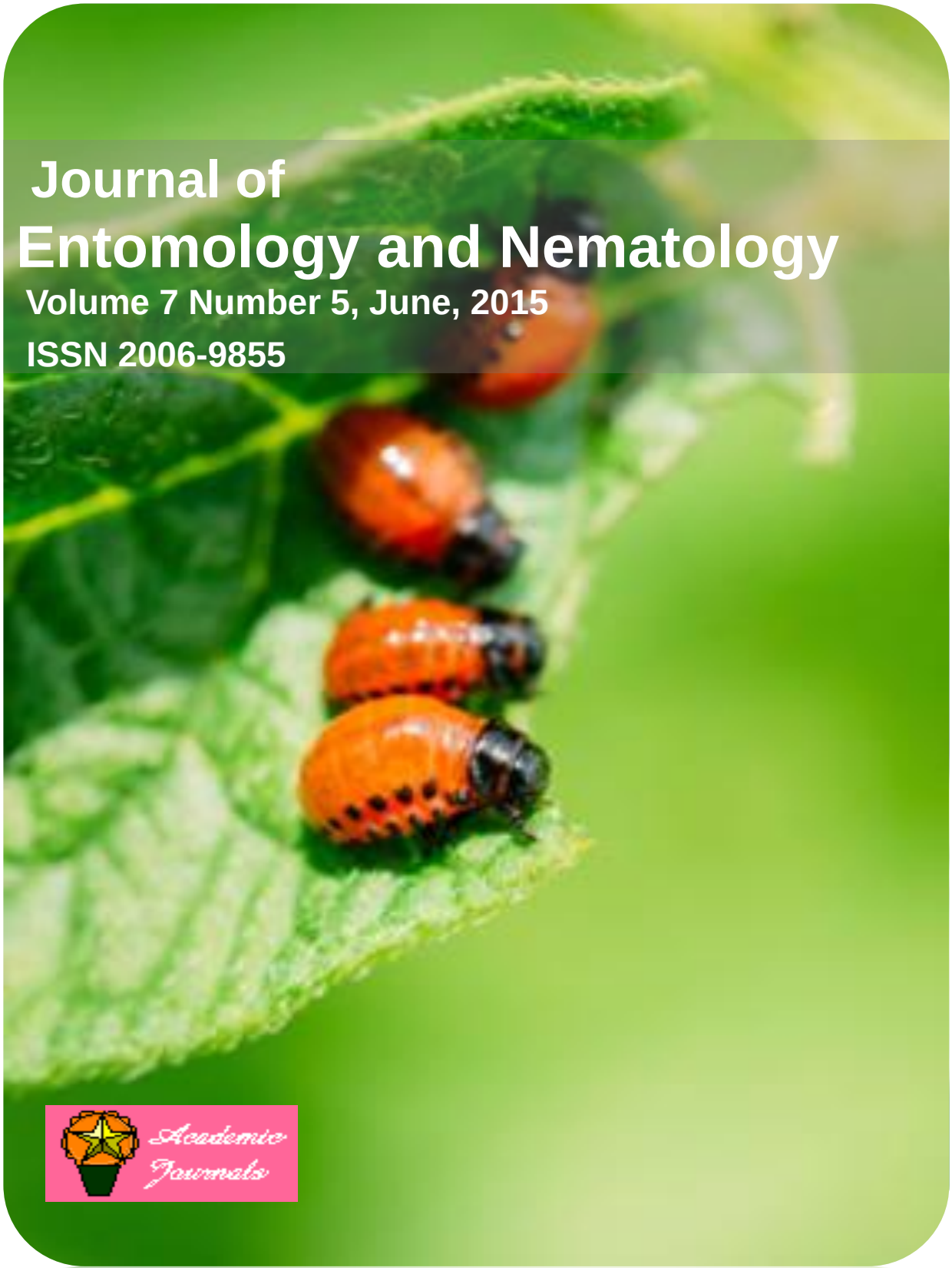

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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

Haemocytes and protein changes in *Schistocerca gregaria* after infection with nucleopolyhedrovirus

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Innate immune response in insects is essential to resist the infections of pathogens. The present study investigates the antiviral immune response against intracellular baculovirus infection in the desert locust, *Schistocerca gregaria*. The desert locust was injected with nuclear polyhedral virus isolated from *Spodoptera littoralis* larvae collected from cotton and maize field in Giza governorates (*Sl*NPV). Haemolymph was perfused 24 h post injection. The hemolymph was taken from control and treated locusts to separate haemocytes from the supernatant plasma to characterize the reaction of the adult *S. gregaria* to the viral infection. Transmission electron microscope revealed that, infected granular plasmtocyte and prohaemocytes possessed phagocytic vacuoles. However, the haemocytes behavior against viral infections does not seem to be consistent. Conversely, the biochemical results demonstrated that the total number of protein bands of *S. gregaria* increased due to treatment with *Sl*NPV 24 h post injection. Analysis of haemolymph protein pattern of control (untreated adult) of *S. gregaria* detected 11 protein bands and 14 protein bands in treated haemolymph pattern. Three common protein bands between untreated and treated haemolymph protein patterns (15.7, 22 and 25 kDa), one protein band (kDa) was increased in its amount percentage due to treatment course 1.4 fold times, and two common protein bands with molecular weight (22 and 25 kDa) were decreased in amount percentage due to treatment course from by 2.4 and 1.04 folds, respectively. The present study provides the basis for supplementary studies of molecular mechanisms underlying baculovirus strategies that have evolved for the suppression or evasion of the antiviral immunity in infected cells.

Key words: Immunity, nuclear polyhedrosis virus, grasshopper.

INTRODUCTION

Insects get infected by a wide array of viruses some of which may be pathogenic to insects or transmitted to vertebrates by insects. The medical and economic importance of virus induced diseases highlights the need to understand the interaction between the pathogenic virus and the insect immune system. The study of insect defense mechanisms is of great practical importance in two contexts. First, the need to assess the

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ways by which insects might avoid destruction by biological control agents. Second, defense mechanisms play a critical but underestimated role in the complex relationships that exist between arthropod pests and its pathogens. A better understanding of these mechanisms may lead to methods for manipulating them to human advantage.

Nuclear polyhedrosis virus is a circular double stranded DNA virus belonging to the family baculoviridae. Baculoviruses, members of the family baculoviridae, include a diverse group of large enveloped DNA viruses that are specifically pathogenic to arthropods, including insects. Baculoviruses possess a large circular double-stranded DNA of approximately 80-180 kbp in size, which encoded 100-200 predicted proteins (Herniou et al., 2012).

Individual baculoviruses act together with different insect cell lines after entry into cells causing numerous types of abortive (non-productive) as well as productive infections (Castro et al., 1997; Morris and Miller, 1993; Shirata et al., 1999, 2004, 2010). The route of infection of baculovirus consists of several successive steps, starting from entry of the viruses into cells, morphogenesis and discharge of budded virus (BV), very late gene expression, until ending with the formation of occlusion bodies (OBs) which contain blocked virus (OV) which is a different phenotype from that of BV (Morris and Miller, 1993; Shirata et al., 1999).

Once pathogens break a surface barrier and gain the entry into insects, the second line of innate immunity at the cellular and molecular levels is activated in the haemocoel. When the immune response be successful, the pathogens were eliminated from the infected insects and prevented their propagation and spread. In contrast, pathogens have developed a variety of mechanisms to suppress or evade the activated innate immunity in the haemocoel. Since insects lack adaptive immunity as in vertebrates, innate immunity is essential for insects to survive infections of pathogens, including viruses. The cellular immune reaction of insects involves the recognition and subsequent phagocytosis or encapsulation of foreign bodies by the haemocytes (Salt, 1970). In case of cells infected with baculovirus, shutdown of global protein synthesis and apoptosis act as two major intracellular innate antiviral mechanisms (Clarke and Clem, 2003a; Clem, 2005; Thiem, 2009). Thus, baculoviruses and insects provide a good model system for studying the co- evolution of animals and their pathogens.

Desert locusts, *Schistocerca gregaria* (Forskal), are the most dangerous of locust species. Under favorable environmental conditions, a few solitary individuals can dramatically multiply, form large swarms able to migrate great distances, and therefore, threaten agriculture (Cressman, 1999). Most studies on insect innate immunity have focused on bacterial and fungal infections (Ferrandon et al., 2007; Kanost et al., 2004; Lemaitre and Hoffmann, 2007).

Nuclear polyhedrosis viruses are considered to be limited in their host range to one species or genus of insects. The narrow host range that characterizes the baculoviruses is regarded as a great advantage over chemical pesticides (Bensimon et al., 1987). They succeeded to transmit *Spodoptera littoralis* nucleopolyhedrovirus to infect *S. gregaria* with dark cheeks disease.

The challenge in the future lies in the implementation of control strategies that protect food security with minimal environmental costs. Therefore, in the current investigation, we address the antiviral immunity in baculovirus-infected desert locust, *S. gregaria*, and determine the humoral antiviral immune response to provide a basis for further molecular mechanisms underlying baculovirus strategies that have evolved for the suppression or evasion of the antiviral immunity in infected cells.

MATERIALS AND METHODS

Colonization of *S. gregaria*

The desert locust *S. gregaria*, was obtained from the Locust and Grasshopper Research Department, Plant Protection Research Institute, Agricultural Research Center, Egypt. Locusts were reared in wooden cages at 32 ± 2°C, 50- 60% relative humidity RH) and 16 h day light in the facilities of Entomology Department, Ain Shams University. The locusts were fed on daily fresh clover plant, *Trifolium repens* on daily bases. Packed moist sterilized sand in suitable glass containers about 7 cm in diameter and 10 cm deep were used for egg-laying.

Virus preparation, injection and sampling haemolymph

Nuclear polyhedral virus isolate was obtained from *S. littoralis* larvae collected from cotton and maize field in Giza governorates. The larvae were fed on castor bean leaf (*Ricinus communis*), after the symptoms of the viral infection appeared in the infected larvae were placed into 1.5 ml 0.1% Sodium dodecyl sulphate (SDS) to homogenize; the homogenate was centrifuged for 100 *g* for 5-10 s and the supernatant was removed into clean tube to centrifuge at 2500 *g* for 5 min. To pellet the virus, the supernatant was discarded, and resuspended in 1.5 ml of distilled water and centrifuged at 2500 – 5000 *g* for 5 min. Finally, the supernatant was discarded and the pellet was suspended into a small volume of distilled water. The concentration of the virus was measured by counting under hemocytometer and stored at -20°C until use.

Stimulation to insect immune response

Injections and sampling of haemolymph

Trials were prepared by injecting each adult grasshopper with 10 µl of 10^7 PIB/ml of SlNPV final concentration using a 10 µl of Hamilton micro-syringe (Miranpuri and Khachatourians, 1993). Locusts were injected with 10 µl viral suspension into the insect's haemocoel by inserting the needle in the last coxal corium. Control insects were injected only with equivalent volume of sterile distilled

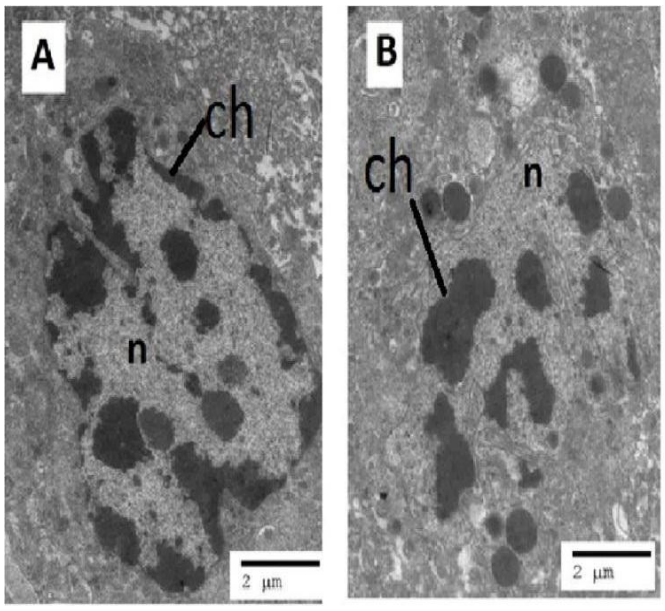

Figure 1 (a, b) . Normal haemocytes of adult *S. gregaria*, showing nucleus (n) with chromatin (ch).

water. Samples of haemolymph were taken from locusts without cooling or anesthesia at 24 h post-injection. The insects were amputated at the hind coax with fine scissors; gentile pressure was applied on the thorax until the haemolymph appeared and obtained with fine–tipped calibrated glass capillary, according to Hoffmann (1980). The perfused haemolymph was centrifuged at 12000 rpm for 15 min and the plasma stored at -20°C to separate haemocytes from the supernatant plasma for using in cellular and molecular study.

Transmission electron microscopy (TEM)

To characterize the cellular antiviral immune response in infected adult *S. gregaria*, intra- thoracic haemolymph was obtained from 5-10 locusts via micropipette.

The haemocytes pellets were separated from plasma. Five drops of 3% gluteraldehyde was added to the haemocytes for 2 h, during which a precipitate was formed. The supernatant was eliminated from the samples, which were washed with 5 drops of PBS-sucrose three times for 10 min. Samples were further fixed with 2% osmium tetroxide for 3h, after which they were washed three times with PBS-sucrose. Samples were then gradually dehydrated with increasing concentrations of alcohol: 10 to 90% for 10 min. Finally, for 30 min in each sample of three changes of 100% alcohol. After this, 100% alcohol and propylene oxide were added in proportions of 2:1, 1:1, and 1:2, finalized with 100% propylene oxide; each stage of these changes in proportion lasted 30 min. Epon 812 propylene was added in proportions of 2:1, 1:1, and 1:2, with changes in proportions every 30 min. With 100% Epon 812, the samples were left open for 4 h to evaporate the solvent. The samples were then polymerized at 60ºC for 24 h. From each polymerized block, semi-fine sections were made using Reichert Om U3 ultra-microtome and transferred to pioloform-treated 200-mesh grids. The sections were examined under SEO PEM-100TEM. Cell types were photographed with transmission electron microscope (TEM) at the facility of Military Medical Academy, Cairo, Egypt.

Protein electrophoresis

To determine the humoral antiviral immune response, the hemolymph of the normal and infected adult locusts was perfused. SDS PAGE gel electrophoresis was used by adding droplets to a tube containing 20 ml of gel buffer [2% sodium dodycel sulphate (SDS), 60 mM Tris Cl pH 6.8, 10% glycerol and 50% protease inhibitor cocktail (one tablet/50 ml, Boehringer Mannheim, Indianapolis, IN)]. The perfused hemolymph was resolved in SDS polyacrylamide gel electrophoresis (SDS-PAGE). SDS PAGE was performed at 200 V, for approximately 3 h. Gel was stained using Brilliant Blue R-250 and followed by distaining process. Images of the gel were captured and analyzed using specialized software; Gel-Pro Analyzer (Media Cybernetics, NT, USA).

RESULTS

Transmission electron microscopy (TEM)

The reaction of the adult *S. gregaria* to the viral infection was characterized by injecting insects with 10 µl of 10^7 PIB/ml of Giza *S*lNPV, and perfuses the hemolymph 24 h later. Ultrathin sections cut through normal and infected haemocytes adult locust revealed a significant changes in the blood cells compared to the normal (Figures 1 and 2). In the normal haemocytes, the nucleus occupies a large size of the whole cell with definite patches of chromatins (Figures 1). However, after 24 h post injection, the haemocytes displayed a phagocytic activity with being able to internalize virus particles inside cytoplasmic vacuoles, particularly in virus-phagocytosing cells (Figure 2b, 2c). Also, some of the infected haemocytes showed an extended protrusion filled with virus particles inside the cytoplasmic vacuoles, seemingly (Figure 2).

Protein electrophoresis

The analysis of protein content extracted from hemolymph of the adult *S. gregaria* revealed an increase in the total number of protein bands in the infected hemolymph when compared with control (Figure 3). Analysis of hemolymph protein pattern of control detected 11 protein bands of size which ranged from 11.5 to 155.6 kDa. However, 14 protein bands were detected in the infected hemolymph that ranged from 13.4 to 96.4 kDa. The new protein bands of infected in comparison to control were 11 protein bands with different amount percentage as shown in Table 1. In addition, eight protein bands disappeared due to treatment with *S*lNPV (11.5, 45.4, 57, 62.5, 69, 82.9, 105.3, and 155.6 kDa) .We observed three common protein bands between control and treated specimens with 10^7 µl of Giza *S*lNPV, one of them (15.7 kDa) showed an increase in the amount percentage by 1.4 fold from 16.9 to 24 due to treatment course. The other two common protein bands with molecular weight (22 and 25 kDa) decreased in amount percentage (from

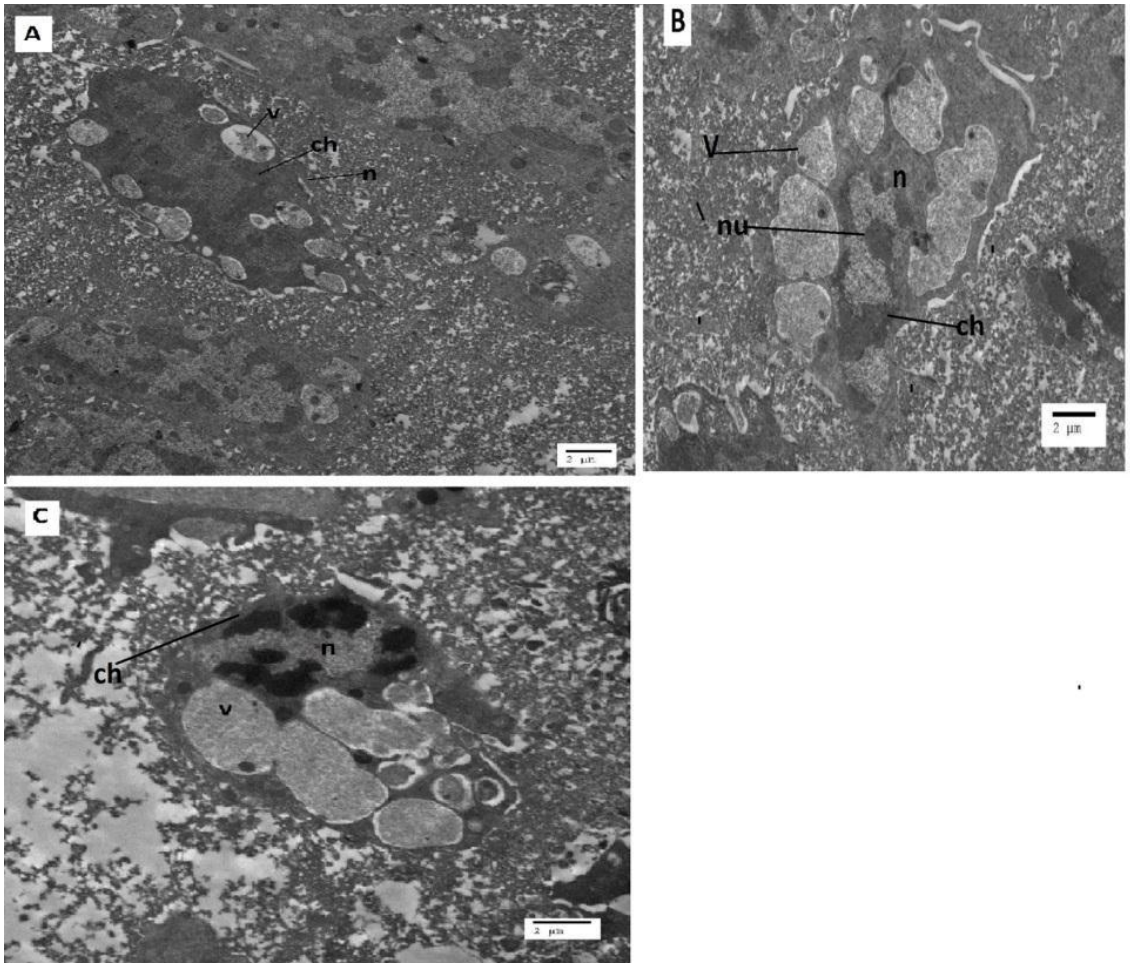

Figure 2 (A, B and C). Haemocytes showing cytoplasmic vacuoles (V). ch =chromatin; n= nucleus; nu= nucleolus.

6.4 to 2.6 amount %, and from 4.9 to 4.7 amount %) by 2.4 and 1.04 folds, respectively.

DISCUSSION

Little is known about insect defense against virus infection; neither cell-mediated nor humoral immunity has been demonstrated against virus infection in insects (Narayanan, 1997).

Recently, advances have been made which demonstrate viral resistance and the ability of an insect to clear viral pathogens (Briese, 1986).

In our work, the haemocytes behavior against viral infections does not seem to be consistent. The hemocytes can have different roles, from actively spreading the baculoviruses, via avoiding being infected, to actively clearing the haemolymph from the viruses. The first description of an effective immune response in insects against viral infection was a study on *Helicoverpa zea* (Lepidoptera: Noctuidae) infected with NPV (Washburn et al., 1996). The haemocytes appeared in aggregation form around midgut-associated tracheae infected by baculovirus. The baculovirus-infected cells were then encapsulated by haemocytes and thereafter removed from the haemocoel.

At the molecular level, there are many studies on the change in the electrophoretic pattern of haemolymph proteins of larvae infected with the nuclear polyhedrosis virus (Watanabe, 1986). In some unsuccessful infections with baculovirus, it is clear that both cellular and viral protein synthesis are dramatically reduced and completely blocked in late infection (Ikeda et al., 2012). This explanation may give a reason for not appearance of which may be the reason of reduction of total protein however, the observed increase in total protein at 24 hpost infection could be due to the interference of the viral protein with the protein of the infected cells. Also, post- infection duration could be not enough shutdown global protein synthesis and may need increased time after the infection 48 or 72 h. The mechanism of shutdown of

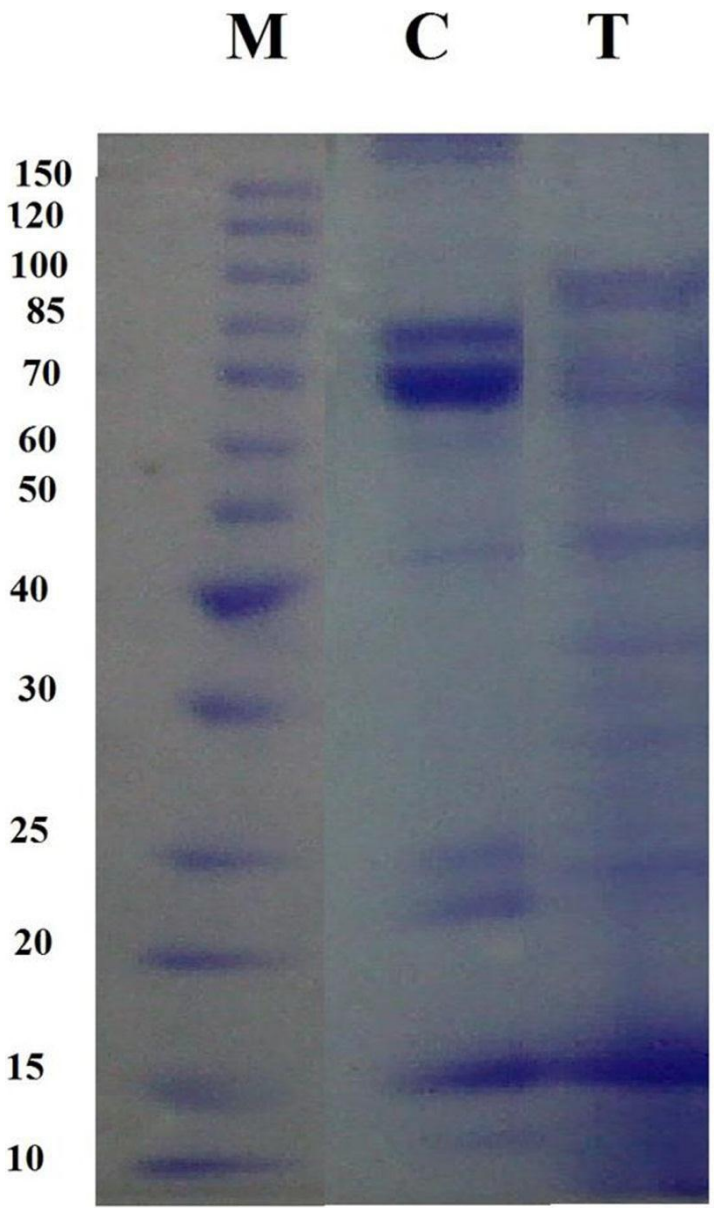

Figure 3. Photograph illustration of electrophoretic hemolymph protein patterns of infected and control adults of *S. gregaria.* M = protein marker (kDa). Lane C= control. Lanes T= Haemolymph protein patterns after infection with 10 µl of 10^7 of *Sl*NPV.

global protein synthesis is generally observed in the late infection with baculovirus and could be distinguished from the shutdown of the host cell protein synthesis (Schultz and Friesen, 2009; Schultz et al., 2009; Xue et al., 2012) as found in *Lymantria dispar* cell line infected with *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV) in which the protein synthesis is globally shut down at late stages of infection.

Conversely, Sarma et al. (1994) found an observable decrease in protein content in NPV infected silkworm larvae, called “hypoproteinemia”. The authors attributed the reduction of hemolymph protein to the decrease of protein synthesis. Finally, the incompatibility between cellular response and protein analysis in our study could be related to that abortive infection which included in virus replication or from activation of innate antiviral responses, and leads to apoptosis and global protein synthesis shutdown in infected cells (Ikeda et al., 2013).

Finally, it could be concluded that this work is considered as a preliminary trial to predict cellular and molecular mechanisms of innate immune response of one of the most dangerous species insect, locusts, towards baculovirus infection. Better understanding of

Table 1. Monitoring, amount percentage and relative fragmentation of haemolymph protein fractions of infected and control adults of *S. gregaria* expressed as molecular weight.

Lane	Marker (mol.w.)	Lane C (mol.w.)	Lane T (mol.w.)	Lane C	Lane T
				Amount %	Amount %
1	150	155.6		0.8	
2	120				
3	100	105.3	96.4	0.68	3.1
4	85	82.9	91.3	9	1.9
5	70	69	73.4	22.4	3.7
6		62.5	67.1	2.3	4.1
7	60	57		0.9	
8					
9	50	45.4	46.9	2.5	3.1
10			43.3		0.8
11	40		35.5		2.3
12			31.5		2.3
13	30		29		4.1
14			25.9		3.1
15	25	25	25	4.9	4.7
16		22	22	6.4	2.65
17	20				
18					
19	15	15.7	15.7	16.9	24.1
20			13.4		6.5
21	10	11.5		8.4	
Sum	71.9			75.6	67.15
In lane	100			100	100

M= Protein marker (kDa). Lane C= control. Lanes T = treated hemolymph protein patterns with 10 µl of 10^7 of *Sl*NPV

humoral defense mechanism and molecular basis against viruses will provide necessary information for better management and modify host range properties of insect viruses in the future.

Conflict of interests

The authors did not declare any conflict of interest.

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Bioactivity of fractionated indigenous medicinal plant extracts of *Phlomis damascena* Born. and *Ranunculus myosuroides* against the cotton whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae)

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Two bioactive methanol (MeOH) extracts of two indigenous medicinal plant species *Phlomis damascena* Born. and *Ranunculus myosuroides* Boiss. & Kotschy were tested for insecticidal activity of their fractions against the cotton whitefly *Bemisia tabaci* (Gennadius) adults under controlled conditions. This study is within bio-prospection context, in form of utilizing local plant species as an alternative in sustainable agriculture development. Organic solvent extraction of the two bioactive crude extracts yielded four fractions each: ethyl acetate fraction, chloroform ($CHCl_3$) – water fraction, $CHCl_3$-methanol (MeOH) fraction of the acid basic layer and MeOH fraction of the aqueous basic layer. The two extracts and their $CHCl_3$-MeOH fractions caused significant decrease in number of live adult whiteflies compared to the control. Fractions of the bioactive $CHCl_3$-MeOH of *P. damascena* were collected and isolated by combinations of repeated chromatography including silica gel chromatography and Thin Layer Chromatography (TLC). The most bioactive fraction was further purified using silica gel column eluted with a gradient of $CHCl_3$: MeOH (9:1) with increasing volume of methanol till total elution; the 5 eluted sub-fractions were analyzed by TLC. The 4th isolated sub-fraction having R_f of 0.41 was eluted with $CHCl_3$-MeOH (9:2) and was found not significantly different in its effect from the bioactive $CHCl_3$-MeOH fraction. This is the first report for the effect on survival of insects for fractions of these two medicinal plant species in comparison to their raw extracts. Thus, the crude extracts and their chemical fractions contribute to the development of insecticidal products based on these plant species and their bioactive chemical components.

Key words: *Bemisia tabaci*, whitefly, plant extract, botanical, endemic species, *Phlomis damascena*, *Ranunculus myosuroides.*

INTRODUCTION

The cotton whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) is polyphagous in nature (Greathead, 1986; Cock, 1986). Repeated spray applications against *B. tabaci* on different crops have

been necessary and often resulted in overuse of these chemicals. Consequently, this pest has developed resistance to numerous conventional insecticides worldwide (Dittrich and Ernst, 1990; Gerling and Kravchenko, 1995; Palumbo et al., 2001). The severity of this pest and other economic pests have increased the need for effective, biodegradable pesticides with greater selectivity, and alternative strategies that include the search for new types of insecticides and the use of traditional botanical pest control agents or their fractionated bioactive components.

Many plants have developed chemical defenses to withstand attacks of herbivores; some plants may be cultivated to provide sources of biodegradable pesticides (Farombi, 2003). The structurally diverse natural compounds of bioactive plant extracts might act as repellents, deterrents, antifeedants, growth inhibitors, and toxins. Lately, consumer awareness for natural over synthetic pesticides is growing. Many indigenous medicinal plants are known in the world as sources of drugs or herbal extracts for various chemotherapeutic purposes (Purbrick, 1998; Farombi, 2004). These plants can be used for other purposes as acaricides, fungicides, rodenticides or insecticides (Mishra et al., 2013). However, there is a need to organize the natural resources of these endemic plants, develop quality control, adopt standardi-zation strategies and modify regulatory mechanisms for use as botanical pesticides (Koul and Walia, 2009).

A few studies have dealt with the use of fractionated medicinal plant extracts or their components as potential pesticides against whiteflies. Two bioactive compounds from the Chilean plant *Calceolaria andina* (Scrophulariaceae), related to the familiar garden 'slipper' plant, have been identified as hydroxynapthoquinone and its acetate, which are effective against a range of commercially important pests including the tobacco whitefly, *Bemisia tabaci*, aphids and the two-spotted spider mite, *Tetranychus urticae* (Khambay et al., 1999). Certain compounds in the plant genus Agalia were found to be effective against a range of resistant insect strains including the B-biotype of the tobacco whitefly, *B. tabaci* (Koul and Walia, 2009). Yadegari et al. (2013) studied the effect of four plant extracts and essential oils of thyme *Thymus vulgaris* L., yarrow *Achillea millefoliun* L., lavender *Lavandula angustifolia* Mill and fennel *Foeniculum vulgare* Mill against eggs and nymphs of the whitefly *Trialeurodes vaporariorum* Westwood. Their results show that there were significant differences between extracts and essential oils of these plants. The best ovicidal extracts were obtained from thyme and the most nymphicidal effectiveness was with essential oils from fennel. They also found that the essential oils of fennel and lavender were strongest and weakest, respectively than other tested essential oils of thyme and yarrow, against the whitefly eggs.

The main objective of our study was to determine the bioactivity of chemical fractions of 2 botanical raw extracts that were found to have significant repellent effect against the adult whitefly *B. tabaci* in a previous study (Hammad et al., 2014). Another objective was the initial determination of the chemical characterization of the bioactive fractions in only one of these bioactive plant extracts.

MATERIALS AND METHODS

All plant extraction and phytochemical procedures were performed at the Department of Chemistry in the Faculty of Arts and Sciences, American University of Beirut (AUB), Lebanon, at room temperature of 18-23°C. Insect rearing and all bioassays with whiteflies were performed under controlled conditions inside a glasshouse at the Faculty of Agricultural and Food Sciences, AUB, at 25 ± 2°C, R.H. of 80 ± 10 % and photoperiod of 16:8 (L:D).

Plant extract preparation

Plant selection, collection and extraction were performed according to a procedure set for testing different bioactivities of plant extracts at the Center of Initiative for Biodiversity Studies in Arid Regions (IBSAR) located at the premises of the American University of Beirut.

Plant material

In our previous study *(*Hammad et al., 2014), 41 extracts of 28 medicinal plant species belonging to 10 botanical families, endemic to Lebanon, were selected and tested against the whitefly *B. tabaci*; five plant extracts out of the 41 extracts showed significant repellency against the adult *B. tabaci* and out of these five extracts only two extracts belonging to the plant species, *Phlomis damascena* Born. (Family Lamiaceae) and *Ranunculus myosuroides* Boiss. & Kotschy (Family Ranunculaceae), are used in the current study. The latter plant species were collected locally from 2 locations Mahmeit Baalabeck and Hasroun, respectively.

Extraction method of plant material

Harvested plants were washed with distilled water to remove any contaminants on the surface of plant parts and were dried in the shade at a temperature of 25-32ºC and R.H. of 50-60% with adequate ventilation for 2 weeks. Due to low availability of the two plant species *P. damascena* and *R. myosuroides*, the whole plant (wp) sample (including leaves, flowers and stems combined), were ground into very small particles (0.3 mm in diam.) by using a grinder (SM 100 Cutting Mill, Brinkman, Germany) at a speed of 1600 rpm at 60 HZ.

Powdered ground material (100 g) of each plant sp. were soaked in methanol for 16 h and placed in a shaker-incubator for the first 2 h at (Harborne, 1998). The methanol solvent was used to extract

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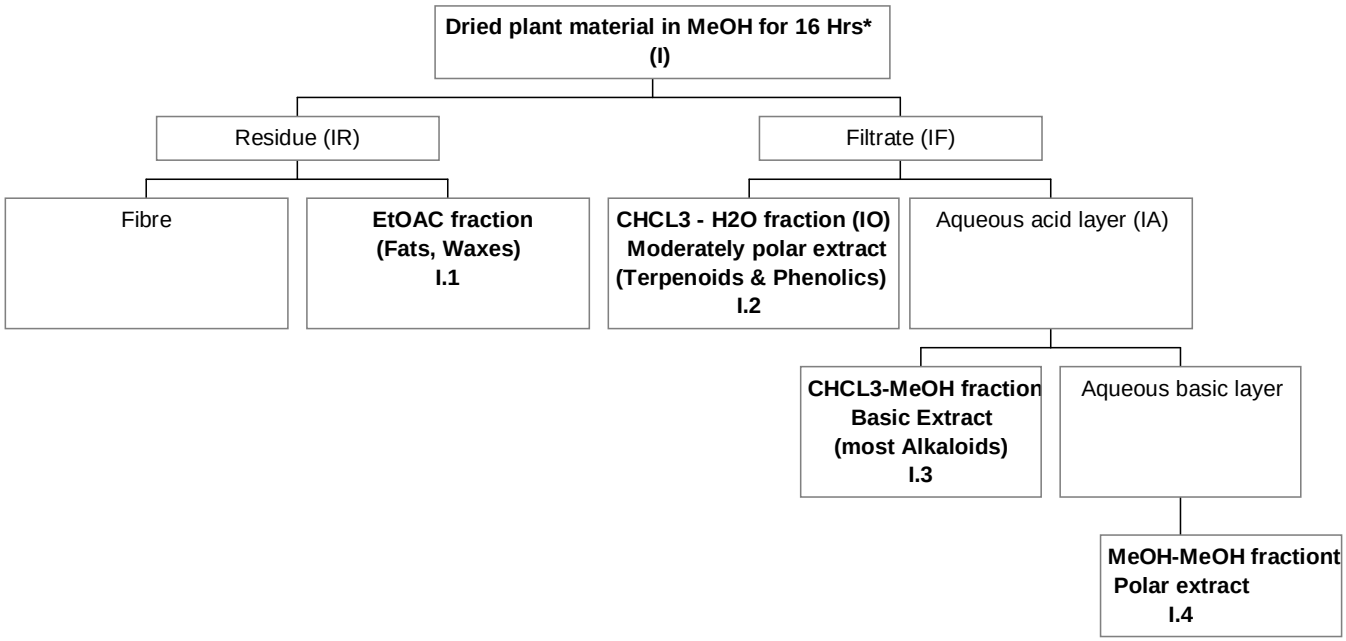

Figure 1. Schematic diagram of organic solvent extraction procedure for a bioactive crude extract. *: (I) = initial extract; IR = initial extract residue; IF = initial extract filtrate; IO = initial extract organic layer; IA = initial extract aqueous layer.

most of the semi-polar and polar constituents. This pure MeOH extraction was performed using the standard plant material / solvent concentration of 1:10 (w: v). The extract was filtered by vacuum pressure through several layers of sterile cheesecloth. Filtrate of each crude extract was stored in 5-10 ml glass vials wrapped with aluminum foil at -20ºC for use in the bioassays and for further phytochemical analysis.

Fractionation of the bioactive plant extracts

Fractions of the two (wp) extracts, *P. damascena* (*Pd*) and *R. myosuroides* (*Rm*) that were selected for their repellent bioactivity against the adult whitefly (Hammad et al., 2014), were collected and isolated according to the schematic procedure (Figure 1).

Chemical and chromatographic materials.

Silica gel (0.035–0.07 mm, 6 nm pore diameter; Acros organics, USA) was used as the stationary phase in the Column Chromatography (CC) analysis. The column used for CC analysis was 2.5 cm ID x 60 cm L (Chromaflex®, Kontes, USA). Thin Layer Chromatography (TLC) glass plates (20×20 cm) precoated with 250 µm layer of Silica gel (Analtech, Uniplate No. 02521, Alltech, Lebanon) were used. The eluting pure solvents in TLC and CC separations were chloroform, methanol and ethyl acetate (CMC, Germany).

Organic solvent extraction of the bioactive crude extracts

Each crude extract was fractionated with different organic solvents (Figure 1). The crude methanol extract (I) was kept at −20°C for 24 h in order to remove the fats from the extract, followed by filtration using a cloth sheet filter and a vacuum pump (Erickson, 1991). The residue (IR) was soaked in ethyl acetate (EtOAc) and then separated by filtration into a residue of fiber (polysaccharide) and a filtrate containing mainly fats and waxes, the filtrate is EtOAc extract (I.1). The filtrate (IF) was acidified to pH 2 using 5M sulfuric acid (H_2SO_4) and then extracted with a mixture of chloroform ($CHCl_3$) and water (H_2O) (2:1) to remove compounds of high polarity. The semi-polar compounds dissolve in $CHCl_3$ whereas polar compounds will remain in the aqueous layer. Both the aqueous (IA) and organic (IO) phases were separated using a separatory funnel. The IA layer is basified to pH 10, by adding ammonium hydroxide (NH_4OH) stepwise to increasingly higher pH, and then extracted with a $CHCl_3$ and MeOH mixture (3:1) to extract alkaloids. Two layers will develop, the $CHCl_3$-MeOH layer (I.3) and the aqueous layer which is again extracted with MeOH to get (I.4). All four fractions of each extract were tested for their bioactivity and the most bioactive fraction was further analyzed chromatographically.

Phytochemical analysis of the bioactive fraction

The $CHCl_3$-MeOH fraction (I.3) of *P. damascena* was found to be the most bioactive compared to the different fractions (I.1, I.2 and I.4) of both plant extracts. The (I.3) fraction was collected in 10 ml vials; each vial was concentrated into 1 ml volume for further use in CC and TLC analysis. The solvents chosen during these analyses were chloroform and methanol. The mobile phase, chloroform-methanol (9:1) was selected after trying several types and combination of different solvents; about 32 different combinations of organic solvents, starting from pure solvents of medium elution power to several combinations between solvents of different polarity (Hahn-Deinstrop, 2000); the choice of technique of separation depends largely on the solubility properties of compounds to be separated. Three TLC plates were tested for each solvent system for precision purposes. The mobile phase is optimized using different solvent combinations tested on silica-TLC plates. The best mobile phase was found to be $CHCl_3$: MeOH (9:1).

CC analysis

CC is a valuable technique for purification of synthetic or natural products. The $CHCL_3$-MeOH fraction of *P. damascena* was separated by CC as well as by TLC, through the same mechanism, by distribution between two phases. One hundred grams of silica gel was used as the stationary phase. A glass column was uniformly packed with slurry of silica gel (45 cm) in the mobile phase solvent system (chloroform-methanol 9:1) and other solvent systems of higher methanol concentration at advanced phases of CC. Thus, the most bioactive fraction material was purified using this silica gel column successively eluted with a stepwise gradient of chloroform: methanol (9:1) with increasing volume of methanol till total elution; a total volume of 4L of $CHCl_3$ and 2L of MeOH were used. The $CHCL_3$-MeOH fraction (0.5 g), originating from crude extract of *P. damascena* (100 g) was dissolved in 10 ml mobile phase (chloroform-methanol 9:1) and was gently applied with a pipette as concentrated band to the top of the column after the mobile phase has been drained until 1 cm above the bed surface. The mobile phase was applied on the top of the column and continuous elution started; volume of each solvent used was 4 and 2 L for chloroform and methanol, respectively. The column was developed by allowing the mobile phase to pass through the silica at a flow rate of ~ 1- 3 ml / min. As different organic compounds elute through the column they were collected in test tubes (20 ml) for further TLC analysis.

TLC analysis

TLC was employed in this study to isolate the compounds present in the most bioactive $CHCl_3$-MeOH fraction (I.3) of *P. damascena*. Silica gel was chosen as stationary phase, as it is an efficient adsorbent for the TLC separation of most plant extracts (Barbetti et al., 1987; Houghton et al., 1996; Wagner and Bladt, 1996). Aliquots of 15-20 µl of the isolated fractions collected by CC were applied as separate spots on a TLC plate about 1.5 cm from the edge (spotting line), using 25 µl Hamilton precision syringes (Hague-Holland). After sample application, the plate was placed vertically into a solvent vapor saturated TLC chamber (12 x 15 x 10 cm). The mobile phase used was chloroform-methanol (9:1) and the spotting line was about 0.5 cm from the developing solution. After the mobile phase had moved about 80% from the spotting line, the plate was removed from the developing chamber and air dried in a fume hood to visualize spots within 1-2 min. on the plate. The migrated spots on TLC plates representing various fractions / compounds were visualized with UV lamp at UV–254 nm. The collected CC eluted material that showed at TLC plates a common pattern for the fractionated compounds were combined, concentrated and tested for their bioactivity against adult whiteflies on leaves of plants. Thus, similar TLC pattern allowed pooling of eluted material into several potent fractions that were isolated and corresponded to bands detected on the bioactive fraction TLC plate.

Bioassays with fractionated bioactive plant extracts against *B. tabaci*

Whitefly colony

B. tabaci colony was raised in a glasshouse compartment under controlled conditions as mentioned above. The colony, originally from a field population was reared on cucumber plants of the variety Beit Alpha (F1 parthenocarpic, dust free and thiram treated seeds; Edena Seeds, USA) in a whitefly proof cage (140 x 85 x 130 cm) covered completely with a fine mesh (270 x 770 µm). Two true leaf seedlings were grown in 12 cm plastic pots to provide a continuous supply of healthy young plants to the whitefly colony and bioassays. Fertilization with Floral® (20-20-20+ microelements; Cifo S.p.A., Bologna, Italy) was applied at a rate of 5g per 10 L through irrigation of the seedlings, about 2 times a week.

Experimental setup with whitefly adults

Cucumber seedlings having two true leaves with detached cotyledons were used in the bioassays. In one bioassay, the treatments included the two crude whole plant extracts of the two plant species *P. damascena* and *R. myosuroides* with 4 fractions for each extract as: EtOAc (I.1), $CHCL_3$ - H_2O (I.2), $CHCL_3$-MeOH (I.3) and MeOH-MeOH fraction (I.4) plus 2 controls: distilled water and 10% methanol. In another bioassay, treatments included the most previously detected bioactive fraction of the two extracts, the $CHCL_3$-MeOH fraction of *P. damascena* with its five fractions and the two controls: distilled water and 10% Methanol.

Each treatment was replicated 9 times during the study. Ten millimeters of each crude extract or its fraction was rotovaped up to 1 ml, after which 9 ml of distilled water was added to homogenize the solution before application to the plant. Each seedling received an average of 9 ml of the extract/fraction or the control by spraying them on the upper and lower sides of the leaves using 10 ml glass-bottle sprayers. Each treated seedling was allowed to air dry and consequently was placed in one plastic cage (28 cm high x 21 cm diam. manufactured locally) having an aeration opening of (15 cm diam.) at the top and two circular openings (5 cm diam.) in body of cage, covered by a mesh net (270 x 770 µm).

Ten adult whiteflies (of about 3 days old) were collected by a hand aspirator (Hausherr's machine, N.J., USA) and introduced into each treated seedling in one plastic cage. Numbers of adult whiteflies dead or alive in each cage were recorded at 72 h after treatment with specification of the location of the insect in the cage as on the plant, walls or top cover of the cage or on soil surface in pot; dead whiteflies were counted to relate to presence of toxic effect, if any, that might be attributed to residues of plant extracts after spraying on plants. Selection of repellency assessment at 72 h after treatment was based on the following observations related to the extracts. A few hours after treatment, adult whiteflies were found landing at the top of the cage, this could be attributed to the fact that the bioactive extracts contained some volatile compounds that repelled the insects from approaching the plant. However, observations at 72 h confirmed the repellent efficacy (Coudriet et al, 1985; Ateyyat et al., 2009; Martini et al., 2012; Yadav and Mendhulkar, 2015) of some extracts to whiteflies, knowing that some botanicals are characterized by reduced stability which indicates that the presence of a pest on treated leaves was more obvious with time (Sundaram, 1996), mostly with non-repellent extracts.

Statistical analysis

All experiments were laid out in a completely randomized block design with treatment as the only factor. Each treatment was replicated 9 times. Data were transformed by sqrt (x+1), x being the number of live adult whiteflies after treatment, to normalize the data. Analysis of variance (ANOVA) was performed over the treatment factor, using the SPSS statistical package (Anonymous 2010). All means were separated by Fisher's LSD test (1949), at a significance level of $\alpha = 0.05$.

RESULTS AND DISCUSSION

Determination of bioactive fractions in two whitefly repellent plant extracts

Results of our study have shown that some fractions of

Table 1. Effect of two bioactive whole plant methanol extracts and their fractions against *Bemisia tabaci* adults under glasshouse conditions.

Treatment*	Extract / Fraction**	Number of live adults*** (Mean±S.E)
Untreated		5.56± 1.33a
MeOH (10%)		5.56± 2.06a
Pd wp I.1	EtOAc	5.55± 2.06a
Pd wp I.4	MeOH-MeOH	5.55± 2.45a
Pd wp I.2	$CHCl_3$-H_2O	5.11± 1.69ab
Rm wp I.2	$CHCl_3$-H_2O	5.11± 2.31ab
Rm wp I.4	MeOH-MeOH	4.66± 2.23ab
Rm wp I.1	EtOAc	4.00± 2.59bc
Pd wp I	MeOH	3.22± 1.33c
Rm wp I.3	$CHCl_3$-MeOH	3.11± 1.83c
Rm wp I	MeOH	2.77± 1.39c
Pd wp I.3	$CHCl_3$-MeOH	2.66± 1.58c

* *Pd* = *Phlomis damascena*; wp = whole plant (leaves + flowers + stems of plant); *Rm* = *Ranunculus myosuroudes*.** EtOAc = Ethyl acetate; MeOH = Methanol; $CHCl_3$ = Chloroform; MeOH-MeOH = out of aqueous basic layer; $CHCl_3$-MeOH = out of aqueous acid layer.*** Means followed by the same letter within a column are not significantly different (Fisher Lsd test; $P<0.05$).

the two crude bioactive extracts of *P. damascena* and *R. myosuroides* were significantly different in number of live adult whiteflies encountered per plant compared to the two controls (Table 1), knowing that the non-encountered whiteflies on the plant were not necessarily dead at the end of the experiment. In this bioassay, the two $CHCl_3$-MeOH fractions (*Pd* wp I.3 and *Rm* wp I.3) and the EtOAc fraction (*Rm* wp I.1) were not significantly different in their effect from the two crude extracts *Pd* wp I and *Rm* wp I, but all these treatments were significantly different in their effect from the two controls. However, the other fractions were not significantly different in their effect from the two controls. The $CHCl_3$-MeOH fraction of *P. damascena* (*Pd* wp I.3) showed the most significant bioactivity numerically as only 2.66 of adult whiteflies remained alive per plant compared to 5.56 in both controls; the former fraction was further not significantly different from the original raw extract of *P. damascena* allowing survival of only 3.22 adult whiteflies per plant. The latter result was further verified and comparable to the previously determined high repellency of this raw extract to the adult insect that allowed encountering of only 2.22 live adult whiteflies per plant at 72 h after treatment (Hammad et al., 2014). However, it seems that the whole plant extract of *R. myosuroides* have two bioactive fractions, the $CHCl_3$-MeOH fraction (*Rm* wp I.3) and the ethyl acetate fraction (*Rm* wp I.1) against adult *B. tabaci* (Table 1); the two fractions were comparable to the original raw extract of *R. myosuroides* allowing encountering of only 2.77 live adult whiteflies per plant. The latter result was further verified and comparable to the previously determined high repellency of this raw extract to the adult insect that caused encountering of only 1.22 live adult whiteflies per plant at 72 h after treatment (Hammad et al., 2014).

Some studies have reported repellency to insects by fractionated plant extracts (Harwood et al., 1990; Tunon et al., 1994; Gkinis et al., 2003; Singh and Metha, 2003). In our study, only the organic fractions *Pd* wp I.3, *Rm* wp I.3 and *Rm* wp I.1 have insecticidal activity against adult whiteflies as very low number of live whiteflies were encountered per plant after treatment in comparison to the control, but the aqueous fractions of both plant extracts further partitioned with $CHCl_3$-H_2O or MeOH were not bioactive. Thus, the former organic bioactive fractions might be enriched with alkaloids as it was found that the combination between two solvents, chloroform and methanol of medium and high polarity, respectively would result in the accumulation of alkaloid-type active compounds (Fried and Sherma, 1994). Similar to our study, Ahn et al. (1997) found in a stepwise extraction procedure with MeOH and EtOAc that the organic phase of the *Ginkgo biloba* L. extract was the only bioactive material when isolating this most potent extract which was tested within 119 methanol extracts of 52 plant sp. against the brown planthopper *Nilaparvata lugens* Stal. (Order: Hemiptera).

Furthermore, in our study, the EtOAc fraction of *R. myosuroides* (*Rm* wp I.1) was of high bioactivity against the adult whitefly allowing insect survival of only 4.0 live adult whiteflies per plant (Table 1). Similar to our study, Neoliya et al. (2003) have found high bioactivity with EtOAc fraction isolated from aqueous-methanolic and acetone extracts of air-dried leaves of *Catharanthus roseus* (Linn) that were fractionated successively with n-hexane, $CHCl_3$, EtOAc and n-BuOH. Their results showed maximum insect growth regulator (IGR) activity

Table 2. Isolated fractions of Pd wp I.3 eluted through CC and analyzed by TLC.

Fractions	R_f*	Weight (mg)	% out of 0.5 g
B1	0.72	35	7
B2	0.62	42	8.4
B3	0.55	87	17.4
B4	0.41	76	15.2
B5	0.72, 0.62, 0.55	12	2.4

*R_f is equal to the distance traveled by the substance divided by the distance traveled by the solvent. Its value is always between zero and one.

against the 6th instar larvae of *Spodoptera litura* Fab. of 84.20 % with acetone extracts followed consecutively by EtOAc of 63.51%. On the other hand, Bhattacharya et al. (1993) dealt with the distillate of 1kg aerial part of *Ranunculus sceleratus* L., another species than *R. myosuroides* that was saturated by adding sodium chloride and extracted with successive portions of diethyl ether. The eluted yellow oily residue fraction caused larval mortality for *Drosophila melanogaster* L. of 100, 56 and 16% at 5, 1 and 0.05% concentrations, respectively, at 24 h after treatment. Furthermore, it is worth mentioning that the *R. myosuroides* whole plant extract besides its insecticidal activity (Hammad et al., 2014), it has been found to have significant antimicrobial activity (Barbour et al., 2004) within a bio-prospection context study; in form of utilizing local plant species as an alternative in sustainable agriculture development.

Effect of isolated sub-fractions of $CHCl_3$-MeOH fraction of *P. damascena* whole plant extract (Pd wp I.3) against whitefly adults

The bioactive $CHCl_3$-MeOH fraction of *P. damascena* whole plant extract (Pd wp I.3) was selected for further fractionation as it caused the lowest significant numerical survival to adult whiteflies among all treatments (Table 1). Analysis of the eluted material of this fraction on TLC plates yielded several sub-fractions. Similar TLC pattern allowed pooling of eluted material into five potent sub-fractions that were isolated and corresponded to bands detected on Pd wp I.3 TLC plate. R_f values of the isolated sub-fractions were 0.72, 0.62 and 0.55 for B1 (35 mg), B2 (42 mg), B3 (87 mg) eluted with chloroform-methanol (9: 1); B4 (76 mg) having R_f value of 0.41 was eluted with chloroform-methanol (9: 2) and B5 (12 mg) having 3 bands with R_f values 0.72, 0.62 and 0.55 were eluted with chloroform-methanol 9:1 (Table 2). Continued elution with chloroform-methanol (9:3) till (9:9) and with 100 % methanol revealed no fractions on TLC for the collected eluted materials. This suggests the necessity of using other solvent types for eluting the materials or using other chemicals for detecting other compounds on TLC plates.

A significant insecticidal activity of the five isolated fractions B1, B2, B3, B4 and B5 was detected against the adult whiteflies (Table 3). There were significant differences in number of live adult whiteflies among all isolated fractions and the controls. However, only the isolated fraction B4 was not significantly different in its effect from the bioactive chloroform-methanol fraction (Pd wp I.3). This indicates that the similar bioactivity detected in the raw extract of *P. damascena* methanol extract and its bioactive $CHCl_3$-MeOH fraction (*Pd* wp I.3) is due mainly to active components found in the isolated fraction B4.

A few studies dealt with bioassay-guided fractionation for certain bioactive plant extracts of *Ginkgo bilboa* (Ahn et al., 1997), *Heliotropium floridum* (Reina et al., 1997), and *Serratula coronata* L. (Odinokov et al*.,* 2002). Different classes of glycosides comprising diterpenoids, phenyl-propanoids, iridoids, and flavonoids had been identified from genus *Phlomis* L.; many of the phenylpropanoids showed significant biological activities. The air-dried powdered leaves of *Phlomis aurea* Decne. were extracted with 70% EtOH. Similar to our methodology applied to *P. damascena* and yielding 5 fractions, the dried ethanolic extract of *P. aurea* was suspended in H_2O and deffated with *n*-hexane, but the MeOH extracts were chromate-graphed by silica gel CC using CH_2Cl_2–MeOH–H_2O (70:30:3) and (80:20:2) and yielding four and three fractions, respectively (Kamel et al., 2000). Iridoid glucosides (IG) were also isolated from the 1-butanol-fraction of a methanolic extract of the aerial parts of another *Phlomis* sp., *Phlomis rigida* Labill. These IG fractions were collected after being chromatographed over Diaion HP-20 column with a stepwise increase of MeOH in water (Takeda et al., 2000). On the other hand, ether extracts of *R. sceleratus* caused significant reduction in larval activity, pupal weight and pupal emergence of *D. melanogaster* at all concentrations tested. Similarly, there were weight reductions and high mortality in treated red flour beetles; no insect could survive at 1 and 5% concentrations of the extracts beyond 15 and 10 d, respectively. These insecticidal properties exhibited by the ether extract were attributed

Table 3. Effect of isolated fractions of Pd wp I.3, eluted through CC and analyzed by TLC, on *B. tabaci* adults.

Treatment	Number of live Adult* (Mean ± S.E)
MeOH	8.33± 1.22 a
Untreated	7.22± 0.66 b
B3	5.66± 1.93 c
B2	5.55± 1.74 c
B5	5.33± 1.73 cd
B1	4.88± 1.05 cd
B4	4.66± 1.65 de
Ap (PD p) I.3	3.88± 0.92 e

*Means followed by the same letter within a column are not significantly different (Fisher Lsd test; P<0.05).

to the isolated compounds: the two lactones, protoanemonin and anemonin, and the glycoside ranunculin (Bhattacharya et al., 1993).

Thus, the bioactive fraction 4 (15.2% by weight) obtained from the bioactive chloroform-methanol fraction of the whole plant methanol extract, after being subjected to successive chromatography and preparative TLC on silica gel might be of the alkaloid-type active compounds. However, these compounds need to be further identified by using other chromatographic techniques as GC/MS analysis and others. Thus, it is in the developing countries that are rich in the endemic plant biodiversity where these botanical pesticides may ultimately have their greatest impact in future integrated pest management (IPM) programs, given their relative safety to non-target organisms and the environment.

Conflict of interests

The author(s) did not declare any conflict of interest.

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