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Evaluation the toxicity of *Viola odorata* extract and Spirotetramat pesticide on the *Agonoscena pistaciae* (Hemiptera: Psyllidea)

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Abstract
The *Agonoscena pistaciae* is one of the most important pests in pistachio plant on which the adults feed the plant sap and causes the plants growth reduction, falling flowering buds and hence results in yield reduction. High reproductive potential and short growth period increase the resistance of this pest against different pesticides. Also pesticides because environmental problems and show biological magnification, therefore, using more safe materials like plant extracts, have been considered in this pest control and management. These materials have extensive range and also have the components which degrade easily within the soil, so they are safe regarding the environmental conditions and can be used as appropriate alternative of traditional pesticide. The present study have evaluate the *Viola odorata* extract and Spirotetramat pesticide toxicity on the *A. pistaciae* (Hemiptera: Psyllidea). The mortality rate results showed that the *Viola odorata* extract was effective on *A. pistaciae* mortality percentage. Also the Spirotetramat pesticide has toxic effect on *A. pistaciae*. The LC_{50} for *V. odorata* extract was approximately equals to 5.76/1000. The LC_{50}, up and down limits of Spirotetramat pesticide on this insect were equal to 0.09/1000, 0.02/1000 and 0.31/1000 respectively.

Keywords: *Agonoscena pistaciae*, Spirotetramat, *Viola odorata*, toxicity.

1. Introduction
Pistacia Vera is one of the most important agriculture crops which has high economic value. In Kerman province, Iran, Rafsanjan Township is the most important pistachio production region in Iran and also in the world (Razavi, 2005) [26]. The *Agonoscena pistaciae* Burckhardt and Lauterer (Hom: Psyllidae) is an important pest in pistachio plant which is found in many pistachio production areas in the world such as Iran, Turkey, Iraq, Armanistan and Mediterranian regions (Burckardt and Lauterer 1989; Lauterer *et al*., 1998; Mar *et al*., 1995; Anagnou–Veroniki *et al.*, 2008) [6, 2, 18]. Among all *Agonoscena* species in Iran, the *A. pistaciae* is the most common and destructive pests in pistachio. The nymph and adult insects feed the plant sap by sticking their mouth part into the leaf tissue and cause pistachio performance reduction qualitatively and quantitatively and also is recognized as Shire Khosk among farmers in Kerman regions (Samih *et al*., 2005) [28]. This pest has six complete generations and one incomplete generation during the year in Rafsanjan region conditions (Hasani *et al*., 2009) [10]. Management of this pest requires pistachio trees pesticide spraying six times during growth period which causes pesticides overuse and also increases the environmental pollution. Common pesticides which are used to control this pest in pistachio garden are the Organic-phosphorous compositions (Fuzalon (Zolone ®), Diazinone, Etion, chlorpyriphos), pyrethroid compositions (Permethrin and Fen- Valerite), Growth regulator (Hexaflomoran (Consult®)), and also Fuzalon+Tephlobenzorane (Marshal®), Formamidines (Amitraz (Mitac®)), and recently Nicotinoid compositions (Imidaclopride (Konfidor®)), Acetamipride (Musiplane®), Tiachlopride (Kalipso® and Biscaya®) and acaricides (Spirodiclophone (Envidor®)) (Lababidi, 2002) [17].
High reproduction potential, short growth period, having many generations in a year and also pesticides overuse during the growing season for this pest management, causes their higher resistance against different pesticides (Berrada *et al.*, 1995) [4]. Today with attention to repeated pesticides application and because of pesticide ineffectiveness on this pest, the *A. pistaciae* resistance problem have been discussed. The sensitivity of different population of *A. pistaciae* have concentrated only on the Fuzalon insecticide as a common and popular pesticide to control this pest so that past studies in Kerman province showed that the Rafsanjan

pest population has lower sensitivity to this insecticide (Talebi *et al*., 2001; Alizadeh *et al*., 2011) [1, 29]. A newer insecticide which is used to control the *A. pistaciae* is Imidachlopride although the resistance of some insects to this Nicotinoid insecticide has mentioned (Zhao *et al.* 1995; 2000; Olson *et al*. 2000) [31, 23].

During the recent years, uncontrolled pesticides usage because of dangerous effects on environment and humans cause the public peoples and scientists serious concerns. Each year, about 2.5 million tons of different pesticides are used in crop pests control process so that the damages resulting from their usage estimated to be 100 million dollars each year (Koul *et al*., 2008) [16]. Also the natural enemies activity cannot prevent the pests contribution in viruses transmission process. Usually the plants oils have extensive range and also have the components which are easily degrade within the soil, so these materials are environmentally safe and can be good alternatives instead of traditional pesticides (Misra *et al*., 1996) [22]. The plant extracts penetrate the waxy cuticle using aliphatic and fragrant components and counteract with the pests neural transmitters, growth hormones and digestive enzymes activities disturbance (Choi *et al*., 2003) [7]. The plant Extracts have different materials which show oviposition deterrency, deterrent effect, anti-feeding, repulsive and toxicity effects on different insects (Isman, 2006) [12]. Different studies have done about the plant's Extract biological activities and result show that the composition has insecticide, fungicide, bactericide and oviposition deterrency (Bouda *et al*., 2001; Ketoh *et al*., 2002; Lee *et al*., 2006; Mahboubi and Haghi, 2008) [33, 5, 20, 19]. There are many plant species in different regions of Iran which, their different parts such as stem, root, leaf, flower and seed are used as drug, but their insecticide effect have not been determined. Evaluation these plants and their effective compositions and based on extensive researches, these compositions can be used in pests and plant diseases control. So, in present study the *viola odorata* insecticide effects have been evaluated.

2. Materials and methods

2.1 Insect rearing

In order to rearing *A. pistaciae pests*, the pistachio seedling, from the zarand cultivar were used which their growth was done within the plastic pots (7×35cm) in appropriate greenhouse conditions. The *A. pistaciae* were collected from contaminated pistachio gardens in Rafsanjan town ship and transmitted onto pistachio pots. The contaminated pots were placed in wooden trays (80×60×50cm) and covered with special mesh (mesh, 40) in a growth room with controlled temperature on 26±2 °C, relative humidity 45±5% and also 16:8 photoperiod conditions. In order to provide same *A. pistaciae adult stage*, 20-25 pairs of adult insects were placed in cylindrical PVC boxes (30×12cm) onto six month pistachio seedling. And after 24 hours, their eggs were used to produce the same adult insects.

2.2 Viola odorata extraction

The *Viola odorata* plants were collected from the Rafsanjan paradise of Vali-Asr University and their aerial parts (Branches, Leaf and Flower) were dried and powdered. After plant identification by specialist botanist, one sample archived in agricultural university herbarium. The Hydro-Alcoholic extract was solved firstly on dry ben-marry and then in D-Methyl-Sulphoxide. In order to prepare the Fractions, 10 g of dried Hydro-Alcoholic extract poured in distilled water and transferred in to separator funnel. Then respectively with adding Ethyl-Acetate and N-Butanol solvents, the soluble materials in Ethyl-Acetate (Ethyl-Acetate Fraction) and after that, soluble materials in N-Butanol (N-Butanol Fraction) were separated and remainder, made the Hydro-Fraction materials. After solving the fraction solvents, the Hydro-Fraction was solved in Sodium chloride (0.9%) and the Ethyl-Acetate and N-Butanol fractions were solved in D-Methyl-Sulphoxide (Sadeghnia *et al*., 2009: Rakhshandah *et al*., 2010) [10, 25].

2.3 Toxicity bioassays

The bioassay tests should be repeatable and needs simple facilities and instruments. Therefore, with doing primary experiments among common methods such as leaf disk, dropping, scattering and immersion, the insect immersion within toxic solutions which had lower death percentage in control population and also had simple method were selected and all biological analyses on different population were done using this method. Because, the technical material is not soluble in water, so firstly, the needed amount to make the basal solution was dissolved in 1ML of Acetone and then, the Twin 80 (concentration=2%) were used to solve this material. The preliminary tests to determine the concentration which cause 25-75% of population death were determined for final test. The bioassay test on adult insects which had lower than 24 hours different and are recognizable by having two black spots in lateral body side and a black spot on body distal end from other pupa ages was done. The treatment method was based on Alizadeh, *et al*., 2011 [1] method, so that the adult insect in each replication were placed on 2×5cm lace stuff (mesh 40) using painting-brush and immersed in insecticide solution two seconds. The adult insects were placed on Filter paper. Before adult insect treatment, the leaf discs with 5CM diameter were cutted and to become moisturized were placed on moisturized Filter paper in Petri-dish and in order to sufficient ventilation and air penetration, some holes were made on Petri-dishes and then covered with stuff mesh. After treatment, adult insects were transferred on leaf discs and Petri-dishes were placed in room temperature, 26±2 °C, relative humidity 45±5% and 16:8 photoperiod. After 24 hours, the adult insects mortality percentage determined and also mortality percentage was corrected using Ebot formula. The insects which had not movement and showed severe toxicity symptoms regarded as dead insects. In order to prove the insects death if it could not move equal to his body length after insect simulation with a needle, it was regarded as dead insect.

2.4 Final Tests

Minimally five concentrations with logarithmic interval between concentrations which cause 25-75% of insect's death were selected and used in concentration- responses (mortality) curves estimation. Evaluation the effect of selected concentration on adult insect were done based on immersion method and using pesticides technical material in preliminary experiments. For each concentration minimally 160 adult insects were tested so that each concentration was evaluated 8 replications and in each replication, 20 insects were selected randomly.

2.5 Data Analysis

Data analysis was done using Polo-Plus software version2 and based on probability method and the insecticides concentration- responses curves were determined on *A. pistaciae pests.* The LC_{50} (effective concentration on 50% of population) of pesticide and plant extract on *A. pistaciae* population were determined and comparison of the mean of mortality % was done using SPSS software version17.

3. Results

The result of present study regarding the mortality % showed that the *V. odorata* extract is effective on *A. pistaciae* mortality. Also the result show that increasing the concentrations, increased mortality percentage (Table1). The highest mortality of adult insects recorded in highest concentration of studied plant extract and Spirotetramat pesticide. Also the lowest mortality was recorded for lower concentration.

The result regarding the LC_{50} of studied plant extract and pesticides on *A. pistaciae* show that they have high toxicity for this pest in adult stage. The LC_{50} of *V. odorata* extract was approximately equal to 5.67/1000. The LC_{90}, up and down limits of this extract were respectively equal to 31.00/1000, 23.38/1000 and 45.19/1000. The LC_{50}, up and down limits of Spirotetramat pesticide on *A. pistaciae* were 0.09/1000, 0.02/1000 and 0.31/1000, respectively. Also the LC_{90} for this pesticide was equal to 0.46/1000.

Table1: LC_{50} and LC_{90} values of Spirotetramat and *Viola odorata* extract on the adult stage of *Agonoscena pistaciae* (Hemiptera: Psyllidea)

	LC_{50} Confidence interval	**LC_{90} Confidence interval**	**Slope ± SE**	**X^2 (df)**	**p-value**
Viola odorata	5.67 (4.94– 6.56)	31.00 (23.38 – 45.19)	1.57±.12	13.14 (4)	0.0001
Spirotetramat	0.09 (0. 02 – 0.31)	0.64 (0.56 –0.79)	3.61±0.38	9.50 (4)	0.0001

With attention to Table 3, the *A. pistaciae* mortality percentages had significant different in different concentrations of Spirotetramat pesticide (F= 36.03, df= 4). The highest adult *A. pistaciae* mortality percentage using Spirotetramat pesticide was related to 0.450/ 1000 treatment (Table 2). The results in Table 2 show that increasing the insecticide concentration, increased the *A. pistaciae* adult mortality %. In all used concentration, morality percentage was classified in different groups and each concentration had significant different with other concentrations. The lowest *A. pistaciae* adult mortality % observed in 0.200/1000 concentration. In probit transformed responses diagram showed that with increasing the concentration, mortality probit increased accordingly (Figure1).

Table 2: Mortality percentage of Spirotetramat by different concentration on the adult stage of *Agonoscena pistaciae* (Hemiptera: Psyllidea).

Concentration	0.200/1000	0.245/1000	0.300/1000	0.367/1000	0.450/1000
Mortality %	29.37±3.94e	41.25±3.50d	52.50±3.56c	61.87±2.48b	78.75±1.82a

Table 3: Analysis of variance of the percentage mortality of different concentration of Spirotetramat on the *Agonoscena pistaciae* (Hemiptera: Psyllidea)

Resources changes	**df**	**Mean Square**	**F**	**P-value**
Concentrations	4	2875.93	36.03	0.0001
Error	35	79.82		
Total	39			

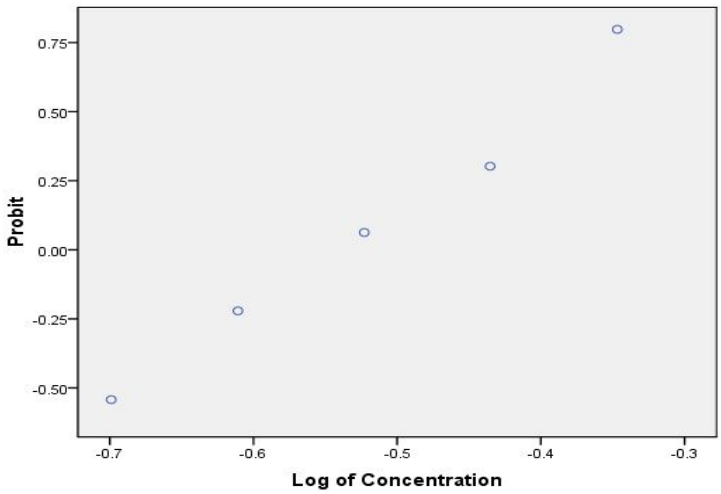

Fig1: Probit transformed responses diagram of toxicity of Spirotetramat on the *Agonoscena pistaciae* (Hemiptera: Psyllidea).

With attention to Table 5, the *A. pistaciae* mortality percentages had significant different in different concentrations of *V. odorata* extract (F= 38.69, df= 4). The highest mortality percentage of adult *A. pistaciae* using *V. odorata* extract was related to 16/ 1000 treatment (80.62±4.37) (Table 4). The results in Table 4 show that increasing the insecticide concentration increased the *A. pistaciae* adult mortality %. The lowest mortality % of *A. pistaciae* adult was observed in 1/1000 concentration (16.25±2.26). In probit transformed responses diagram *V. odorata* extract showed that with increasing the concentration, mortality probit increased accordingly (Figure2).

Table 4: Mortality percentage of *Viola odorata* extract by different concentration on the *Agonoscena pistaciae* (Hemiptera: Psyllidea)

Concentration	1/1000	2/1000	4/1000	8/1000	16/1000
Mortality %	16.25±2.26c	20.62±3.19c	47.50±5.08b	58.75±5.72a	80.62±4.37a

Table 5: Analysis of variance of the percentage mortality of different concentration of *Viola odorata* extract on the *Agonoscena pistaciae* (Hemiptera: Psyllidea)

Resources changes	df	Mean Square	F	P-value
Concentrations	4	5769.68	38.69	0.0001
Error	35	149.10		
Total	39			

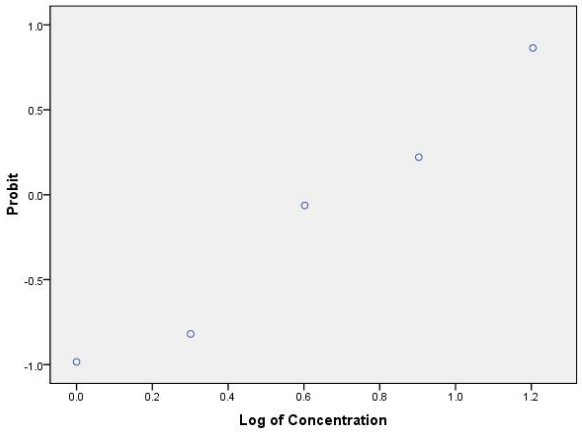

Fig 2: Probit transformed responses diagram of toxicity of *Viola odorata* extract on the *Agonoscena pistaciae* (Hemiptera: Psyllidea).

4. Discussion

Among insects, Psylla is regarded as an important pest in agricultural crops are in the world. Although there are several methods to control the psylla, but the most common method is based on pesticides application in many regions of the world, such as Iran. However, due to the gradual resistance evolution in natural populations of these psylla, its chemical control is less effective. On the other hand population density of psylla on host plant leaves have changed and complicated the pest control methods. Also with providing suitable condition, psylla is protected against pesticides treatments. In recent decades necessity the use of other methods has greater importance such as plant extracts application due to their volatility and very short-term persistence in the environment. Since the plant extracts usage reduce plant products pollution in major agricultural areas, the use of plant extract as alternatives to chemical pesticides have received important position. Because of *V. odorata* extract high medicinal value and importance and its cultivation adaptability in Iran country, cultivation and studying it's different features are regarded as main priorities in medicinal centers. In present study, with attention to this point that dangerous effects of chemical pesticides in recent decode have confused public minds and also many scientist are concentrated on appropriate solutions because of high biological and environmental pollutions and chemical toxic residual effects on agricultural crops, insect resistance against chemical insecticides valuable and useful insect reduction and finally nature imbalance and human's ever increasing needs. Based on different investigations on this subject, scientists are trying to use alternative methods for pests control such as biological control, producing resistant plants against different plant pests and diseases, hormonal control, pheromone application and using plant extract. Today there is this perspective that the insecticides can be produced using natural sources. Some of these resources are plants, micro Organisms and marine technologies (Isman 1996) [11]. The insecticide effects of *V. odorata* extract probably is because of cyclotie compositions. The first report of cyclotides insecticide activity was in the year 2001 which in it, the Kalata-B1 showed the Helicoverpa larva killing effects and their growth control, using an artificial feeding regime containing cyclotides (Jennings *et al*., 2001) [13]. When in one feeding regime, there is approximately same concentration of it in natural leaf tissue (approximately 8% µm/gr), Kalata-B1 effects as strong insecticide factor. These results have proved in different studies regarding Kalata-B2 (Jennings *et al*., 2005) [14] from *O. offinis* and also Cter M from *Clitoria ternatea* extract (Poth *et al*., 2011) [24]. It seems that the insecticide activity mechanism is membrane disturbance in middle parts of larval intestine which have eaten Cyclotides, are determined using electron microscopes studies (Barbeta *et al*., 2008) [3]. Although it is assumed that the insecticide activity, is primary biological performance of cyclotides (Craik, 2009) [8] but until new there is not regular reports about their experimentation on extensive range of insect species and also there is not probable synergistic effects of Cyclotides, with attention to this point that each plants can express different Cyclotides in their tissues commonly (Gruber *et al*., 2007) [9]. Evaluation the cross resistance of these insecticides with Neo-Nincotinoid insecticides seem essential and important. The *A. pistaciae* sensitivity to the pesticides reported by Talebi *et al*., 2001 [29] for the first time in Kerman province which was evaluated in relation to Fuzalone insecticide. Their results showed that the lowest sensitivity is related to Kerman and Rafsenjan population and the highest sensitivity is related to the Jabal-Barez region insect populations and also, resistance level for samples of Rafsenjan township was twofold that sensitive population. The result of our study showed that the *V. odorata* extract had strong killing effect on *A. pistaciae*. Also different studies showed that this plant extract has insecticidal effects, antimicrobial and antioxidant (Ghorbani *et al.,* 2012; Toiu *et al*., 2007) [27]. Recent researches showed that this plant is containing Cyclotide materials that has cellular killing effects (Saether *et al*., 1995). Totally our study had valuable finding regarding the killing effects of *V. odorata* extract and Spirotetramat pesticide on the *A. pistaciae* adult stage. Therefore with attention to these plant extract safely for human and other mammals, and their low stability in natural ecosystem, they can be appropriate alternatives of chemical pesticides and can be used as effective composition for *A. pistaciae* controlling. Also this plant extract and pesticide integration method can be used in IPM of *A. pistaciae.*

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