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EVALUATION OF INSECTICIDE RESISTANCE AND ALTERNATIVES FOR BED BUG CONTROL

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Abstract Resistance to insecticides is one of the multiple factors related to sudden bed bug reemergence. In this work, we evaluated synthetic repellents and the attractant role of cuticular compounds as potential alternatives to conventional strategies used in pest control management. On the one hand, we found DEET and δ-dodecalactone had a significant and variable repellency among bed bug colonies, while IR3535 did not elicit repellence significantly. On the other hand, we studied cuticular hydrocarbons and their role in conspecific communication. The *n*- alkanes with 26 to 36 C atoms (linear and monomethyl-branched) were the most abundant cuticular compounds. Finally, bed bugs were affected by chemical stimuli derived from cuticle extracts of males and females. However, the major cuticle components (straight-chain alkanes of 29 and 31 Carbons) delivered a significant response only in male bed bugs. Repellents and attractants compounds need further attention before incorporating into resistance management programs. Also, improving the design of controlled release systems and new formulations allows increasing the efficacy of bed bug control infestations.

Key words *Cimex lectularius*, insecticide resistance, behavior modifiers, integrated pest management

INTRODUCTION

For more than two decades, bed bugs infestations becoming a worldwide concern. Among the multiple causes associated with this phenomenon, the resistance to insecticides is considered a critical factor in the spreading and proliferation of bed bug populations (Alizadeh et al., 2020). Also, control failures have become more frequent and challenging those traditional pest management programs. In Argentina, we started to fill the gaps in the scant knowledge of bed bugs in the local scene. A high prevalence of *Cimex lectularius* (L) was detected in the hospitality sector and dwelling houses. Besides, field-collected colonies exhibited high resistance ratios to pyrethroids and insecticides with a different mode of action (Cáceres et al., 2019). Also, we investigated the activity of xenobiotic degradation enzymes as a resistance mechanism, showing high activity levels of cytochrome-P450 and glutathione S-transferases in resistant bed bug colonies. Therefore, research efforts are required to develop innovative bed bug control techniques. Behavioral-modifying compounds (BMC) could be an alternative to traditional insecticides treatments against urban and crop insect pests (Weeks et al., 2011). BMCs allow the manipulation of insect behavior using a chemical stimulus that induces the activation or inhibition of the behavioral response of the target organism. Selecting the most suitable chemical cue depends on the behavior intended to modify (Foster & Harris, 1997). Different strategies implemented in hematophagous insects mainly consist of disrupting the orientation towards the host (repellents) or attracting compounds that allow insects to be captured competitively with the natural compounds emitted by a host or by its conspecifics (kairomones and pheromones) (Weeks et al., 2011). Here we evaluated the repellence by synthetics chemicals on *C. lectularius* susceptible and resistant strains. Also, we studied the cuticle components in susceptible and resistant colonies and the potential role of cuticular extracts in conspecific bed bugs communication.

MATERIALS AND METHODS

Insects We used five bed bug colonies for behavioral assays and chemical analysis. The Harlan colony (HH-S) is the susceptible strain that has been maintained in laboratory conditions without insecticide exposure for more than four decades (Feldlaufer et al., 2014). Field-collected colonies were initiated with specimens sampled in hotel infestations from different Argentinean cities (Cáceres et al., 2019). Insects kept in plastic vials with corduroy fabric inside as refuge and oviposition surface and kept at 25 ± 1.5 ºC, 40 ± 15% relative humidity (RH), and 12:12 (L:D) h. The feeding procedure consisted of a pigeon-blood source offered weekly and supervised by the CIPEIN Institutional Animal Care and Use Committee (Cáceres et al., 2019). Stage IV and V nymphs were collected from both colonies and fed *ad libitum* to guarantee their molting. Afterward (7-10 d), freshly molted nymphs 5th and males and females (1:1) were used for repellence test and attractants bioassays, respectively.

Repellence assays The experiments were performed with nymphs 5th of HH-S and four resistant strains. The arena was a circle filter paper (Whatman N°1, 110 mm) divided into two zones with equal areas(35, 26 cm^2): an inner circle and an outer ring. A glass ring was placed on the paper avoiding bed bugs to escape. Technical grade chemicals purchased by Sigma-Aldrich: N,N-Dietil-meta-toluamide (DEET) 97%, ethyl 3-[acetyl(butyl)amino]propanoate (IR3535) 99%and δ-dodecalactone 97,5%. Acetone (Sintorgan SA) was used as a solvent and applied alone in control treatments. The outer zone of the arena was impregnated with 0.5 ml of repellent solution (1.40 mg/cm^2). Five nymphs were placed in the middle of the arena and recorded individual positions every five minutes for 1 hour. We calculated a Repellence Index for each strain and repellent: [(Nc–Nt)/Nc]x100, where Nc is the number of individuals in the outer zone in the control arena, and Nt is the number of individuals in the outer zone in the treated paper. Three replicates were performed for each treatment.

CHC identification and behavioral assessment For extraction and purification of cuticular compounds, male and female specimens were analyzed separately. Samples (5 insects each) were washed with redistilled water to remove any water-soluble contaminants. Then, they were transferred to a glass tube and submerged in 150 µL hexane (Merck) for 5 min each to extract the total lipids. The hexane volume was reduced under a nitrogen stream. For identification of CHC, the reduced extracts under a nitrogen stream were resuspended in 2 µL of hexane and analyzed by GC-MS using a Shimadzu GC-2010 coupled to a QP2010 mass spectrometer. The operative procedure consisted of: injection port in splitless mode at 320ºC, a non-polar DB-5MS (J&W Scientific, Agilent Technologies) column (30 m x 0.25 mm ID x 0.25 µm film), and helium as the carrier gas (1.6 mL/min flow rate). The oven temperature was programmed to 50ºC for 1 min and increased to 200ºC (50ºC/min rate), then further increased to 280°C (3ºC/min rate) and held for 10 minutes, and finally increased to 320ºC (3ºC/min rate), and held for 25 min. The mass spectrometer detector was set at 70 eV with the transfer line and the quadrupole at 320ºC and 150ºC, respectively. The Kovats retention index (KI) was calculated for each hydrocarbon peak after measuring the elution times of the alkane standards (Aldrich Chemical Standards, C10 – C40 Alkane standard mixture for performance, 99%) run under similar conditions. The hydrocarbon peak areas were calculated for each chromatogram and expressed as a percentage of the total peak area. Interpretation of the mass spectra was performed as described previously (Carlson et al., 1998, Feldlaufer and Blomquist, 2011; Calderón-Fernández and Juárez, 2013).

Male and female cuticular extracts and four alkanes identified by GCMS were assessed in a choice test arena with adult bed bugs. The experimental arena consisted of filter paper (Whatman N°1, 90 mm), divided into two equal areas: a control area (solvent) and the treated area (extract/alkane). The extracts were obtained following the extraction protocol previously described. The sample reduced by nitrogen stream was resuspended in 40 µl of solvent and applied to a piece of filter paper (3.75 cm^2) placed in the treated area. A similar filter paper was impregnated with 40 µl of the solvent and placed in the control zone. We evaluated cuticular extracts obtained with different sets of insects: 8, 16, 32, and 64 individuals. Also, four n-alkanes (C 28 to C31) at three different concentrations were analyzed to find any behavioral response to the stimuli. Insects were individually placed in an open vial in the center of the arena and acclimatized for 10 min. After that time, each insect was released and recorded their movements for 15 min with an HD Webcam C525-Logitech placed 25 cm above the arena. The video-tracking recorded were analyzed with

Ethovision XT 10.0 software. The time spent in each sector (control/treated) of the arena was obtained and used to estimate a Preference Index (PI): IP = Nt /(Nt + Nc), where Nt is the time spent in the area with the stimulus, and Nc is the time spent in the control area. The index assumes values between 0 and 1, where 0 indicates that insects stay longer in the zone without stimuli and 1 means that insects preferred the treated zone (Gonzalez et al., 2015).

Table 1. CHC profile obtained of cuticular extracts of *C. lectularius* adults identified by GC-MS.

KI	Name	Abundance(%)	ID mehtod
1215	Decanol	>0,1	MS, IK, B
1416	Dodecanol	>0,1	MS, IK, B
1947	Hexadecanoic acid	>0,1	MS, IK, B
1952	Octadecanoic acid	>0,1	MS, IK, B
2500	nC25	0,10	MS, IK, B
2600	nC26	0,22	MS, IK, B
2663	2-Met C26	0,32	MS, IK, B
2692	4,8-Dimet C26	>0,1	MS, IK, B
2700	nC27	5,55	MS, IK, P
2762	2-Met C27	0,04	MS, IK,
2772	3-Met C27	0,08	MS, IK,
2800	nC28	3,66	MS, IK, P
2828	12- + 14-Met C28	>0,1	MS, IK, P
2863	2-Met C28	4,20	MS, IK,
2893	2,12- + 2,14-Dimetil C28	0,05	MS, IK,
2900	nC29	19,42	MS, IK,
2932	11- + 13- + 15- + 9-Met C29	0,11	MS, IK, P, B
2962	2-Met C29	0,31	MS, IK,
2972	3-Met C29	0,35	MS, IK,
2977	5,9-Dimet C29	>0,1	MS, IK,
3000	nC30	7,74	MS, IK,
3028	13- + 14- +15-Met C30	>0,1	MS, IK, P
3064	2-Met C30	14,23	MS, IK,
3088	Cholesterol	2,27	MS, IK,
3100	nC31	16,25	MS, IK,
3124	15- + 13- + 11-Met C31	0,32	M, P, B
3149	15,19- + 13,17-Dimet C31	0,11	IK, M, P
3160	4-Met + 7,11-Dimet C31	0,27	IK, M
3171	3-Met C31	0,80	IK,M
3200	nC32	5,31	IK, M, P
3221	9,13,17,21-Tetrametil C31	>0,1	IK, M
3255	2-Met C32	10,03	IK,M
3279	3-Met C32	1,01	IK, M
3300	nC33	2,78	IK, M
3317	11- + 13- + 15-Met C33	0,71	IK, M,
3339	13,17- + 15,19-Dimet C33	0,24	IK, M,
3369	5,17- + 5,19-Dimet C33	0,37	IK, M
3400	nC34	0,87	IK, M, P
3420	12-Met C34	0,19	IK, M
3453	4-Met + 12,16-Dimet C34	0,84	IK, M
3475	4,20- + 4,18-Dimet C34	0,37	IK, M
3486	6,10,14-Trimet C34	0,38	IK, M
3513	4,8,12- + 4,8,16-Trimet C34	0,19	IK, M
3598	nC36	>0,1	IK

Identification methods: B (GC-MS libraries), IK: Kovats retention index, M (Mass spectra), P (analytical standard)

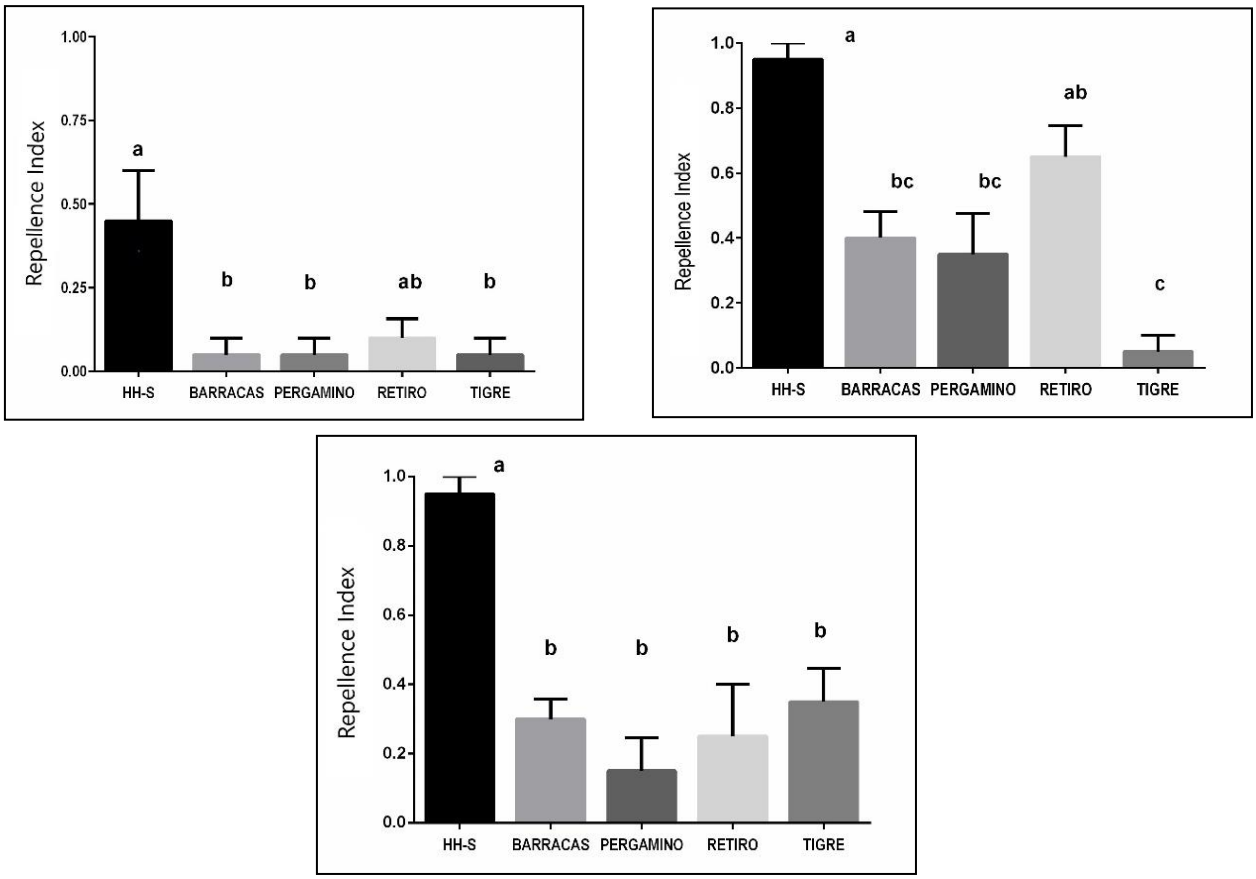

Figure 1. RI values in colonies

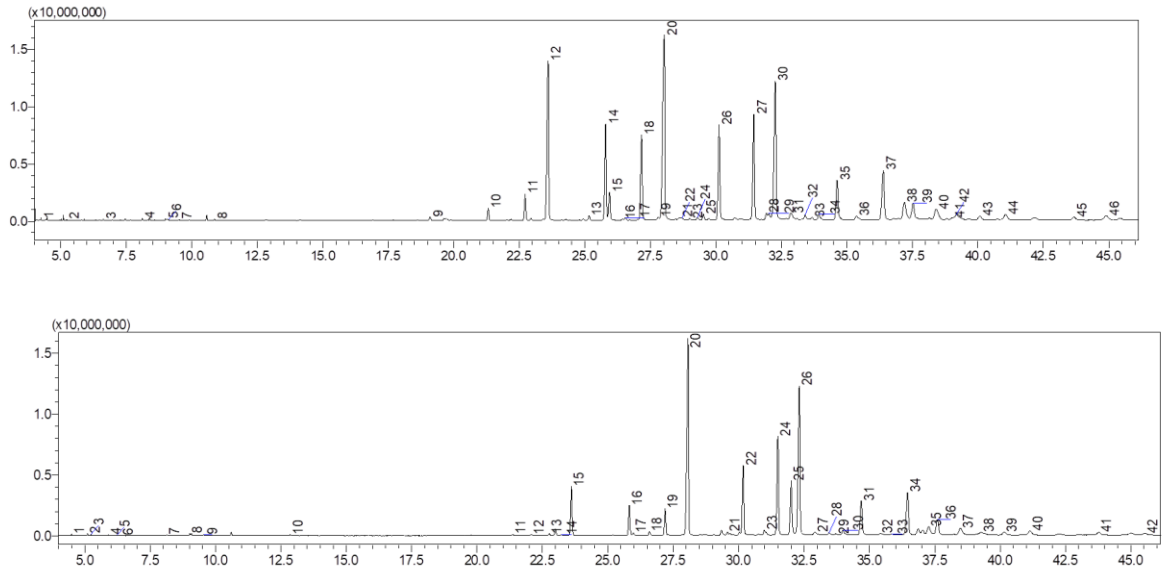

Figure 2. Identification of cuticle compounds of *C. lectularius* for five colonies of bed bugs.

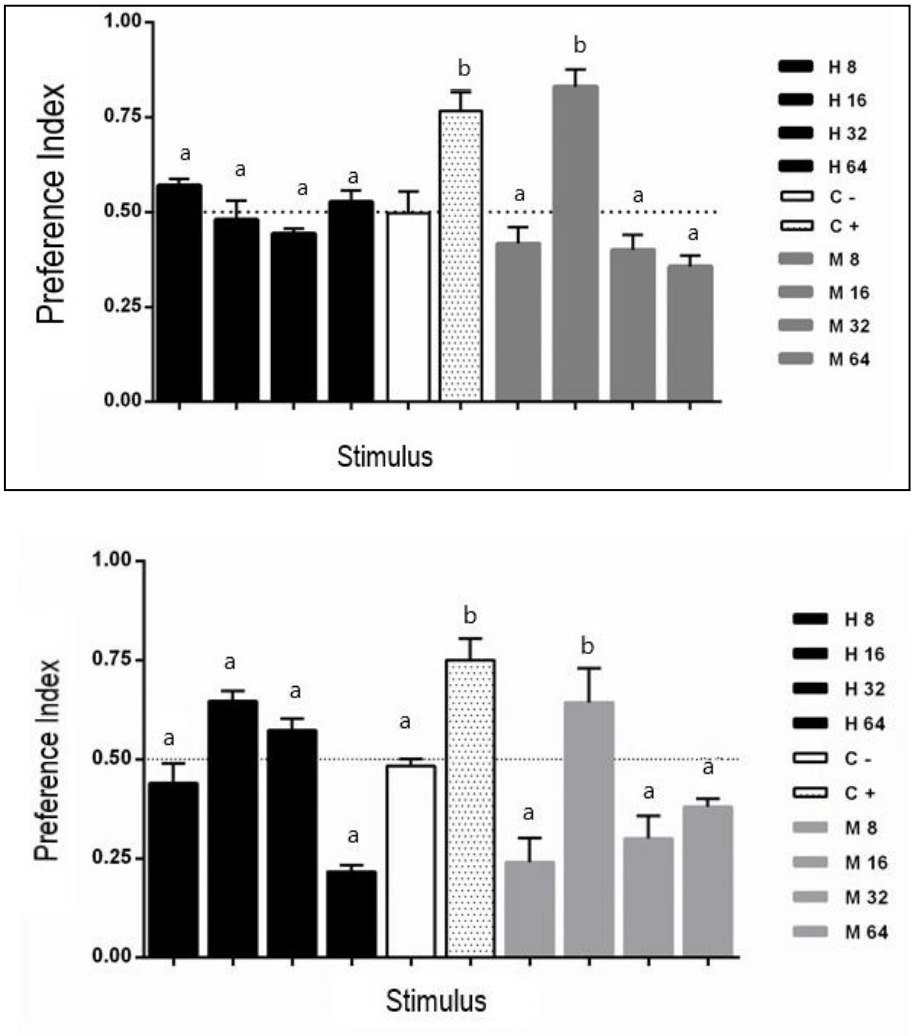

Figure 3. Males and females of *C. lectularius* preference area with stimuli.

Statistical analyses RI data were analyzed by factorial ANOVA, which was previously transformed with the logarithm function to ensure normality and homogeneity of variances assumptions. A Tukey test allowed us to detect differences between the colonies for each repellent, with a level of significance $p < 0.05$. Differences in the relative amounts of cuticle hydrocarbons between sexes were tested by ANOVA. Statistical significance between means was assessed by Tukey's test ($p<0.05$). Also, factorial ANOVA was performed to determine the preference of adult bed bugs (male and female) to the different cuticular extracts of both sexes. Differences between the behavioral response of males and females to the presence of chemical stimuli in cuticular extracts of both sexes were evaluated using Fisher's LSD test ($p<0.05$). All statistical analyses were made using the statistical software Infostat (DiRienzo, et al., 2018).

RESULTS AND DISCUSSION

In all bed bug colonies, IR3535 showed RI values significantly lower than DEET and δ-dodecalactone ($F= 17.6$; $P= 0.0001$), while DEET presented higher repellence in most of the colonies (Figure 1). Furthermore, the repellent effect of IR3535, DEET and δ-dodecalactone was significantly different between colonies ($F= 4.35$; $p= 0.0006$). Four colonies showed significantly lower CR than the susceptible colony HH-S. The mean value was 0.15 ± 0.2 (mean ± SD) in the Barracas, Pergamino, and Tigre colonies and 0.25 ± 0.2 (mean ± SD) in the Retiro colony. The RI of DEET in the susceptible strain was 0.95 ± 0.12 (Mean ± SD) and was significantly different from the repellency observed in

the Barracas, Pergamino, and Tigre colonies (Figure 1b). In contrast, RIs in the resistant colonies were significantly lower (Figure 1b). A similar repellent effect was observed for δ-dodecalactone on nymphs from the susceptible colony. Figure 1c shows that the repellency was greater than 90% in HH-S, while the resistant colonies showed a significantly different behavior, with mean RI values that ranged between 20-40%.

The identification of cuticle compounds of *C. lectularius* was performed by GC-MS for five colonies of bed bugs in our insectary (Figure 2). The cuticle of adult bed bugs is composed, in decreasing order, of n-alkanes > monomethyl alkanes > dimethylalkanes > trimethylalkanes > tetramethylalkanes. Of a total of 34 identified cuticular compounds, the most abundant 58% corresponds to the series of homologous n-alkanes, from n-C 26 to n-C 34 (Table 1). In all cuticular profiles (sex and colonies), the most abundant compound was n-nonacosane (n-C29), with an average abundance of 25%, followed by n-hentriacontane (n-C31), which together represented between 35 -55% of the total identified hydrocarbons. Among the branched hydrocarbons, 2-methyl triacontane (2MeC30) represented between 9-11% of the total hydrocarbons. The 2-methyl alkanes were identified by their characteristic fragmentation patterns with strong M/Z 43 ion (Feldlaufer and Blomquist 2011). Also, the presence of cholesterol (eluting at 31.9 minutes, just before n-C31) was present in all samples with a relative abundance of 5% (Table 1). For most colonies, the mean content of n-C29, n-C31, and 2-Methyl C30 was slightly higher than HH-S.

The behavior of adult bed bugs in the presence of paper impregnated with the cuticular extract was significantly different depending on the extract composition ($F= 9.25$, $P=0.0003$). The males and females of *C. lectularius* had a significant preference for the area with the stimuli extracted from 16 males/females (Figure 3). Similarly, individuals of both sexes responded with a significant preference for the treated area in the positive control (a piece of paper with contact chemical cues obtained from individuals for one week). In contrast, the response to extracts concentration, a higher or lower than groups of 16 insects was not significantly different from the negative control (absence of stimuli). Previously, Feldlaufer and Blomquist (2011) made a preliminary identification of the cuticular composition but did not evaluate the possible differences between colonies and sexes. Here, it was possible to extend the aforementioned data from the 17 identified compounds to a total of 34 components. The bed bug cuticle components identified here are mostly long-chain branched hydrocarbons. This sort of compound in the cuticle of insects is considered a significant contribution to protection against dehydration (Juárez and Fernández., 2007). These results are consistent with the particular long survival of bed bugs, in which they remain hidden and aggregated in close contact, thus maintaining humidity while they do not feed. Furthermore, no differences were observed between the sexes. Similar studies carried out in *R. prolixus* and *T. infestans* demonstrated the absence of sexual dimorphism at the level of the cuticle in both species, and suggest that sexual attraction would not be mediated by recognition at the level of the cuticular surface (Juárez and Fernández., 2007). Bed bugs do not possess sex pheromones specifically involved in recognition and copulation (Usinger, 1966). In contrast, the traumatic insemination strategy is well known, whereby the male tries to copulate with females and even with other males and nymphs of stages IV or V when they have just fed (Reinhardt and Siva-Jothy, 2003).

CONCLUSION

We studied the behavioral responses to natural and synthetic compounds as modifiers of bed bug behavior that could be included as helpful technics for preventing and monitoring bed bug infestations. On the one hand, IR3535 does not produce a significant repellent effect on bed bugs. In contrast, DEET and δ-dodecalactone demonstrated greater repellency in bed bug colonies. However, among resistant bed bug colonies, the effect of these repellents was lower compared to susceptible insects. On the other hand, the profile of the hydrocarbons components of the cuticle of adult bed bugs shows a higher proportion of n-alkanes, which is an indicator of their role in the regulation of water loss. However, the behavioral response of adult bed bugs preferentially to the cuticular extract suggests its role in communication between adults. The efficacy of these synthetic or natural compounds could produce effective tools for personal protection and help to decrease the selection pressure of resistant insects. Also, a detailed examination of the biology and ecology of bed bugs can achieve more complete knowledge to design satisfactory strategies for protection, as well as for the control of pests in the environments inhabited by these insects.

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