.

Chemicals

All the reagents and solvents were procured from Sigma Aldrich and Merck Millipore for the collection of the volatiles and their subsequent fractionation through GC (Gas Chromatography).

Collection of Pheromone

Separated females were subjected to adsorbent method for the collection of the volatiles. The adsorbant setup was fabricated by Durga Scientific Pvt. Co., Vadodara, in the Division of Entomology, Department of Zoology, The M. S. University of Baroda in the year 2009 for carrying out the pheromone isolation work. This method deals with the introduction of insect pest into an airborne collection apparatus which is 125 mm in length and 11.5 mm in diameter. Females were fed on the plant sap by maintaining them on cotton balls and cotton leaves. One end of the enclosed glass chamber contains activated charcoal which filters the air supplied continuously through the aerator and the other end is provided with the adsorbent (Tenax TA 60/80 mesh, 1.5 grams, Sigma-Aldrich) to trap the volatiles. So the filtered air flowed from one end was passed over the adult females and the volatiles released by the pest females got trapped in the adsorbent trap. Insects were exposed in the collection apparatus for 3-4 days. The number of insects used for collection of volatile in adsorbant setup was 1000.

The adsorbents were eluted with n-hexane (40 ml/ adsorbent trap) and then transferred in glass vials (8cm in length× 1.5 cm in breadth). Elutes were then processed for chemical fractionation through GC and GC-MS. Volatiles were stored in -30°C in refrigerator immediately after their elution until use.

GC-MS

GC-MS is helpful in fractionation and identification of the key components of female pheromone responsible for attraction of their male counterpart. Volatiles traped in the Tenax eluted with n-hexane were subjected to GC-MS for chemical fractionation. Gas chromatography coupled to mass spectrometry were done using Perkin Elmer, Auto system XL GC+, Turbo mass 4.1- software, in SICART, India. One microliter of the volatile eluted with n hexane was injected into the system. The column used was silica base capillary column (250 mμ diameter x 100m length, 0.25 μm thickness and cp- silica 8). The injector temperature was set at 210°C with a split ratio of 1:10. Initially the temperature of the oven was kept at 50°C which continued for 1 min and then programmed to increase to 250 at the rate of 5°C / min. Helium was used as the carrier gas with a course of 1.2 ml/min.

Behavioral Bioassays

Attractiveness of the male individual of *Maconellicoccus hirsutus* towards the crude extract isolated from adult virgin female was calculated by a simple bioassay (Arai, 2000). Bioassay was performed by using glass petri plates (10 cm in diameter and 1.5 in height) and filter paper which is Watman filter paper 1 in our case (Diameter – 25 mm, thickness – 180 μm, pore size – 11 μm). Two sets were maintained with only difference in solvent used to conduct the experiment. One of them is treated with the crude extract obtained from adsorbant method and the other one was kept as control. The latter one was attained by dipping the filter paper in the solvent i.e. n-hexane minus crude extract. Both the papers i.e. the one treated with the test samples and the control one were then placed in separate petri plates. 50 numbers of males were then released in the petri plate containing filter paper treated with both the extracts using soft paint brush. The same procedure was repeated with the female individual of the same species. The experiments were repeated thrice for avoiding any error. The number of insects including both the sexes was recorded on the basis of their response towards the volatiles.

Attractive index of each group was then quantified using the following formula

AI= (No. of insects responded to test material - No. of insects responded to control) ÷ (No. of insects released - No. of insects responded to control)

The collected data were statistically analyzed for χ^2 (chi square) goodness-of- fit.

Results and Discussion

Volatiles collected from 1000 virgin adult females were trapped in Tenax and subsequently isolated using n hexane as solvent. Crude extract isolated from insect pest through Adsorbant method was fractionated using Gas chromatographic studies on Florisil. Volatiles eluted in n-hexane showed strongest activity after 15 minutes of retention time. Highest peaks were evident at 15.02 and 15.10 minutes (Fig. 1) which indicated the proper elution of different components present in the complex chemical mixture of the sample volatiles. Indeed the presence of chemical groups in the sample was confirmed. So to uncover the major components responsible for attraction of male individuals of Phm towards the volatiles isolated from virgin adult females were further subjected to GC-MS. Results of mass spectrometric analysis of GC peaks indicated the presence of phenolic component in the volatiles. Chemically phenol is an aromatic organic compound which consists of phenyl group and hydroxyl group.

The major compounds obtained from the chromatographic assay at 15.02 retention time (Fig. 2) are as follows:

Butylated Hydroxytoluene ($C_{15}H_{24}O$) is a chemical derivative of Phenol having a molecular weight of 220 Da.

PHENOL, 4,6-DI(1,1-DIMETHYLETHYL)-2-METHYL- ($C_{15}H_{24}O$) with a molecular weight of 220 Da

PHENOL, 2, 4, 6- TRIS (1-METHYLETHYL)- ($C_{15}H_{24}O$) with a molecular weight of 220 Da

The major compounds reported from the chromatographic assay at 15.10 (Fig. 3) retention time are as follows:

PHENOL, 2,4-BIS(1,1-DIMETHYLETHYL)- ($C_{14}H_{22}O$) with a molecular weight 206 Da

PHENOL, 3,5-BIS(1,1-DIMETHYLETHYL)-($C_{14}H_{22}O$) having a molecular weight 206 Da

EI mass spectrometric results give us the idea about the base peak and values of molecular ion (M^+) and M+1 of the given pheromonal sample. Base peaks are considered as most stable component present in the sample whereas the molecular ion is likely to be unstable because of the fragmentation of molecules in the course of mass spectrometry procedure. The base peaks were found to be 205 m/z and 191 m/z at the retention time 15.02 and 15.10 respectively. Moreover, 220m/z were reported as the M^+ value and 221 m/z was theM+1 value at 15.02 RT. At 15.10 RT the values of M^+ and M+1 were 206 m/z and 207 m/z respectively.

Havoc created by the armored scale insects are not unknown to the world anymore. They were considered as one of the major pests of different plants ranging from economically important crops to different ornamental crops. They have earned the status of a serious pest almost six decades ago (Butcher, 1958). Controlling the pest using chemical pesticides is the most common practice but their hazardous effects asked for a better replacement. Biorational fits the category which deals with the isolation of pheromone for controlling the pest species. In a recent study conducted in Japan, presence of sex pheromone from Grey Pineapple mealybug was confirmed by behavioural bioassay where monoterpenes were identified as the major component (Tabata and Ichiki, 2015). *Dysmicoccus grassii*, another major agricultural pest was reported to contain terpene in their sex pheromone (de Alfonso *et al.,* 2012). Terpenoid was reported as sex pheromone in *Acutaspis albopicta* which is an invasive scale species and known to infest a number of exotic plants in California (Millar *et al.,* 2012). In a similar study, irregular terpenoid structure was reported to be present in the sex pheromone of Madeira mealybugs (Ho *et al.,* 2009). Japanese mealybug, *Planococcus Kraunhinhe* was reported to contain esters of butyric acid in their sex pheromone which was confirmed by behavioural bioassay studies (Sugie *et al.,* 2008). Similar bioassay studies were conducted in obscure mealybug i.e. *Pseudococcus vibruni* known to infest a great range of ornamental crops, grape vines etc. was described to contain sex pheromone which was further fractionated and identified as monoterpenoids (Millar and Midland, 2007). Akin studies were performed on passionvine mealybug where the sex pheromone was found to contain a class of terpenoids (Ho *et al.,* 2007). Citrus mealybug also known to release sex pheromone which contains alcohol and acetate group (Bierl-Leonhardt *et al.,* 1981).Keeping another devastating pest i.e. *Pseudococcus cryptus* in mind a research programme was undertaken in Japan to implement an eco-friendly means for controlling this pest. Female volatiles were isolated and confirmed as sex pheromone (Arai, 2002). Later the sex pheromone was further fractionated by GC-MS which was seen to contain ester group (Arai *et al.,* 2003). *Planococcus ficus*, a great agricultural pest produces sex pheromone which contains monoterpene and a corresponding ester where the latter was shown to attract more males than the monoterpene (Hinkens *et al.,* 2001). Sex pheromone in

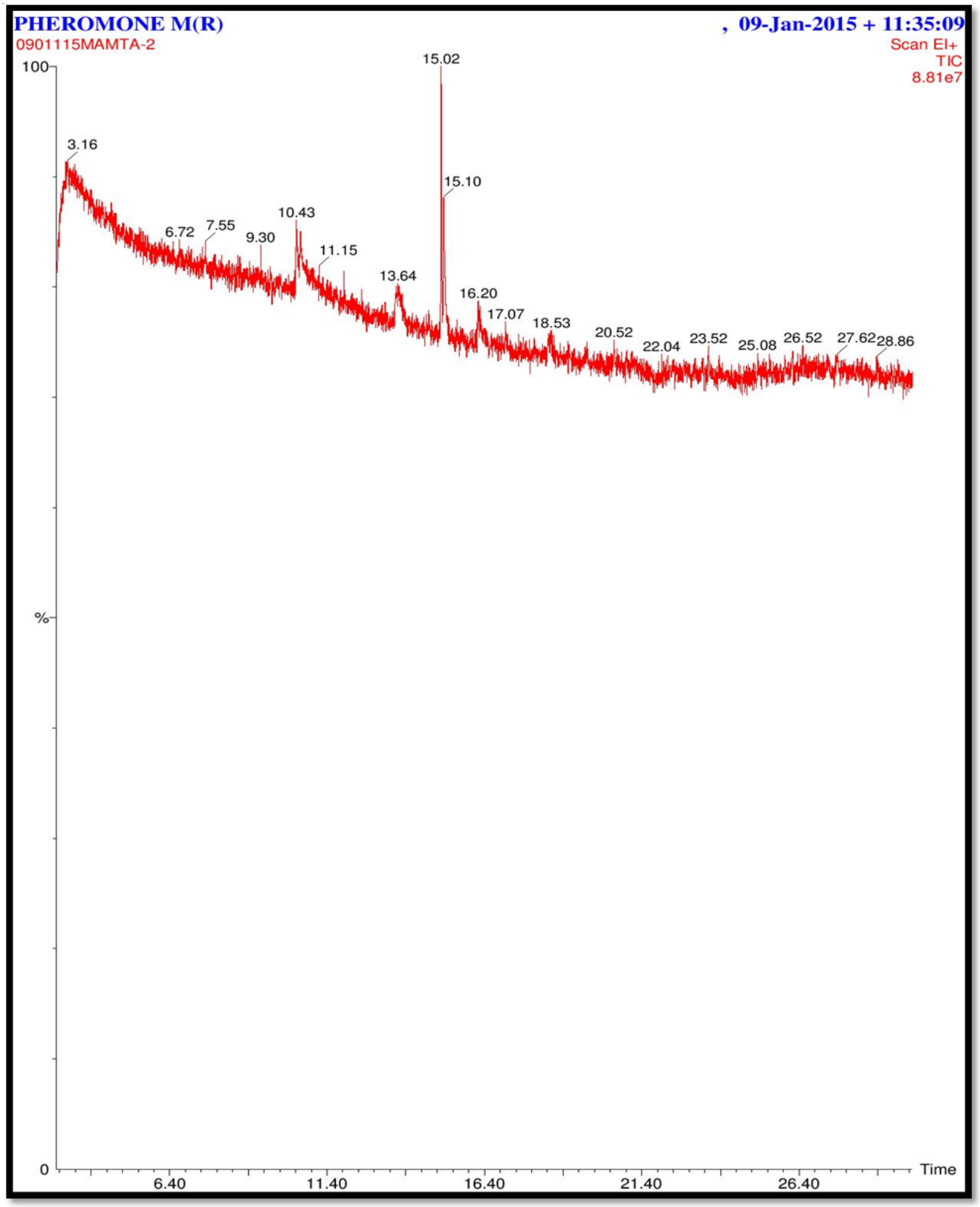

Fig. 1. Representation of Gas Chromatographic (GC) Results of the Female Volatiles Isolated from *Maconellicoccus hirsutus*

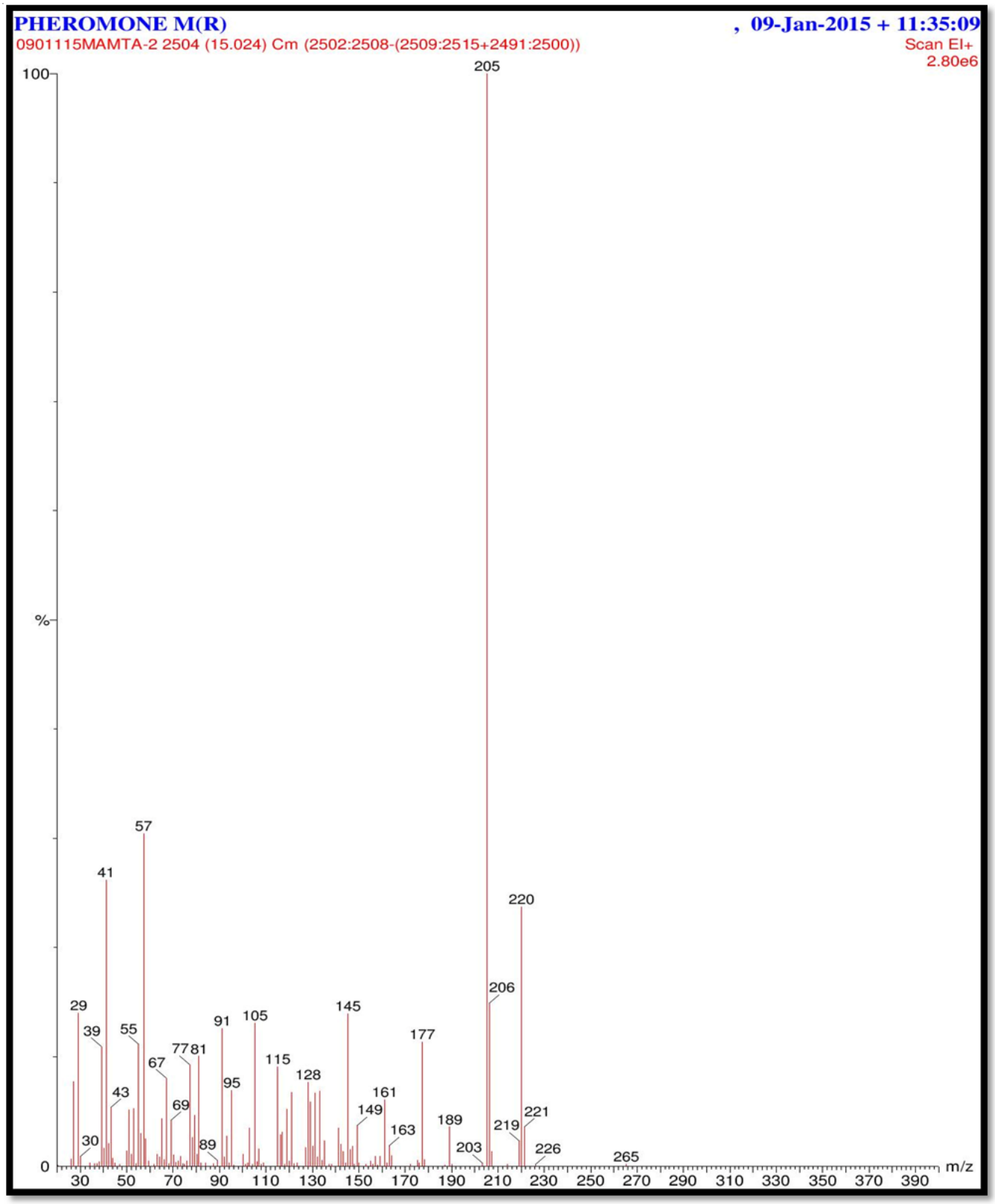

Fig. 2. Representation of EI Mass Spectrum Results of the Female Specific Volatile at 15.02 RT

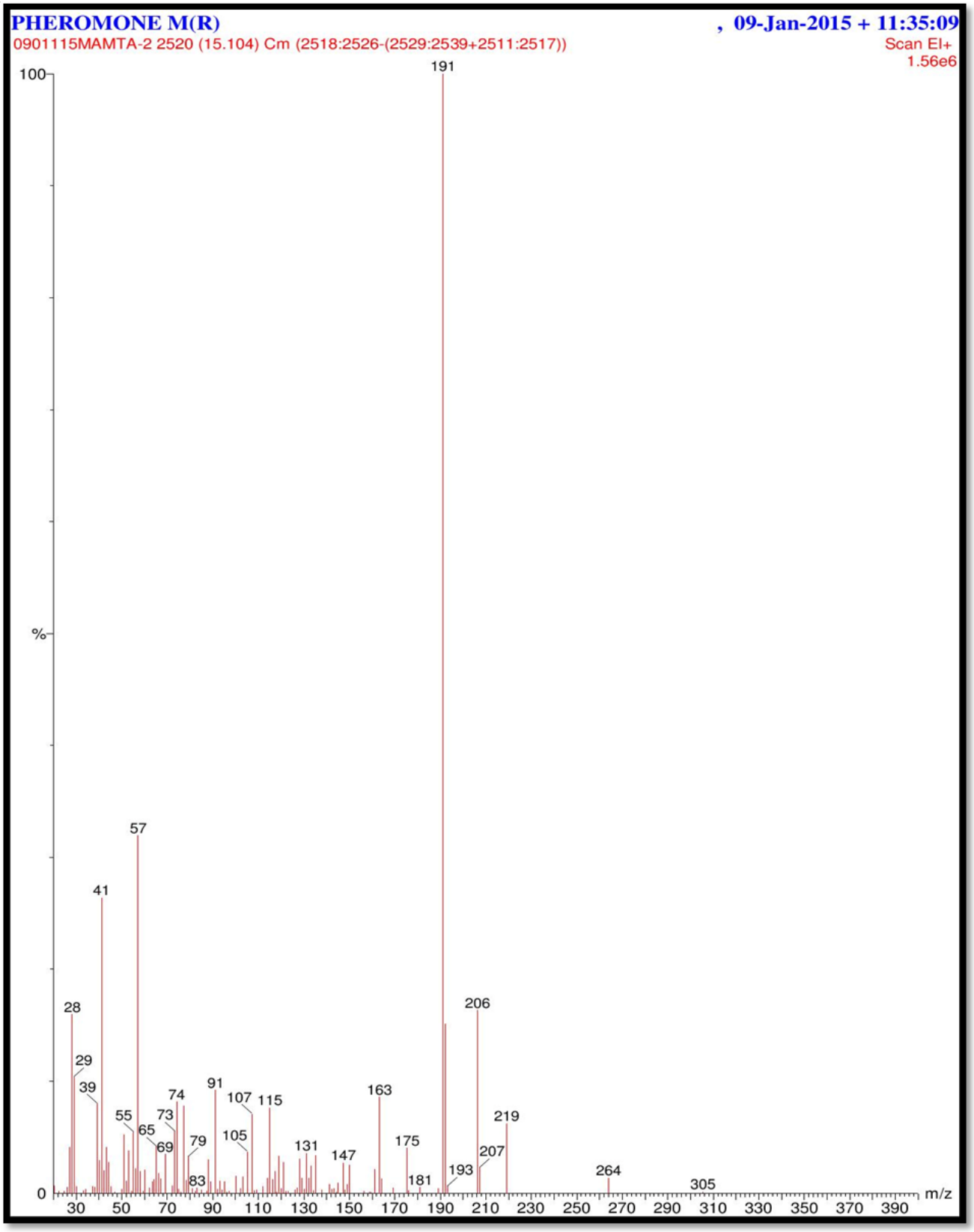

Fig. 3. Representation of EI Mass Spectrum Results of the Female Specific Volatile at 15.10 RT

Comstock mealybugwas found to contain 2,6-dimethyl-1,5-heptadien-3 ol acetate as the major pheromonal component (Fall *et al.,* 1986, Bierl-Leonhardt *et al.,* 1982). In a similar study conducted in Vadodara, Gujarat, esters and terpenes were isolated and identified from the sex pheromone of one of the major pests of Cotton i.e. *Phenacoccus solenopsis* (Singh and Kumar, 2015).

Isolation of pheromone from pest species and their subsequent use as biolure is not confined to the Hemipteran pests.Apart from the scale insects, isolation of pheromone has also been done in *Spodoptera litura*, a lepidopteran pest whose larval statges known to create havoc in agricultural fields, sex pheromone has been isolated and reported to contain alcohol and carbonyl compound (Tamaki *et al.,* 1973). Moreover, female volatiles from house fly,one of the major disease vector, confirmed the presence of (Z)-9-Tricosene in their sex pheromone (Carlson *et al.,* 1971)which is a very well-known biopesticide available today. Thus, this can be efficiently used to control the concerned pest in the prone areas. This presents a suitable example of commercialization of pheromones for controlling the pest.

The presence of potent pheromonal compound in the sample volatile was further investigated and confirmed by behavioral bioassay studies. In first set of experiment, 50 females were released in Petri plates containing filter paper soaked with sample volatile. Among which only 9 in first set, 11 in second set and 8 in third set insect pests were attracted towards the test volatile. This shows that sample volatiles were ineffective in attracting the female individuals. A control set was also maintained along with the test sample. But the result showed insignificant attraction where only 0, 2 and 1 females were seen to move towards the test sample and that too for a small time.

In second set of experiment, 50 males were released in test sample to assess the efficiency of crude extract where 38, 44, 43 males were recorded to show attractive behavior towards the sample volatile in the first, second and third set respectively. At the beginning of the experiment the males showed no response towards the volatiles. With the increase in time their movement is also reported to be increased. The graph again went down towards the end of the study time. So the responsiveness can be positively correlated with the time. Thus the results demonstrated the efficiency of crude extracts in drawing the males towards it. Same experiment was repeated with the control. The results were similar to that of control experiment performed with females. Insignificant number of individual i.e. 1, 1, 2 of the triplicates maintained, moved towards n-hexane.

Responsive percentage and attractive index (Table 1) were calculated using the raw data obtained from behavioral bioassay study and further subjected to statistical analysis. The highest value i.e. 0.88 AI was recorded for the sample volatiles isolated through adsorbant method which lured the male insect. The responsive percentage of the same was 87.75. All the triplicates of second set showed higher values of AI (i.e. 0.85, 0.88 and 0.88) and RP (85.41, 87.75and 75.51) in comparison to the volatiles of the first set where females were taken as the test organism. These results led us to conclude that the volatile that was isolated from the *Maconellicoccus hirsutus* was a sex pheromone which attracted the male individuals of the species.

The results of AI and responsive percentage were further supported by the values of Chi square goodness–of–fit test. The values of N and H were found insignificant (Table 2) in comparison to the values of V which concluded that the test volatile was efficient in attracting more males towards it leaving a small population of non

Table 1. Results of Behavioral Bioassay Studies of *Maconellicoccus hirsutus* and their Statistical Analysis

Adults released in experimental set up					Responsive Percentage	Attractive Index
Sex	**Samples**	**No of insects released**	**No of insects responded**	**No of nonresponsive insects**		
♀	*Volatiles*	*50*	*9*	*41*	*18*	*0.18*
	n-hexane		*0*			
♀	*Volatiles*	*50*	*11*	*37*	*18.75*	*0.19*
	n-hexane		*2*			
♀	*Volatiles*	*50*	*8*	*41*	*14.29*	*0.14*
	n-hexane		*1*			
♂	*Volatiles*	*50*	*38*	*11*	*75.51*	*0.76*
	n-hexane		*1*			
♂	*Volatiles*	*50*	*44*	*5*	**87.75**	**0.88**
	n-hexane		*1*			
♂	*Volatiles*	*50*	*43*	*5*	*85.41*	*0.85*
	n-hexane		*2*			

Table 2. Values of chi square and their significance

Sex	Mean			□² Value and significance			
	V	H	N	V/H	V/N	H/N	♀/♂
♀	*9*	*1*	*40*	*6.40*	*19.61*	*37.09*	*21.35*
♂	*42*	*1*	*7*	*39.09*	*25.00*	*4.50*	

(*V= Insects responded to test volatile, H= Insects moved towards n-Hexane, N= Non responders)

responders which either moved towards the control group or those that did not respond to any group. The outcome of χ^2 (Table 2) value also confirmed it as a sex pheromone as very few female individuals were attracted towards the crude extract.

The combined result of GC-MS and bioassay conducted in *Maconellicoccus hirsutus* adapted in the environmental conditions of Vadodara city, Gujarat which experiences extreme heat and moderate cold, motivated to take up the study one step ahead. The main idea behind the study was to come out with a solution which can be proved as harmless and ecofriendly. Major concern regarding an insecticide before commercialization is its effect on environment and non-target organism including humans. Thus the present study can be an inspiration for further progress in the concerned field to address this deadly pest.

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