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DISTURED ACETYLCHOLINESTERASE ACTIVITY IN HAEMOLYMPH AND FAT BODIES OF *SCHISTOCERCA GREGARIA* (FORSKAL) (ORTHOPTERA: ACRIDIDAE) BY EXTRACTS OF POMEGRANATE *PUNICA GRANATUM* LINN. AND TOOTHPICK WEED *AMMI VISNAGA* L.

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ABSTRACT

The present study was carried out to investigate the effects of Punica granatum peel extracts and Ammi visnaga fruit extracts on the Acetylcholinesterase (AChE) activity in haemolymph and fat bodies of last instar nymphs and newly emerged adult females of Schistocerca gregaria. The extracts of both P. granatum peel and A. visnaga fruit predominantly enhanced the enzyme activity in haemolymph of nymphs and adults with few exceptions. The disturbance induced due to P. granatum peel extracts on AChE activity in fat bodies depended on both nymphal age and developmental stage. Where, although remarkable increase in AChE activity was detected in the mid- and late-aged nymphs, serious decrease was noticed in early-aged nymphs and adults. A. visnaga fruit extracts prohibited the early-aged nymphs to attain the normal enzyme activity in their fat bodies but promoted the nymphs of other ages to gain high activity. In fat bodies of adults, n-butanol extract of A. visnaga caused a pronounced inhibition of the enzyme activity but other extracts promoted for considerable increasing activity.

Keywords: Adult, Ethanol, Fat bodies, Haemolymph, Petroleum ether, N-butanol, Nymph.

Contribution/ Originality

This study documents the probable candidates for the development of biopesticides, as alternative to the hazardous synthetic insecticides, from extracts of Punica granatum peel and Ammi visnaga seeds for controlling the dangerous desert locust Schistocerca gregaria by the disturbance of acetylcholinesterase activity and all physiological process depending it.

1. INTRODUCTION

The desert locust *Schistocerca gregaria* is a destructive pest for several crops, particularly which are considered as the main food sources for man and animals. In some cases, a single swarm

contains billions of adult locusts, with up to 80 million per square kilometer over an area of more than 1,000 square kilometers (Steedman, 1988). Plagues of this pest have been recognized as threat to agricultural production in Africa and western Asia for thousands of years (Showler, 1995; 1996; Ceccato *et al*., 2007). Damage is caused as a consequence of its polyphagous behaviour, high population density, and the nature to aggregate and swarm. Each individual gregarious locust can consume roughly its own weight of foliage daily (Lindsey, 2002). Invasions of this locust are the cause of calamity because they can result 100% crop loss (Meinzingen, 1993; FAO, 2012). Therefore, it is necessary to search and develop some effective control strategies for suppressing the population density aiming to suppress the phase transition into gregaria or to prevent the outbreak of the mobile swarms. Because of the difficulty to predict locust outbreaks, the concerned countries usually apply pollutant chemical pesticides for control (Gruys, 1993). As reported by Lecoq (2001), the current locust control operations are mainly based on organophosphorus pesticides. Often huge quantities of such pesticides are used (Cirad, 2004). The use of such amounts of pesticides represents a real hazard for the environment. Also, repeated use of a particular insecticide may result in the development of resistance (Bell *et al*., 2001; FAO, 2003). Locusts remain a serious problem despite these large doses of applied insecticides (Ouali-N'goran *et al*., 2013).

Taking these drawbacks of synthetic pesticides into account, many institutions have intensified their efforts in the search for integrated locust control measures. Much attention has been devoted to use plant extracts or plant products that have insecticidal effects (Schmutterer, 1990a; Schmutterer, 1990b); Krall and Wilps (1994). The botanical control agents are generally pest-specific and relatively harmless to non-target organisms. Also, they are biodegradable and consequently harmless to the environment (Rembold, 1994). Majority of botanicals are still at the experimental stage. Unfortunately, the large scale production is problematic and the difficulties that facing the registration of variable products will limit adoption (Meinzingen and Kooyman, 1997). Otherwise, prior results on the effects of plant extracts on the desert locust were encouraging their implementation as an alternative measure to chemical control (Abbassi *et al*., 2003).

Pomegranate (*Punica granatum* Linn., Lythraceae) is one of the oldest cultivated plants in the world (Lye, 2008). It was a symbol of immortality and love in oriental regions (Ageel *et al*., 1991). It is cultivated in Central Asia and the drier parts of Southern Asia (Holland *et al*., 2009), as well as in Mediterranean, tropical and subtropical areas (Mars, 2000). It was introduced into Latin America, California and Arizona (Khan and Hanee, 2011). From the medical point of view, pomegranate is of a great interest to research in pharmaceutical and new drug development fields because of its distinctive bioactivities (Singh *et al*., 2002; Negi *et al*., 2003; Vasconcelos *et al*., 2006; Lansky and Newman, 2007; Reddy *et al*., 2007; Jurenka, 2008; Tayel *et al*., 2009; Augusta *et al*., 2010; Abdollahzadeh *et al*., 2011; Dkhil, 2013; Eldiasty *et al*., 2014). For pest control, aqueous

extract of *P. granatum* fruit rind was toxic against tapeworms (Hukkeri *et al*., 1993) and extracts of bark exhibited molluscicidal activity (Tripathi and Singh, 2000; Tripathi *et al*., 2004). Also, the fruit rind was effective on some parasitological parameters of *Schistosoma mansoni* (Osman *et al*., 2013). With regard to insect pests, available literature reported the insecticidal effects of *P. granatum* extracts and its disruptive effects on growth and development (Alanis *et al*., 2005; Melendez and Capriles, 2006; Liu *et al*., 2007; Sharma and Rajguru, 2009; Mahmood, 2010; Mansour *et al*., 2010; 2012; Ghandi and Pillai, 2011; Mansour *et al*., 2012; Mohammad, 2012; Eldiasty *et al*., 2014; Ghoneim *et al*., 2014a). Also, *P. granatum* peel extracts affected the adult performance and transaminase activity in *S. gregaria* (Ghoneim et al., 2014b; 2014c). Toothpick weed (*Ammi visnaga* Lamarck, Apiaceae= Umbelliferaceae) is commonly known as khella or Al-khillah, especially in Egypt. It is native to Europe, Asia and North Africa but can be found throughout the world as an introduced species. The *A. visnaga* extracts have been used in the traditional medicine and their chemical components have been used in the modern medicine (Duarte *et al*., 1999; Khan *et al*., 2001; Cordero *et al*., 2004; Whitton *et al*., 2008; Lee *et al*., 2010). However, the research work on using this plant, or some of its chemical constituents, in pest control; is unfortunately scarce. The available literature reported an ovicidal activity of its extracts against Hessian fly *Mayetiola destructor* (Lamiri *et al*., 2001) and a larvicidal activity against some mosquito species (Amer and Mehlhorn, 2006a; 2006b; Pavela, 2008). These extracts had been reported, also, as a grain protectant against weevils (Abdel-Latif, 2004; Ahmed and Al-Moajel, 2005). Recently, fruit extracts exhibited disruptive effects on the growth, development, general metabolism and phosphatase activity in *S. gregaria* (Ghoneim *et al*., 2014d; 2014e; 2014f).

Acetylcholinesterase (AChE, EC 3.1.1.7) is a key enzyme catalyzing the hydrolysis of the neurotransmitter, acetylcholine, in the nervous system (Grundy and Still, 1985; Wang *et al*., 2004; Zibaee, 2011) and is primarily responsible for termination of cholinergic neurotransmission at synapses in both humans and insects (Fournier and Mutero, 1994; Carlier *et al*., 2008). AChE is known to be the target of many organophosphate- and carbamate-based insecticides which cause modifications of the active site of the AChE enzyme leading to the inhibition of AChE activity and block the hydrolysis of acetylcholine (Oppenoorth and Welling, 1979). Thus, AChE activity is one of the main resistance mechanisms in various insect species against the organophosphrous or carbamate- resistant insects (Hemingway *et al*., 1986; Zhu and Gao, 1999; Kozaki *et al*., 2001; Stumpf *et al*., 2001; Li and Han, 2002; Yoo *et al*., 2002; Fournier, 2005; Yu, 2006).

For medical purposes, Barbosa Filho *et al.* (2006) reviewed 309 plants and 260 chemically defined natural molecules reported in the literature, which have been evaluated for AChE inhibition. The isolated and identified compounds belong to the classes of alkaloids, monoterpenes, coumarins, triterpenes, flavonoids, benzenoids, diterpenes, *etc.* A comprehensive review of cholinesterase inhibitor phytoconstituents was presented by Ahmed *et al.* (2013). Recently, Mathew and Subramanian (2014) screened the methanolic extracts of 20 plants used in

Indian Ayurvedic system of medicine for the same purpose. In respect of the pest control, AChE activity was affected by some plant extracts (Senthil Nathan *et al*., 2008). According to Begum *et al*. (2011), ethanol extracts of seeds of *Annona squamosa* and *Calotropis procera* inhibited the enzyme activity in different developmental stages of *Musca domestica*. Also, ethanol senescent leaf extracts of *Jatropha gossypifolia* and *Melia azedarach* inhibited the detoxification enzymes and AChE activities in *Spodoptera frugiperda* larvae (Bullangpoti *et al*., 2012). The present study aimed to investigate the effects of different extracts of *P. granatum* peel and *A. visnaga* fruits on the AChE activity in two tissues of *S. gregaria* nymphs and adults.

2. MATERIALS AND METHODS

2.1. Experimental Insect

The desert locust *Schistocerca gregaria* (Forskal)(Orthoptera: Acrididae) was used as an experimental insect in the present study. The present culture was originated by a sample of gregarious nymphs from Plant Protection Research Institute, Ministry of Agriculture, Giza. As designed by Hunter-Jones (1961) and improved by Ghoneim *et al*. (2009), insects were reared in wooden cages (60 x 60 x 70 cm). The bottom was furnished with a sandy layer (20 cm depth) provided with 10-15% humidity suitable for egg laying. An electric bulb (100 watts) was adjusted in each cage to maintain a continuous photoperiod of 12 L: 12 D as well as an ambient temperature (32±2°C). The insects were reared and handled under the crowded conditions. The feces, dead locusts and food remains were removed daily before introducing fresh food. Care was seriously taken to clean these cages at regular intervals and the sand was sterilized in drying oven (at 140°C for 24 hours) to avoid contamination with any pathogenic microorganisms. Fresh clean leaves of clover *Trifolium alexandrinum* were provided as a food.

2.2. Plant Extraction

A weight of 1.5 Kg *P. granatum* peel was purchased from the Egyptian market and thoroughly cleaned with tap water for disposing of impurities. The peel was shade dried and then finely grinded by a micro-mill. The pulverized powder was macerated with ethanol in a stoppered container for a defined period with frequent agitation until soluble matter is dissolved as adopted from Ncube *et al*. (2008). The ethanol extract was divided into two parts, one part was evaporated for obtaining 37 gm dried extract and the other was concentrated into 300 ml by rotary evaporator, and then diluted with 300 ml distilled water. Using a separating funnel, the dilute was fractionalized by petroleum ether (300 ml X 5) and n-butanol (300 ml X 5) gave 29 and 34 gm, respectively. From each of ethanolic crude fractionalized petroleum ether and n-butanol extracts, a series of concentrations was prepared; 80, 40, 20, 10, 5 and 2.5%. *A. visnaga* fruits (1.5 Kg) was purchased from an Egyptian market and thoroughly cleaned with tap water for disposing of impurities. The fruits were shade dried and then finely ground by a micromill. Solvents of

different polarities were used for the extraction, where pulverized powder was macerated with ethanol in a closed container for a defined period with frequent agitation until soluble matter was dissolved as adopted from Ncube *et al.* (2008) and treated as previously mentioned for *P. granatum*. From each of ethanolic crude fractionalized petroleum ether and n-butanol extracts, six concentrations were prepared: 80.0, 40.0, 20.0, 10.0, 5.0 and 2.5%.

2.3. Nymphal Treatments:

In a preliminary experiment, different concentration levels of ethanol, petroleum ether, and n-butanol extracts of *P. granatum* peel and *A. visnaga* fruits had been applied on the newly moulted penultimate (4th) instar nymphs of *S. gregaria* through the fresh food leaves of *Trifolium alexandrinum* that dipped once in each extract for 3 minutes. LC_{50} values were calculated in 36.7, 22.2 and 40.7% of ethanol, petroleum ether and n-butanol extracts of *P. granatum* peel, respectively. LC_{50} values were calculated in 21.0, 12.0 and 22.5% of ethanol, petroleum ether, and n-butanol extracts of *A. visnaga* fruits, respectively. After treatment with these LC_{50}s, the successfully moulted last instar nymphs and newly emerged adult females were used to determine the effect on AChE activity in two tissues: haemolymph and fat body. Three ages of last instar nymphs were used: early- (1-day old), mid- (4-day old) and late-aged (7-day old) nymphs.

2.4. Tissue Preparation and Enzyme Assay

For the determination of AChE activity, samples of haemolymph had been collected from the last instar nymphs and newly emerged adult females. The haemolymph was obtained by amputation of one or two hind legs of the nymph and adult with fine scissors. Gentle pressure was done on the thorax until a drop of haemolymph appeared at the point of amputation. Haemolymph was drawn into Eppendorff Pipetman containing few milligrams of phenoloxidase inhibitor (phenylthiourea) to prevent tanning or darkening and then diluted 5x with 0.7% saline solution. For whole assays, the diluted haemolymph was frozen for 20s to rupture the haemocytes. The haemolymph samples were then centrifuged at 2000 r.p.m. for 5 min, and only the supernatant fractions were used directly or frozen until the use. Three replicates were used and the haemolymph of two individuals were never mixed. Also, samples of fat bodies (parietal and visceral) were collected from nymphs (of the same ages) and newly emerged adult females. The fat body was weighed and then homogenized in a saline solution (fat body of one insect/1 ml of saline solution 0.7 %) using a fine electric homogenizer. Tissue was grinded for 2 min. Homogenates were centrifuged at 4000 r.p.m. for 15 min. The supernatant was used directly or frozen until the use. Three replicates were used and the fat bodies from two individuals were avoided to be mixed. AChE activity was determined according to the method of Weber (1966) using a kit of Diamond company. The enzyme was measured at wave length 405 nm by spectrophotometer.

2.5. Statistical Analysis of Data

Obtained data were analyzed by the Student's *t*-distribution test; and refined by Bessel correction (Moroney, 1956) for the test significance of difference between means.

3. RESULTS

3.1. Effects on Ache Activity in Haemolymph of *S. Gregaria* Nymphs and Adults

As clearly shown in Table (1), *P. granatum* peel extracts predominantly enhanced the AChE activity in haemolymph of the last nymphal instar and newly emerged adults. An exceptional case of AChE inhibition was recorded in adults after treatment only with n-butanol extract (4.6% reduction). The most potent enhancing action was exerted by ethanol followed by petroleum ether and n-butanol extracts in early- and mid-aged nymphs but reversed action was exerted on the late-aged nymphs (38.3, 26.3 and 12.0% increments by n-butanol extract, petroleum ether extract and ethanol extract, respectively).

The disturbance of AChE activity in haemolymph that induced due to *A. visnaga* fruit extracts were arranged in Table (2). A prevalent stimulatory effect had been obviously exhibited on the enzyme activity, regardless the extract. An exceptional case of the enzyme inhibition was estimated in the late-aged nymphs as a response to the petroleum ether extract (0.5% reduction). However, the enzyme activity increased in nymphs and adults in no certain trend except in haemolymph of the early-aged nymphs which attained increasingly raising enzyme activity by ethanol extract followed by petroleum ether and n-butanol extracts.

3.2. Effects on Ache Activity in Fat Bodies of *S. Gregaria* Nymphs and Adults

In the light of data assorted in Table (3), the disturbance induced due to *P. granatum* peel extracts on AChE activity in fat bodies depended on both nymphal age and developmental stage. Where, remarkable increase in AChE activity was detected in mid- and late-aged nymphs. That is to say, both of n-butanol and petroleum ether extracts exhibited the most pronouncedly promoting effect on the enzyme activity in fat bodies of mid- (1367.8±133.2 vs. 1253.1±167.2 U/L of control trials) and late (1267.4±136.5 vs. 955.0±219.2 U/L of control trials) aged nymphs, respectively. In contrast, the enzyme activity was seriously inhibited in fat bodies of both early-aged nymphs (25.4, 28.1 and 13.2% reduction by ethanol, petroleum ether and n-butanol extracts, respectively) and newly emerged adults (4.7, 3.4 and 14.3% reduction by the same sequence of extracts).

To a great extent, similar effects on the enzyme activity in fat bodies had been exhibited by *A. visnaga* fruit extracts as shown in Table (4). Where, as all extracts prohibited the early-aged nymphs to attain normal enzyme activity (12.4, 7.6 and 26.9% reduction by ethanol, petroleum ether and n-butanol extracts, respectively), n-butanol extract only prohibited the newly emerged adults to attain normal enzyme activity (8.4% reduction). On the contrary, nymphs of other ages

had been induced to gain higher enzyme activity, regardless the extract. In addition, ethanol extract and petroleum ether extract enhanced the adults to gain higher enzyme activity (10.7 and 7.7 increments, respectively).

4. DISCUSSION

Determination of AChE activity has been used routinely as a biomarker of exposure to certain groups of contaminants, such as organophosphate and carbamate insecticides (Grue *et al*., 1997). These two groups of insecticides are known as important AChE inhibitors suppressing the enzyme action (Thompson, 1999; Yoo *et al*., 2002; Wang *et al*., 2004). Moreover, the enzyme activity was disturbed in *Plutella xylostella* and *Spodoptera exigua* larvae by flupyrazofos (pyrazole organophosphorous insecticide), depending on the larval instar and the time of determination (Lee *et al*., 2003).

In the present study, *P. granatum* peel and *A. visnaga* fruit extracts predominantly enhanced the AChE activity in haemolymph of nymphs and adults with few exceptions. In harmony, similar response was observed on different stages of *S. gregaria* following the treatment with LC_{30} or LC_{50} of abamectin (El-Aziz, 2010), Neemazal or *Nigella sativa* extracts (Hamadah, 2009) and *Fagonia bruguieri* extracts (Ghoneim *et al*., 2012). Similar findings was noticed on the 4th instar larvae of *Spodoptera littoralis* that treated with camphor plant oil (Fetoh and Asiry, 2013)

Also, the disturbance in AChE activity due to the aforementioned treatments was affected by the nymphal age and developmental stage. Although, remarkably activity enhancement was detected in the mid- and late-aged nymphs but a seriously inhibition was determined in early-aged nymphs and adults. The *A. visnaga* fruit extracts prohibited the early-aged nymphs to attain normal level of enzyme but promoted the nymphs of other ages to gain high enzyme level. In fat bodies of adults, n-butanol extract of *A. visnaga* caused pronounced inhibition of the enzyme activity but treatment with other extracts resulted in considerably increasing activity. However, similar trend was stated in some insect pests treated by several plant extracts and plant secondary metabolites, such as house fly and Madagascar roach (Grundy and Still, 1985), *M. domestica* and *Blatella germanica* (Naqvi, 1986), *Tribolium castaneum* (Ryan and Byrne, 1988), *S. frugiperda* and *Leucophaea maderae* by *M. azedarach* extracts (Breuer *et al*., 2003), *Periplaneta americana* by azadirachtin (Azt.) (Shafeek *et al*., 2004), *Nilaparvata lugens* and *Leodelphax striatellus* by a mixture of insecticide carbosulfan and the plant extract wood vinegar (Kim *et al*., 2008), in *N. lugens* (Senthil Nathan *et al*., 2008), *S. frugiperda* by *M. azedarach* senescent leaf extracts (Bullangpoti *et al*., 2012), *etc.*

The prevalent AChE inhibition in fat bodies and exceptional cases of the enzyme inhibition in haemolymph of *S. gregaria* by *P. granatum* peel extracts and *A. visnaga* fruit extracts, in the present study, may be due to certain toxic components leading to accumulation of acetylcholine at the synapses, so that the post-synaptic membrane is in a state of permanent stimulation, which

results in paralysis, ataxia, general lack of co-ordination in the neuro-muscular system, and eventually death (Aygun *et al*., 2002; Massa *et al*., 2008; Senthil Nathan *et al*., 2008; Begum *et al*., 2011). In addition, the prohibited enzyme activity in *S. gregaria* may indirectly indicate the damage of their nerve cells, which induce the AChE photoin activation and death caused by the disruption of normal nerve conduction (Yin *et al*., 2008). On the other hand, the promoting effects of *P. granatum* peel or *A. visnaga* fruit extracts on the AChE activity in haemolymph of nymphs and adults or fat bodies of adults of *S. gregaria*, in the present study, can't be unfortunately explicated albeit some triterpenoids, steroids, glycosides, saponins, alkaloids, flavonoids, tannins or polyphenols in *P. granatum* peel extracts (Li *et al*., 2006; Bhandary *et al*., 2012) and khellin, visnagin or some pyranocouramins in *A. visnaga* fruit extracts (Ziment, 1998) may be responsible for the AChE induction. Further investigation should be planned and conducted in future for exploring the active ingredients and their mode of action.

In conclusion, this is the first report of disruptive effects of pomegranate and khillah plant extracts on AChE in *S. gregaria*. Disturbance of AChE activity in *S. gregaria* haemolymph and fat bodies by *P. granatum* peel extracts and *A. visnaga* fruit extracts suggest that these plants may prove to be probable candidates for the development of biopesticides to control the populations of the present pest as safer, ecofriendly and economic alternatives to the synthetic pesticides.

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