Extraction and identification of bioactive substances from fruit peels and evaluate the activity against *Icerya aegyptiaca* (Douglas)

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Abstract: For reducing the adverse environmental impacts of synthetic pesticides, the present study aimed to evaluate the insecticidal efficacy of five different extracts from two fruit peels, *Citrus sinensis* and *Punica granatum* against the Egyption mealybug, *Icerya aegyptiaca* (Douglas). The ethanol extract of *Citrus sinensis* peels was the most effective for all concentrations during all periods after application (LC_{50}= 1.51%), concentrations, 7 and 9% from this extract caused 100% mortality after 4 days of application. The other peel extract (*Punica granatum*), after 7 days of application, 0.3% ethanolic extract and 20% aqouse extract gave 73.33% mortality without significant different. Ethanolic extract (LC_{50} = 0.08%) was more toxic than the aqouse extract (LC_{50} =6.6%). Chromatography – mass spectrometry (GC-MS) analysis from the effective ethanolic *Citrus sinensis* extracted identified 4H-1-Benzopyran-4-one,2- (3,4-dimethoxyphenyl)- 5,6,7-trimethoxy, as a major component (17.64%), followed by its derivative 4H-1-Benzopyran-4-one,5,6,7-trimethoxy-2-(4-methoxyphenyl) by (6.38%). Saturated and unsaturated fatty acid as Hexadecanoic acid and Ricinolic acid, respectively, were recorded in the sample. Fural derivatives and Gibberellic acid were also detected. This study showed that, this botanical insecticide which extracted from fruit peels, *Citrus sinensis* and *Punica granatum* are preferable to control this insect pest.

Keywords: *Citrus sinensis, Punica granatum, Iceyra aegyptiaca* (Douglas), fruit peel extraction, Chromatography-mass spectrometry (GC-MS) analysis

1.Introduction:

The Egyptian fluted scale, *Icerya aegyptiaca* (Douglas) which belonged to Hemiptera: Monophlebidea is one of the most important Icerya species all over the world **(Senthilkumar and Barthakur, 2005)**. It is commonly known as Egyptian mealybug, because it is original description in Egypt and recorded in Egypt in coccid list of **Ezzat and Nada (1986)** This insect causes remarkable damage on all tropical fruit trees. it attacks many ornamentals and crop trees, such as *Citrus* sp., *Mangifera indica*, *Ficus* sp., *Annona* sp., *Morus alba* (mulberry), *Psidium guajava* (guava) and *Ficus virens*, **(Meena, *et al*. 2012 and Eman, 2015)**. Since1980s, *I. aegyptiaca* was listed as one of the most dangerous garden pests in Guangzhou and China **(Zhang, 2011)**. Now, it became a pest of garden plants and widely distributed in tropical and subtropical regions **(Liu and Shi, 2020)**, This genus *Icerya* includes about 35 species which distributed all over the world and ommonly known as fluted scales. **(El-Sobky, 2020)**.

The high population densities of *Icerya aegyptiaca* may cause leaf drop symptoms (i.e. leaf yellowing and defoliation), reducing plant growth and also may cause dieback of the branches due to feeding stress, also the insect could often introduce plant pathogens such as viruses and fungi into the host **(Akintola and Ande, 2008)**.

Mealybugs are often difficult to eradicated by conventional insecticides because of decrease of insecticides absorption into their body due to cushion of waxy scales on their dorsal body surface. So, there is no effective synthetic compounds available to control mealybugs **(Mani and Shivaraju, 2016)**. Therefore, farmers use blind, silly and excessive use of synthetic chemicals to control mealybugs, sometimes, the farmers doubled and tripled the application rates, leading to increase crop phytotoxicity, environmental pollution and human health hazards and other non-target benefit insect and natural enemies (predators and parasitoids). In addition, the extensive use of pesticides leads to improve pest resistance **(Badshah, *et al.*, 2017)**.

This urges mankind to use naturally biological substances occurring compounds which is environmentally ecofriendly management methods, **(Bhattacharyya, *et al.*, 2009)**, such as endogenous plant extracts **(Mamoon, *et al*., 2016)** and botanical pesticides are sustainable products which are safe to the environment with no or minimum non-target effects **(Kabir, *et al.*, 2017)**. New pesticides based on plants play a major role in pest management in defendable agriculture production. Many investigators reported that, the kingdom planta is considered as one of the richest sources which can be used as pest control bioagents **(Nakatani *et al*., 2001)**. In Egypt, several studies have been achieved to monitor the insecticidal activity of different plant extracts against many pests **(Sadek, 2003)**.

Citrus are the most commonly grown fruits around the world, and the yield of citrus fruits in 2015 was more than 130 million tons **(FAO, 2017)**. However, the industrial process of citrus gave a large amounts of waste (peel, pulp, and seeds), where these waste lead to consider pollution problems especially in the developing countries. In addition, citrus fruits have been of concern for extraction of essential oil as previously conducted by many studies, but the peels have been less studied and need more works **(Cholke, *et al*. 2017)**.

The present study aimed to evaluate the potential toxic effect of five extracts from two peel fruits, *Citrus sinensis* and *Punica granatum* against *Icerya aegyptiaca* and identify the bioactive components present in crud peel extract of *Citrus sinensis* using GC-MS.

2.Materials and Methods:

2.1. Tested insect

Adult female mealybugs were collected into glass petri-dishes from non-sprayed *Ficus nitida* infested trees located in the Faculty of Agriculture Farm, University of Alexandria, located in Abis region using a camel hair brush to be used in all toxicity bioassays.

2.2. Fruit peels extraction

2.2.1. Sweet orange peels extract

According to **Majeed, *et al.* (2018)**, fruit peels of sweet orange (*Citrus sinensis*) were washed by clean tap-water and dried in shadow for two weeks, and then ground into powder using an electric blender. Soxhlet apparatus was used for the extraction process using three solvents acetone, ethanol and water, after eight hours of extraction, the collective solvents were evaporated to obtain crude extract by rotary evaporator. The tested concentrations of the three extracts were, 2, 3, 5, 7 and 9% of crude extract.

2.2.2. Pomegranate peel extract

According to **Al-Anany, *et al.* (2017)**, peels of pomegranate (*Punica granatum*) were collected, cleaned, air dried in shadow and ground into powder. Ethanol and sterile distilled water were used for the extraction. With ratio 1:4 w/v. The peel powder was soaked in ethanol and shaken for 24 hr using an electric shaker, filtrate and ethanol was evaporated using electric fan until dryness. Using distilled water at the rate of 1:15 w/v, the peel powder was exposed to 70° C for one hour and was shaken for 24 hr using an electric shaker, filtrated and dried to obtain the extracted crude**.** The tested concentrations were, 2.5, 5, 10, 20 and 30% for water extracts and for ethanol extract were 0.025, 0.05, 0.1, 0.2 and 0.3% **(Al-Anany, *et al*. 2017)** with slight modification.

2.3. Bioassay experiments

Bioassay experiment were carried out on adult female of mealybugs with two kind of peel extracts, each with two or three types of solvent i.e. ethanol, acetone and distilled water, five concentrations of each extract were tested against the insect with three replications for each treatment. Experimental design was completely randomized (CRD). Distilled water was used to prepare serial dilutions of plant extracts. Bioassay was performed using freshly cut *Ficus nitida* leaves which were washed and completely air dried before treatments. A slightly moisturized filter paper was placed in each petri dish to keep the leaf moisture during the experiment, ten adult female of *Icerya aegyptiaca* were released on the Ficus leaves using camel-hair brush and treated with small sprayer. Number of died insects were daily recorded for 7 days after treatments and the percentage of mortality were estimated relatively to control treatment which contains the same amount of solvent in treatments.

2.4. Chemical analysis

TSQ 8000 Evotriple quadrapole GC-MS/MS instrument coupled with a thermoscientific "TRACE" 1300 GC mass spectrometer was used. This analysis was carried out on National Institute for Oceanography and Fisheries, Quaitbay, Alexandria. Sample introduction was performed to thermoscientific "TRIPLUS" RSH autosampler and chromatograghic separation using a Thermoscientific TraceGold 15m x 0.25mm I.D. x 0.25µm film capillary column (P/N 26096-1300) was conducted. Trace 1300 GC with Acquisition Mod-GENERAL, MS transfer line temperature was 280^{o} C with an ionizing energy of 70 e.v. The ion source temperature was 250º C. Temperature program was started at 60º C (1 min); then elevated to 320º C at rate 20º C/min. The flow rate of carrier gas through the column was 1.2 ml/min. with PTV splitless mode.

2.5. Statistical analysis:

Statistical analysis of the obtained data was done using the statistical software package CoStat program **(Cohort software, 2005)** and means were compared for significance by the Student–Newman Keuls test at the probability of 0.05. Also, The LC_{50} values were calculated with Ldp line software

3.Results

3.1. Efficacy of the natural extracts

The percentage of mortality against adult female of *Icerya aegyptiaca* are present in Table (1). The results revealed that, the ethanol extract of *Citrus sinensis* peel was the most effective one with all concentrations within all times after application (LC_{50}= 1.51%). For example, concentrations 7 and 9% from this extract caused 100% mortality after only 4 days from application, for this notice this extract was chosen to GC-MS analysis. Also, within all times of the experiment the concentrations 5, 7 and 9% resulted the same percent of mortality with significant different between them followed by 9% aqueous extract of *Citrus sinensis* peel. (LC_{50} of aqueous extract = 1.96%). In addition, the concentration 2% ethanol extract from *Citrus sinensis* peel, 5% and 7% of aqueous extract of *Citrus sinensis* peel gave 73.33%, 80.00% and 92.37% respectively with no significant difference. On the other hand, acetone extract of Citrus peel had the lowest effect against adult female of *Icerya aegyptiaca* throw all the experiment, until 5 days after treatment, there was no effect of this extract on the adult female of *Icerya aegyptiaca* but at the end of the experiment the highest concentration 7 and 9% gave only 38.49% and 46.19% mortality, respectively (LC_{50}= 10.47%). As for the other peel extract (*Punica granatum*), at the end of the experiment (7days after application), the concentration 0.3% ethanolic extract and 20% aqouse extract recorded 73.33% mortality according to control without significant different. So, the ethanolic extract (LC_{50} = 0.08%) is more toxic than the aqouse extract (LC_{50} =6.6%) due to the value of LC_{50}. Also, the concentration 0.05% and 0.1% ethanolic extract gave 53.33% mortality without significant different. These result with agree with **Al-Anany, *et al.* (2017)**, who reported that, the ethanolic extract of *Punica granatum* gave 56.4±7.3% mortality but the aqouse extract gave the lowest mortality by lowest mortality % on the adult of *P. citri*. Also, **Majeed, *et al.*, (2018)**, tested the Insecticidal effect of some plant extracts on citrus mealybug, his data revealed that, least effective botanical extracts against adult female mealybugs after 48 h of exposure were ethanol extracts of *citrus aurantium* seeds (LC_{50}=144.76%) and *citrus sinensis* peels (LC_{50}=131.71%) after 72 h of exposure. Increase of percentage of mortality depend on the time after treatment, in general 7 days after treatment was the best time to kill the adult of female mealybugs. In addition, **AL-Hefny, *et al.* (2011)** reported that, petroleum ether extract of sour orange highly reduction the population of the different stages of the mealybug by 97.01% after 8 days from treatment with the assayed materials at 5% concentration. **Amin, *et al*. (2017)**, examined the effect of different solvent extract from Citrus peel against the 4th instar larvae of *Spodoptera littoralis*, the result showed relatively strong contact action, moderate repellency, weak ingestion and antifeedant activity against this insect.

3.2. GC-MS analysis

GC-MS analysis of ethanol fractions of the crude extract of Citrus sinensis:

Figure (1) and Table (2) illustrate GC-MS analysis of ethanol extract of *Citrus sinensis* which showed twenty-two peaks indicating the presence of the same number of compounds including, 4H-1-Benzopyran-4-one,2-(3,4-dimethoxyphenyl)-5,6,7-trimethoxy, which was the major compound by 17.64%, followed by its derivative 4H-1-Benzopyran-4-one, 5,6,7-trimethoxy-2-(4-methoxyphenyl) by 6.38%. The 3rd compound was the mono-terpene, Androst-4-ene-3,17-dione,12-(trimethylsilyl)oxy] -, bis (O-methyloxime), 6.26% followed by, 5-Hydroxymethylfurfural by 2.13%, followed by Vitamin- E (2.4%) and 5 Hydrxoy methylfur fural (2.13%). These data agree with the data reported by Kumar and Bhaskar, 2012 who founded, 5-Hydrxoymethylfurfural, 4H-1-Benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-5,6,7-trimethoxy, its derevatives 3,5-Dihydroxy-6-methyl-2,3-dihydro-4Hpyran-4-one and 5 Hydrxoy methylfur fural from ethanolic peel extraction of Citrus reticulate using GC-MS. In addition, the GC-MS analysis showed fatty acids, Furfural is used in agriculture/horticulture as a herbicide and it is the active as nematicides, **(Eseyin and Steele, 2015)**. Also Ricinolic acid by 1% (It is an unsaturated fatty acid) and n-Hexadecanoic acid by 0.86%(palmitic acid , saturated fatty acid),. Gibberellic acid was also reported from GC-MS analysis by 0.72% which caused perturbation in development and mortality of

Table (1): The response of adult female of mealybugs to different plant Pell extract as number of died insect

peel extract as number of died insect								
Time after application								
aqouse extract of Punica granatum								
concentration	**24hr**	**48hr**	**73hr**	**4day**	**5day**	**6day**	**7day**	
	mean	mean	mean	mean	mean	mean	mean	total mean
0.0%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.000 n
2.5%	0.00	0.00	0.00	0.00	0.00	0.00	2.67	0.381 lmn
5.0%	0.00	0.00	0.00	0.67	0.67	0.67	4.67	0.952 jk
10.0%	0.00	0.00	0.00	1.33	1.33	2.00	6.00	1.523 hi
20.0%	0.00	0.00	2.00	2.00	3.33	2.00	7.33	2.381 fg
30.0%	0.00	0.00	2.67	2.67	3.33	3.33	8.00	2.857 f
ehanolic extract of Punica granatum								
0.025%	0.00	0.00	0.00	0.00	0.00	0.00	2.00	0.286 mn
0.050%	0.00	0.00	0.00	0.00	0.00	1.33	5.33	0.952 jk
0.100%	0.00	0.00	0.67	0.67	2.67	3.33	5.33	1.810 h
0.200%	0.00	0.00	0.67	0.67	2.67	3.33	6.67	2.000 gh
0.300%	0.00	0.00	1.33	1.33	3.33	4.00	7.33	2.476 fg
Citrus sinensis aquas extract								
2.00%	0.00	0.00	0.00	0.00	0.00	3.33	4.00	1.047 hi
3.00%	0.00	0.00	0.00	0.00	0.67	4.67	6.67	1.714 h
5.00%	0.00	0.00	0.00	0.00	4.67	6.00	8.00	2.666 f
7.00%	0.00	0.00	0.00	1.33	4.67	5.33	8.00	2.761 f
9.00%	0.00	0.00	0.00	3.33	5.33	8.00	8.00	3.523 e
Citrus sinensis ethanolic extract								
2.00%	0.00	0.00	0.00	2.00	4.67	6.00	7.33	2.857 f
3.00%	0.00	0.00	0.67	4.67	8.00	8.67	8.67	4.380 d
5.00%	0.00	0.67	1.33	8.67	10.00	10.00	10.00	5.810 c
7.00%	0.00	1.33	5.33	10.00	10.00	10.00	10.00	6.666 b
9.00%	0.00	2.00	9.33	10.00	10.00	10.00	10.00	7.333 a
Citrus sinensis aceton extract								
2.00%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.000 n
3.00%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.000 n
5.00%	0.00	0.00	0.00	0.00	0.00	1.33	2.67	0.571 klm
7.00%	0.00	0.00	0.00	0.00	0.00	2.67	3.33	0.857 jkl
9.00%	0.00	0.00	0.00	0.00	0.00	3.33	4.00	1.047 hi
Total mean	0 f	0.153 f	0.923 e	1.897 d	2.897c	3.820 b	5.615 a	

LSD 0.05 for treatments = 0.492 LSD (0.05) for time = LSD 0.05 = 0.255

Spodoptera littoralis and *Locusta migratoria migratoria* insect which may be caused by antifeedant effect, **(Abdellaoui, *et al.*, 2009)**. The di carboxylic acid, and Phthalic acid, di(2-propylpentyl) ester represented by 0.64%. The rest of the compounds reported in table (2) with its percentage and retention time. It could be concluded that, the type of extraction solvent is a main factor in determining chemical composition and physicochemical properties of citrus and pomegranate peel extracts. Citrus and pomegranate peel wastes are promising as a natural biopesticide. They are sustainable, non-persistent in the environment, and relatively safe to natural enemies, non-target organisms, and human beings. As well as this study may be useful as a part of an integrated pest control program derived from biological agents and improves the use in organic farming systems. However, further studies on the mode of action and synergism with pesticides under field conditions are needed.

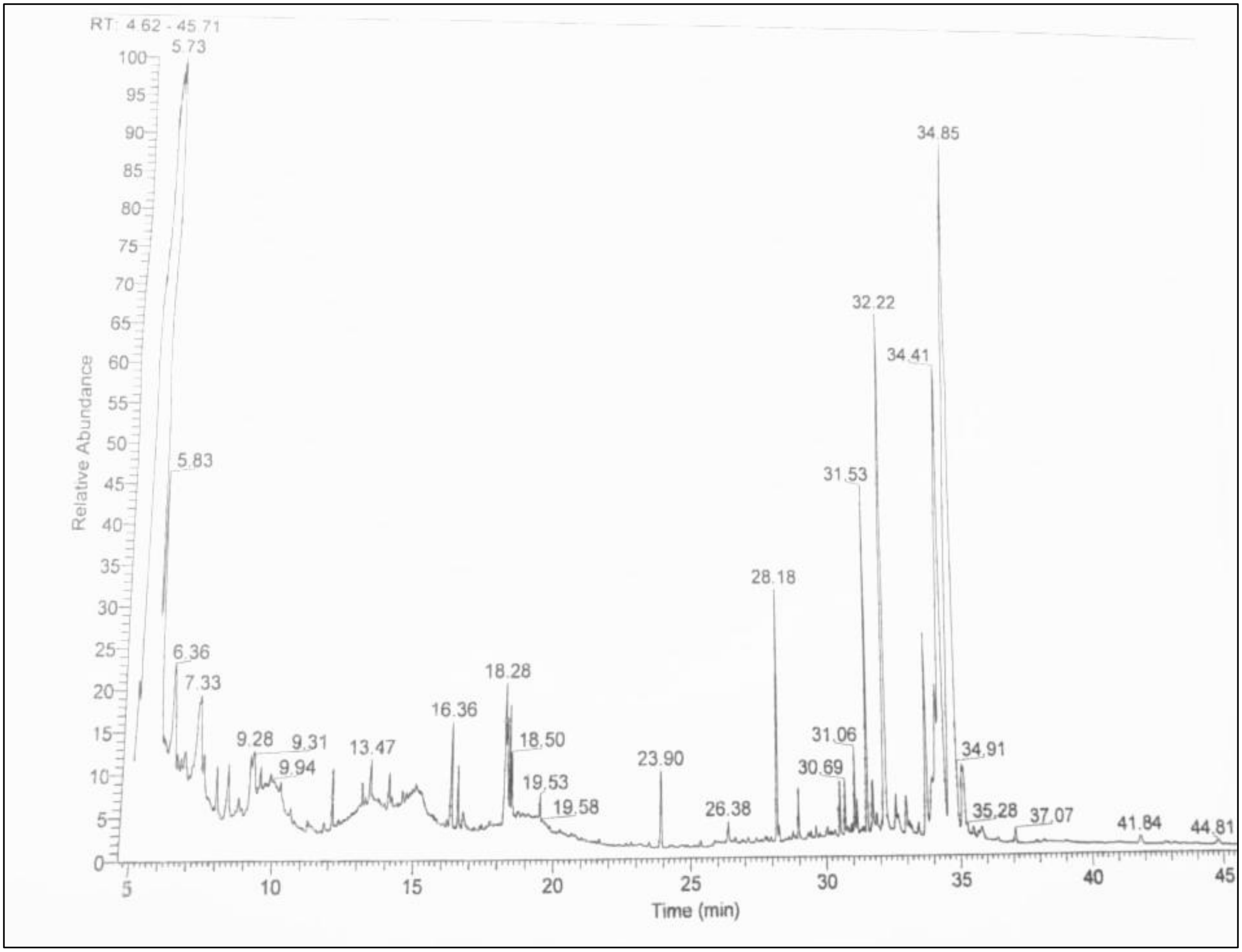

Fig. 1. GC-MS pattern of ethanol fraction of the crude extracts of *Citrus sinensis* peel.

Table (2): **GC-MS analysis of ethanol fraction of the crude extracts of *Citrus sinensis***
RT* =Retention Time

NO.	R.T*	%Area	Compound
1	5.73	2.13	5-Hydroxymethylfurfural
2	5.83	1.84	Ascaridole epoxide
3	7.33	1.41	Acetic acid, 2-(7-hydroxy-2,2-dimethylperhydro[1,3]dioxolo[4,5-c]pyran-4-yl)-, ethyl ester
4	9.31	0.72	Gibberellic acid
5	9.9	0.15	à-D-Glucopyranoside, O-à-D-glucopyranosyl-(1.fwdarw.3)D-fructofuranosyl
6	13.47	0.4	2-Bromotetradecanoic acid
7	16.36	0.86	n-Hexadecanoic acid
8	18.28	1	Ricinoleic acid
9	18.5	0.35	Ethyl iso-allocholate
10	19.53	0.16	5,8,11-Eicosatriynoic acid, tert-butyldimethylsilyl ester
11	23.9	0.64	Phthalic acid, di(2-propylpentyl) ester
12	28.18	1.53	Ethyl iso-allocholate
13	30.69	0.32	ç-Tocopherol
14	31.06	0.51	Octadecane, 3-ethyl-5-(2-ethylbutyl
15	31.53	2.4	Vitamin E
16	32.22	6.38	4H-1-Benzopyran-4-one, 5,6,7-trimethoxy-2-(4-methoxyphenyl)
17	34.41	6.26	Androst-4-ene-3,17-dione, 12-[(trimethylsilyl)oxy]-, bis(O-methyloxime)
18	34.85	17.64	4H-1-Benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-5,6,7-trimethoxy
19	35.28	0.02	Pregn-16-ene-11,14,18,20-tetrol, 3,9-epoxy-3-methoxy-, 11,20-diacetat
20	37.07	0.16	Tricyclo[20.8.0.0(7,16)]triacontane, 1(22),7(16)-diepox
21	41.84	0.11	Rhodopin
22	44.81	0.07	Prednisolone Acetate

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استخلاص والتعرف على المواد النشطة الطبيعية المستخلصة من قشور الفاكهة وتقدير مدى كفاءتها ضد البق الدقيقي المصري
Icerya aegyptiaca (Douglas)

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1- قسم بحوث سمية المبيدات للنباتات - المعمل المركزي للمبيدات - مركز البحوث الزراعية –محطه بحوث الصبحيه – الإسكندرية

2- قسم بحوث الحشرات القشرية و البق الدقيقى - معهد بحوث وقاية النبات - مركز البحوث الزراعية – محطه -بحوث الصبحيه – الإسكندرية

3- قسم بحوث الإختبارات الحيوية - المعمل المركزي للمبيدات - مركز البحوث الزراعية –محطه بحوث الصبحيه

الملخص العربي

تلعب الان المبيدات من اصل نباتي دورا هاما في الحد من الأثر البيئي السلبي لاستخدام المبيدات الكيمائيه ، لذلك تهدف الدراسة الحالية إلى اختبار التأثير الابادى الحشري لخمسة مستخلصات من قشور نوعين من الفاكهة وهما البرتقال الحلو (*Citrus sinensis*) والرمان (*Punica granatum*) ضد البق الدقيقي المصري (*Icerya aegyptiaca* (Douglas)) البالغ الضرر على أشجار الفاكهه واشجار الزينه وكذلك التعرف على المركبات الموجوده في مستخلص الايثانول للبرتقال الحلو باستخدام تقنيه ال GC-MS . وقد أظهرت النتائج ان مستخلص الإيثانول من قشور البرتقال هو المستخلص الأسرع والاكثر فاعلية مع جميع التراكيزات خلال التجربه . فعلي سبيل المثال فان التركيزان ٧ و ٩٪ من هذا المستخلص أعطى نسبه موت ١٠٠٪ من الافه المستهدفة بعد اربعه أيام من المعامله (الأسرع في قتل الحشره وهذا سبب اختياره لعمل تحليل GC-MS). بينما كان مستخلص الاسيتون هو الأقل فاعليه في قتل الحشره. أما عن مستخلص قشر الرمان، فقد كان مستخلص الإيثانول هو الأكثر فاعليه وذلك في نهايه التجربه بعد سبعه أيام من المعامله حيث اعطى التركيز ٠,٣% من مستخلص الايثانول وكذلك تركيز ٢٠% من المستخلص المائي نسبه موت تساوي ٧٣,٣٣% بدون فرق معنوى وعل هذا يكون مستخلص الايثانول لقشر الرمان هو الأفضل بناءا على قيم ال LC_{50} والتي كانت تساوي ٠,٠٨% لمستخلص الايثانول بينما أعطت ٦,٦% للمستخلص المائي. وقد اظهر تحليل GC-MS لمستخلص الايثانول لقشور البرتقال والذى كان الأسرع فاعليه وجود 4H-1-Benzopyran-4-one,2- (3,4-dimethoxyphenyl) - 5,6,7-trimethoxy, بنسبه تواجد في العينه 17.64%, يليه مشتقه 4H-1-Benzopyran-4-one,5,6,7-trimethoxy-2-(4-methoxyphenyl بنسبه تواجد ٦,٣٨%. أيضا تم التعرف على وجود أحماض دهنية مشبعة (حمض هيكساديكانويك) وغير مشبعة (حمض الريسينوليك). كما تم اكتشاف مشتقات الفيورال وحمض الجبريليك.