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Identifying Females of Three Carder Bee Species of Genus *Anthidium* (Hymenoptera: Megachilidae) Using Chemical Compounds of Cuticle

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Abstract

Precise identification of *Anthidium* species as beneficial pollinators is vital for preserving and protecting them. The identification and quantification of cuticular chemical compounds during the present study were performed on a gas chromatography–mass spectrometry (GC/MS). GC/MS analysis indicate that the cuticular chemical profiles of the females of the three *Anthidium* species contains 42 chemical compounds. Fatty acid is the most diverse and abundant class (19 compounds) followed by hydrocarbons (6 compounds), glyceride (4 compounds), organosiloxane (3 compounds), alcohols (3 compounds), two ketones, two sterols and three unclassified compounds. Female *A. amabile* exclusively distinguished by having 10 compounds not found in the other two females, while female *A. pullchelum* distinguished by having two compounds and female *A. testelatum* by having one alcohol compound. The current investigation aimed to evaluate using chemical structure of insect cuticle (body wall) as taxonomic tool helping to identify females of three carder bee species of genus *Anthidium.* The obtained results found that there are qualitative and quantitative variations concerning chemical cuticular compounds among different females of species of genus *Anthidium.* The present study concluded that, chemical structure of insect cuticle can be used, side by side with morphological characters, as chemotaxonomic tool.

Keywords: *Chemotaxonomy, Bees, Egypt, Cuticle, GC-MS, Identification.*

Introduction

Pollination is the main vital element for sustaining agriculture and ecosystem (Klein *et al.,* 2007). The principal pollinators are bees, it has been estimated that 73% of agricultural crops are pollinated by different species of bees (Abrol, 2009). Solitary bees are the most common bee species (about 85% of all bee species) (Batra, 1984). Solitary bees are the most effective pollinators, evener than honeybees, for different crops (Garratt *et al.,* 2014) this is being arises by the innate behaviour to collect huge amounts of pollen grains and nectar for their brood and, therefore, tend to visit numerous flowers (Bosch and Kemp, 2001).

Many classes of lipids have been found in insect cuticle such as hydrocarbons, fatty alcohols, wax esters, triglycerides, and aldehydes (Kühbandner *et al.,* 2012). Chemical structure of insect cuticle makes it suitable to protect against penetration of pathogens and desiccation, used in communication and recognition of mates (Menzel *et al.,* 2019).

Cuticular hydrocarbons are obviously sexually dimorphic (sexes of the same species show different characteristics), in some insect species (e.g., *Drosophila melanogaster*), with many of the compounds present in one sex lacking in the other. (Thomas and Simmons, 2008, Ferveur and Cobb, 2010). Species belong to one genus use compounds similar in structure in communication of pheromone (Wyatt, 2014).

Biochemical studies have indicated that insects have the potential to synthesize numerous hydrocarbon compounds (Soroker and Hefetz, 1995). Hydrocarbons were used as chemo-taxonomic for the first time in 1970 (Jackson and Baker, 1970) and Bagnères and Wicker-Thomas (2010) confirmed species-specificity of hydrocarbons. The profile differences between species often being both qualitative and quantitative.

Stephen *et al*., (2008) confirmed that the hydrocarbons profile can represent characteristic ‘fingerprints’ that are remarkably consistent within a species. Therefore, the cuticular hydrocarbon structure is an output of an insect’s genotype and eligible for use in chemotaxonomy (Lockey, 1991).

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The recent trend is to use the integrative taxonomy, and accordingly, the biochemical investigations support the other taxonomic characteristics such as morphological and molecular.

Schlick-Steiner *et al.*, (2010) concluded that, more "integrative taxonomy" must establish to be convenient with new concepts and approaches. A significant number of studies belong to the "integrative taxonomy" have been published (Fonseca *et al.*, 2008; Gibbs, 2009; Glaw *et al*., 2010; Lumley and Sperling, 2010). The present study aims to evaluate using the chemical structure of an insect cuticle as a taxonomic tool that helps in identifying females of three carder bee species of genus *Anthidium*.

Materials and Methods

Insects: The present investigation is carried out on preserved insects of females of three species of genus *Anthidium*. The specimens collected using sweeping nets throughout different field trips in Egypt, then, killed in poison bottle. A stereo light microscope was used to examine the specimens. Taxonomic keys used for identification, latter, confirmed in the entomological collection of the Plant Protection Research Institute, Giza, Egypt.

Gas Chromatography – Mass Spectrometry (GC/MS): Insects immersed into n-hexane for two minutes and kept at 4°C for later analysis. Extracts analyzed by gas chromatography-mass spectrometry (GC-MS) as described by Wurdack *et al*., (2015).

GC/MS analysis have been done in Al-Azhar centre for mycology, Cairo, Egypt. The analyzing and quantification of chemical compounds were performed on a Thermo Scientific™ TRACE™ 1310 Gas Chromatograph equipped with a DB-5 (Length 30 m. x Internal diameter 0.25 mm. x film thickness 0.25 μm) with helium as carrier gas (flow rate 1 ml/min.). One microliter of sample was injected into the injector in pulsed split less mode. The injector temperature was at 300 °C.

The initial GC temperature of 40 °C (held for 5 min.) then temperature raised to 275 °C (5 min.) at rate 5 °C/min. spectrometric detection was performed in electron impact mode with an ionizing energy of 70 eV. The ion source temperature was 300 °C. The electron multiplier voltage (EM voltage) was maintained 1650 v. above auto run. The instrument was manually turned using perfluorotributylamine (PFTBA). all compounds were identified by comparison with Computer Library (WILEY & NISTMASS SPECTRAL DATABASE) attached to the GC–MS instrument and by comparison to literature relative retention indexes.

Results

Extraction and analyzing the chemical composition of the cuticle of females of three species of genus *Anthidium* collectively showed mixtures of miscellaneous categories of chemical compounds, fatty acids, hydrocarbons, glycerides, organosiloxane, alcohols, ketones, sterols, and other chemical compounds (Table 1, Figure 1), the total number of the identified compounds is 42. There is a variability between the cuticular chemical profiles of the extracts of the females of the three species. The females of the three species of genus *Anthidium* commonly shared fourteen compounds belong to varies classes, six fatty acids (1-Hexadecanol; Hexadecanoic Acid, Methyl Ester; 9,12-Octadecadienoic Acid (Z,Z); Oleic Acid; Octadecanoic Acid and 10Z-nonadecenal); four hydrocabons (Benzene, (1-Butylhexyl); Benzene, (1-Pentylhexyl); Benzene, (1-Butyloctyl) and Benzene,(1-Methyldodecyl); three Organosiloxane (Cyclooctasiloxane, hexadecamethyl; Cyclononasiloxane, octadecamethyl and Cyclodecasiloxane, eicosamethyl) and one alcohols (Ethyl Iso-Allocholate). Generally, oleic acid is the most abundant compound among the chemical constituents of the cuticle (24.96%) in female *A. amabile*.

Fatty acids: The most diverse class, represented by 19 compounds in the three females together (Table 1). The fatty acids ranged from C14 to C28. The composition of the *A. amabile* contains 17 fatty acids: 12 in *A. pulcellum* and 8 in *A. testelatum*. Oleic acid is the most abundant fatty acid compound.

Hydrocarbons: Totally represented by 6 compounds in the three females, four of them common among the three females, Benzene, (1-Butylhexyl); Benzene, (1-Pentylhexyl); Benzene, (1-Butyloctyl); Benzene,(1-Methyldodecyl); while Benzene,(1-Ethyloctadecyl) not included in *A. pulchellum* and Heneicosane, 11-Phenyl exclusively included in *A. amabile*. The hydrocarbons ranged from C26 to C48. Benzene, (1-Methyldodecyl) is the most abundant Hydrocarbon compound.

Glycerides: Collectively represented by 4 compounds all included in cuticle of female *A. amabile*, and 9,12-Octadecadienoic acid (Z, Z)-2-hydroxy-1(hydroxymethyl)ethyl ester, only included in *A. pulchellum* and totally absent from the females of *A. testelatum*. The Glycerides ranged from C21 to C39. Octadecenoic Acid (Z)-,2-Hydroxy-1-(Hydroxymethyl) Ethyl Ester is the most abundant Glyceride compound.

Organosiloxane: Represented by 3 compounds, included in the cuticle of females of all species. The Organosiloxane ranged from C16 to C20. Cyclononasiloxane, octadecamethyl is the most abundant organosiloxane compound.

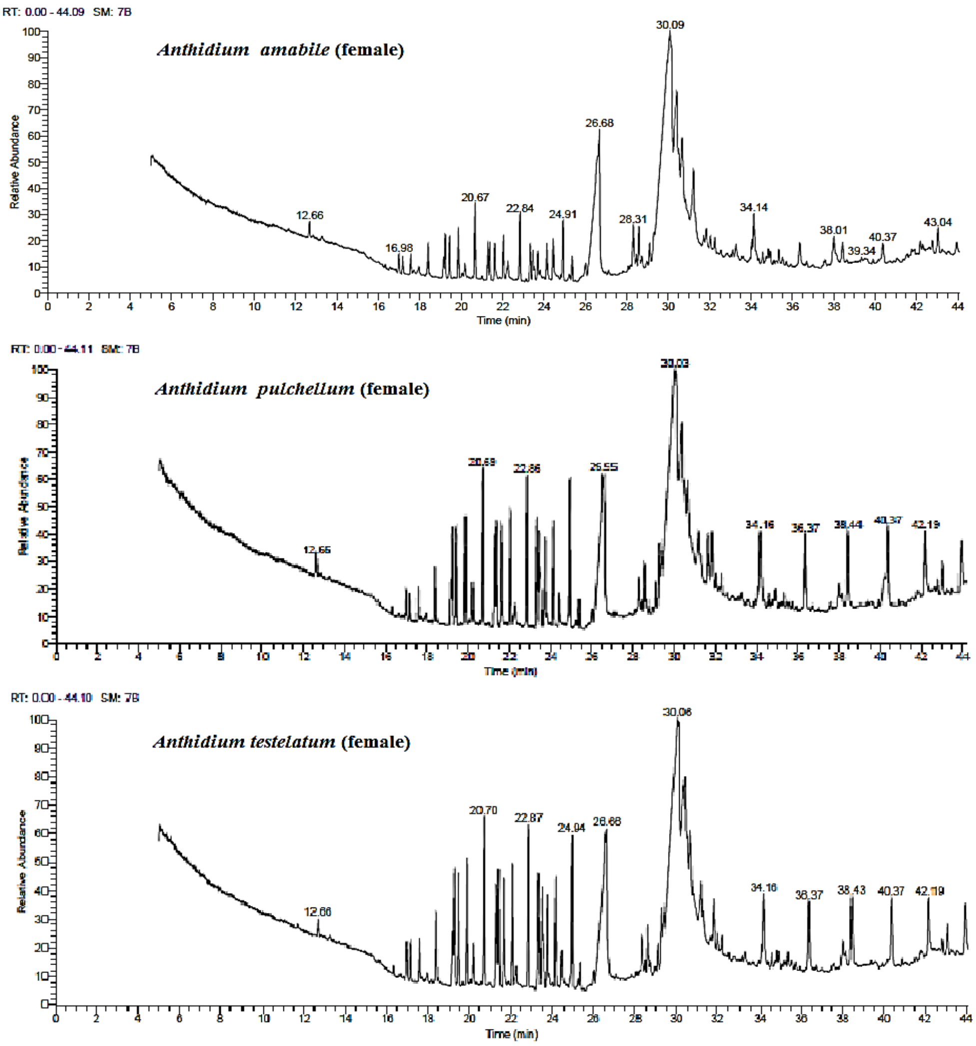

Figure 1: **Chromatogram of GC / MS analysis for cuticular chemical profile of females of three species of genus *Anthidium* Fabricius, 1804.**

Alcohols: Represented by 3 compounds, Ethyl Iso-Allocholate shared among three species, 1-Hexadecanol found only in cuticle of female *A. testelatum* and Hexadecane, 1,1-bis (dodecyloxy) only in *A. pulchellum*. Alcohol compounds ranged from C16 to C40. Hexadecane, 1,1-bis (dodecyloxy) is relatively abundant alcohol compound.

Ketones: Represented by 2 compounds, 2-Propoxybenzaldehyde and Decanophenone, the first absent from females of *A. testelatum* and the later found only in *A. pulchellum*.

Sterols: Represented by 2 compounds, Stigmast-5-En-3-Ol, (3á,24s) and Cholesta-8,24-Dien-3-Ol,4-Methyl-, (3á,4à), the first absent from females of *A. testelatum* and the later found only in *A. amabile*.

Other compounds: 9-Benzyl-2-fluoro-9H-purin-6-amine (C12) absent only from *A. amabile*; Tributyl Acetylcitrate (organooxygen compounds) (C20) in *A. pulchellum* and Cis-2-Phenyl-1,3-Dioxolane-4-Methyl Octadec-9, 12, 15-Trienoate (benzenoid aromatic compound) (C28) found only in *A. testelatum*.

Comparison of Cuticular Chemical profile of the three females:

Female ***A. amabile*** distinguished from other two females by having 10 compounds, seven fatty acid (Tetradecanoic Acid; 2-Oxooctadecanoic acid; Octadecanoic Acid, 4-Hydroxy, Methyl Ester; Eicosanoic acid; Docosanoic acid; Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl) and 9-Octadecenoic Acid, (2-Phenyl-1,3-Dioxolan-4-Y L) Methyl Ester, Cis; single hydrocarbon compound (Heneicosane, 11-Phenyl); four glycerides (9,12-Octadecadienoic acid (Z,Z)-2-hydroxy-1(hydroxymethyl)ethyl ester; 2,3-Dihydroxypropyl Elaidate 9-Octadecenoic Acid (Z)-,2-Hydroxy-1-(Hydroxymethyl)Ethyl Ester; Hexadecanoic acid,1-(hydroxymethyl)-1,2-ethanediyl ester and 2-Hydroxy-3-[(9e)-9-Octadecenoyloxy] Propyl (9e)-9-

Octadecenoate) and single sterol compound (Cholesta-8,24-Dien-3-Ol,4-Methyl-, (3á,4à). This female also characterized from other two females by lacking, 9-Benzyl-2-fluoro-9H-purin-6-amine; 1-Hexadecanol, 2-Methyl and n-Hexadecanoic acid.

Table 1. Cuticular chemical compounds in three females of genus *Anthidium*

Chemical formula	Compound name	*A. amabil*	*A. pullchelum*	*A. testelatum*
Fatty acids				
C14H28O2	Tetradecanoic Acid	0.82	-	-
C16H32O	1-Hexadecanol	1.60	1.04	0.90
C16H32O2	n-Hexadecanoic acid	-	2.74	2.73
C17H34O2	Hexadecanoic Acid, Methyl Ester	0.96	0.78	0.66
C17H36O	1-Hexadecanol, 2-Methyl-	-	1.04	1.06
C18H32O2	9,12-Octadecadienoic Acid (Z,Z)-	2.89	1.83	1.84
C18H34O2	Oleic Acid	24.96	1.40	1.25
C18H34O3	2-Oxooctadecanoic acid	0.43	-	-
C18H36O2	Octadecanoic Acid	4.53	3.70	3.47
C19H36O2	8-Octadecenoic Acid, Methyl Ester	1.64	1.40	-
C19H38O2	Heptadecanoic Acid, 9-Methyl, Methyl Ester	0.85	0.75	-
C19H38O3	Octadecanoic Acid, 4-Hydroxy, Methyl Ester	0.58	-	-
C19H36O	10Z-nonadecenal	1.64	1.40	1.25
C20H40O2	Eicosanoic acid	0.82	-	-
C21H34O2	5,8,11,14-Eicosatetraenoic Acid, Methyl Ester, (All-Z)	3.09	1.83	-
C21H38O4	9,12-Octadecadienoic acid (Z,Z)-, 2-hydroxy-1-(hydroxymethyl) ethyl ester	0.73	1.83	-
C22H44O2	Docosanoic acid	1.91	-	
C27H42O4	Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl)	1.01	-	-
C28H44O4	9-Octadecenoic Acid, (2-Phenyl-1,3-Dioxolan-4-Y L) Methyl Ester, Cis	0.67	-	-
Hydrocarbons				
C16H26	Benzene, (1-Butylhexyl)-	0.68	0.75	0.95
C17H28	Benzene, (1-Pentylhexyl)	0.72	1.19	1.30
C18H30	Benzene, (1-Butyloctyl)-	0.70	0.86	2.72
C19H32	Benzene,(1-Methyldodecyl)	1.25	2.60	3.20
C26H46	Benzene, (1-Ethyloctadecyl)	1.77	-	1.01
C27H48	Heneicosane, 11-Phenyl-	1.45	-	-
Glycerides				
C21H38O4	9,12-Octadecadienoic acid (Z,Z)-2-hydroxy-1(hydroxymethyl)ethyl ester	0.73	1.84	-
C21H40O4	2,3-Dihydroxypropyl Elaidate 9-Octadecenoic Acid (Z)-,2-Hydroxy-1-(Hydroxymethyl)Ethyl Ester	2.83	-	-
C35H68O5	Hexadecanoic acid,1-(hydroxymethyl)-1,2-ethanediyl ester	0.49	-	-
C39H72O5	2-Hydroxy-3-[(9e)-9-Octadecenoyloxy] Propyl (9e)-9-Octadecenoate	1.97	-	-
C16H48O8Si8	Cyclooctasiloxane, hexadecamethyl	0.61	1.27	1.05
C18H54O9Si9	Cyclononasiloxane, octadecamethyl	0.42	1.88	1.44
C20H60O10Si10	Cyclodecasiloxane, eicosamethyl-	1.04	1.53	2.22
Organosiloxane				
C16H48O8Si8	Cyclooctasiloxane, hexadecamethyl	0.61	1.27	1.05
C18H54O9Si9	Cyclononasiloxane, octadecamethyl	0.42	1.88	1.44
C20H60O10Si10	Cyclodecasiloxane, eicosamethyl-	1.04	1.53	2.22

Table 1. Continued

Chemical formulas	Compound name	*A. amabil*	*A. Pullchelum*	*A. testelatum*
Alcohols				
C16H34O	**1-Hexadecanol**	**-**	**-**	**0.90**
C26H44O5	**Ethyl Iso-Allocholate**	**0.67**	**1.00**	**0.93**
C40H82O2	**Hexadecane, 1,1-bis (dodecyloxy)-**	**-**	**1.02**	**-**
Ketones				
C10H12O2	**2-Propoxybenzaldehyde**	**0.61**	**3.05**	**-**
C16H24O	**Decanophenone**		**-**	**2.73**
Sterols				
C28H46O	**Cholesta-8,24-Dien-3-Ol,4-Methyl-, (3á,4à)-**	**0.64**	**-**	**-**
C29H50O	**Stigmast-5-En-3-Ol, (3á,24s)-**	**1.23**	**1.00**	**-**
Other compounds				
C20H34O8	**Tributyl Acetylcitrate (organooxygen)**	**-**	**1.52**	**-**
C28H40O4	**Cis-2-Phenyl-1,3-Dioxolane-4-Methyl Octadec-9, 12, 15-Trienoate (benzenoid aromatic compound)**	**-**	**-**	**1.85**
C12H10FN5	**9-Benzyl-2-fluoro-9H-purin-6-amine**	**-**	**2.86**	**1.16**

The most abundant compound in the cuticle of this female is Oleic Acid (24.96%) followed by Octadecanoic Acid (4.53%) and 5,8,11,14-Eicosatetraenoic Acid, Methyl Ester, (All-Z) (3.09 %), while 2-Oxooctadecanoic acid and Hexadecanoic acid,1-(hydroxymethyl)-1,2-ethanediyl ester, represented by traces (0.43% and 0.49% respectively).

Female ***A. pullchelum*** characterized from other two females by having two compounds, alcohol compound Hexadecane, 1,1-bis (dodecyloxy) and organooxygen compound Tributyl Acetylcitrate. This female also characterized from other two females by absence of Benzene, (1-Ethyloctadecyl). Cuticular compounds contribute in somewhat equal proportions, but the relatively abundant compounds are, Octadecanoic Acid (3.70%) and 2-Propoxybenzaldehyde (3.05%), Hexadecanoic Acid, while the lesser compounds are Methyl Ester (0.75%); Heptadecanoic Acid, 9-Methyl, Methyl Ester (0.75) and Benzene, (1-Butylhexyl) (0.78%).

Female ***A. testelatum*** distinguished from other two females by having one alcohol compound, 1-Hexadecanol and one ketone, Decanophenone. This female also characterized from other two females by absence of 8-Octadecenoic Acid, Methyl Ester; Heptadecanoic Acid, 9-Methyl, Methyl Ester; 5,8,11,14-Eicosatetraenoic Acid, Methyl Ester, (All-Z); 9,12-Octadecadienoic acid (Z, Z)-, 2-hydroxy-1-(hydroxymethyl) ethyl ester and 9,12-Octadecadienoic acid (Z,Z)-2-hydroxy-1(hydroxymethyl)ethyl ester. Cuticular compounds contribute in somewhat equal proportions, but the relatively abundant compounds are, octadecanoic Acid (3.47%); Benzene, (1-Methyldodecyl) (3.20%) and n-Hexadecanoic acid (2.73%) while the lesser compounds are Hexadecanoic Acid, Methyl Ester (0.66%) and 1-Hexadecanol (0.90%).

Discussion

Although the chemical composition of insect cuticles is known in some species (Lockey, 1988) knowledge about this composition in many Egyptian species is still rare and require sufficient studies.

The investigation of chemical classes obtained by GC/MS to the cuticle of three females which belong to genus *Anthidium,* agree with that previously recorded by many authors e.g. Al-Dawsary (2014) (Hydrocarbons, Carboxylic acid, Alcohols, Ketones, Aldehydes, Esters and Amines), Alnajim *et al.* (2019) (fatty acids, hydrocarbon waxes, sterols), Lockey, (1988) (normal and branched saturated and unsaturated hydrocarbons, free fatty acids, free alcohols, alkyl esters, glycerides, sterols and aldehydes) (Lockey, 1988).

Although cuticular hydrocarbon recorded in the current study is relatively represented by a small number of compounds (6 compounds, Benzene, (1-Butylhexyl); Benzene, (1-Pentylhexyl); Benzene, (1-Butyloctyl); Benzene, (1-Methyldodecyl; Benzene,(1-Ethyloctadecyl) and Heneicosane, 11-Phenyl) which is questionable, Drijfout *et al.* (2010) estimated the number of cuticular hydrocarbon compounds in insects as ranged between five and fifty.

Fatty acids are important constituent of cuticle and are commonly engaged in forming more complex lipids, such as triglycerides, which stores energy (Desbois and Smith 2010). Fatty acids are most diverse and abundant in the cuticle of the three females (19 compounds, representing 45.24% of all obtained compounds). This result matches with Alnajim *et al.* (2019) in the investigation of cuticular lipids of *Rhyzopertha dominica* (Fabricius) and *Tribolium castaneum* (Herbst).

Despite the small number of chemical compounds distinguishing *A. pulchellum* and *A. testelatum* females, there are quantitative differences for some compounds that can be used taxonomically. This was adopted by many authors and it supports the importance of the quantitative differences of chemical compounds, such as hydrocarbons, whereas quantitative variation in the same hydrocarbons is considered population level variation (Garratt *et al*., 2016 and Pizzi and Rehan ,2021).

Although these species have been studied by traditional taxonomy, the addition of supplementary characters through biochemical, molecular, ecological, and other biological sciences, makes the identification of species more accurate and confident, which is what the integrative taxonomy aims at (Yeates *et al.,* 2010). The present study is an attempt to complement the previous traditional taxonomic investigation and biochemical approach, toward integrative taxonomy.

Conclusion

Precise identification of *Anthidium* species as beneficial pollinators is vital for preserving and protecting them. Although the traditional taxonomy (the use of morphological characters) is of great importance in identifying and distinguishing insect species, in some cases it is difficult to distinguish species and other insect categories and even between sexes of the same species from each other. Many attempts to include additional methods that support morphology.

There is a tendency to use the integrative taxonomy (using several approaches, such as morphological, genetical, biochemical) to obtain conclusive results in distinguishing the different taxonomic categories.

Sexual characters often overlap with the taxonomic characters of insects. In conclusion, the present study aimed to investigate the qualitative and quantitative differences between cuticular chemical profile (using GC/MS technique) of females of three species of a genus *Anthidium* to differentiate between both types of characters. The study showed that there are differences in the chemical composition of the cuticle (body wall) of the investigated females and it was possible to use the biochemical structure as chemotaxonomic characters which is complementary to the traditional approaches.

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الملخص العربي

تعريف ثلاثة أنواع من النحل القاطع للأوراق باستخدام المحتوي الكيميائي للجليد

يعتبر النحل القاطع للأوراق من جنس *Anthidium* من اهم الملقحات للنبات ولذا يجب الاهتمام بتعريفه على نحو جيد. في هذا العمل تم الاستعانة بتحليل الجليد لهذه الأنواع عن طريق استخدام GC/ MS والذي أوضح أن جليد الإناث من ذلك الجنس يحتوي علي 42 مركب كيميائي، كانت الأحماض الدهنية هي الأكثر انتشارا (19 مركب) ، ثم الهيدروكربون (6 مركبات)، تلاها الجليسريد و الاورجانوسيليكون ب(4 و 3) علي التوالي ، ثم اثنان من الكيتون واثنان من الستيرول و اظهر التحليل أيضا وجود ثلاث مركبات غير مصنفة. تتشارك إناث هذا الجنس في أربعة عشرة مركبا، وتتميز انثي النوع *amabile* بوجود عشر مركبات غير موجودة في النوعين الأخرين. وتهدف الدراسة الحالية الي استخدام التحليل الكيميائي لجليد الحشرات بجانب الصفات الظاهرية للوصول الي تعريف دقيق لهذه الأنواع.