Journal of Agricultural Chemistry and Environment, 2017, 6, 165-174
http://www.scirp.org/journal/jacen
ISSN Online: 2325-744X
ISSN Print: 2325-7458

Evaluation of the Repellency Effect of Neem (*Melia azedarach*) Plant Extracts Based on the Mittler & Dadd Method

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How to cite this paper: Bina, Sh., Javadi, I. and Iravani, O. (2017) Evaluation of the Repellency Effect of Neem (*Melia azedarach*) Plant Extracts Based on the Mittler & Dadd Method. *Journal of Agricultural Chemistry and Environment*, **6**, 165-174.
https://doi.org/10.4236/jacen.2017.64011

Received: July 30, 2017
Accepted: November 5, 2017
Published: November 8, 2017

Abstract

Using insect repellent compounds, especially during the warm months, provides comfort for people and prevents spreading of the insect-borne diseases such as dengue fever, malaria and Leishmaniasis. Poisons derived from plants contain alkaloids, glucoside and some essential oils that are taken from plant tissues. They may have repellent and insecticide properties with little toxicity to humans and animals. In this study, Neem plant[1] from Bandar Abbas city was used to assess its repellency effect. Therefore, after drying and powdering using maceration method, final Neem extract concentration of 5%, 10% and 20% was provided. Mittler & Dadd methodology was hired to assess this effect. In this method, the antifeedant effect of the Neem plant extract was evaluated on cabbage-feeding aphid (*Brevicoryne brassicae*). After 24 and 48 hours, the reduced numbers of feces spot of aphids showed that the ingestion of the provided food mixture contained Neem plant extract was significantly decreased. Here it was shown that the Neem plant extract could be used as an efficient repellent.

Keywords

Neem, Insect Repellent Composition, *Anopheles stephensi*

1. Introduction

Insects can directly affect the health and comfort of human through the transmission of diseases. Diseases such as malaria, dengue fever and many viral conditions are transmitted through mosquitoes. Sometimes they do not transmit

[1]*Melia azedarach*.

any disease but they just annoy individuals with their bites. Their sting may be poisonous and cause itching and irritation [1]. Mosquitoes are from the dipterous insect family. Anopheles, Colexes and Aedes are among their important categories [2].

Repellents are substances used to protect humans, animals and plants from insects. These substances can repel insects from human by changing the host's smell. These materials are used to protect human from mosquito bites. Chemical repellent compounds usually have some unwanted effects. Synthetic toxins have also lost their importance, due to their carcinogenic effect [3]. Scientists think of using new repellent and insecticides that are derived from natural sources and do not have any carcinogenic effect. Herbal insecticides exist in the nature on their own. They are the most effective repellent and insecticides. It is because they have repellency and insecticides properties with minimal toxicity to animals and human [3]. One of the most famous plants in this field is Neem with the scientific name of *Melia azedarach*. There are several formulations of this plant available in the pharmaceutical market in the world. It is native to India. This plant is abundantly found in the southern Iran as an ornamental plant. There is another species of this plant in the north of the country that is called Bitter Olive. The olive is native to Iran. The two mentioned plants belong to Meliaceae and Terebinthales groups. Meliaceae contains 45 types and more than 750 species in the form of trees or shrubs that grow in tropical regions. This category has only one type in Iran that is called Melia. Two species of Neem and Bitter Olives are the only known species in the country. Bitter Olive with the scientific name of *Melia indica* is a tree with a height of 10 - 15 meters, which is native to the Himalayan region. It is also spread in the north of Iran and coastal forests of Caspian Sea, from Lahijan and Ramsar to Mazandaran and Miyan Darreh of Gorgan [3]. It is seen around rural houses and is also ornamental in some gardens. The last root shell of the tree is the most active part for medical and pharmaceutical purposes. The flowers and leaves of this tree are locally used to treat tension headaches in traditional western medicine. Fruit pulp and flowers ointment are used to eliminate lice, parasites and head pimples. The bark of the trees is anti-worm but nauseous if used in high doses. Its fruit with a Saccharin-like flavor is used to treat leprosy and tuberculosis. The oily compound obtained by breaking the seed has anti-fertility and anti-parasitic properties. It is also useful as local treatment for joint infections and resistant ulcers.

Neem (*Melia azedarach*) is a tree with a height of 15 - 30 meters, with a stem diameter of 30 - 90 cm. This tree is a native of South Asia and is widely distributed in India, Pakistan, Sri Lanka, Thailand, Malaysia and Indonesia. In Iran, it grows in the southern part of the country, including Bandar Abbas and Chabahar. This plant is also cultivated in the northwest and Moghan plain in Iran.

The bark of the Neem is used for traditional medicine in India and China to treat fever. Its fruit is used as a strengthening herbal medication [3]. Neem leaves are used as a disinfectant for animal wounds, insect repellants and fungicides, as

well as cosmetics. The bark of the tree enhances muscle tonicity. Its leaflets are useful for treatment of swollen lymph nodes. Azadirachtin is its main active compound, which can disrupt the physiological processes in arthropods [4]. Azadirachtin with brand name of Nimarin contains oil that is extracted from the seeds and leaves of the tree. It is one of the oldest and most popular plant pesticides due to its non-toxic effect to mammals as well as its low sustainability. This combination is welcomed by farmers who are interested in the healthy environment due to its inhibitory effect on worms, thrips and white flies. Neem is presented in various physical forms such as powder, oily extract, emulsion, etc. Applying freshly provided Neem solution is found to be effective on plants and insects in humid conditions in the evening. This solution loses its effect when exposed to sunlight within 8 hours after preparation. Spraying with Nimarin can be repeated every two weeks. Its molecular weight is 720 g/mol and its melting point is 160 centigrade. Its formula is $C_3H_{44}O_{16}$. The high value of Azadirachtin is due to its rapid decomposition in the environment with very low toxic effect on vertebrates. Its LD50 in mice is 500 milligrams per kilogram. Interestingly, insects stop feeding after eating a few nanograms of this compound. The Azadirachtin has often digestive effects on small insects while it has no similar effect on big insects like spiders and parasitoids [5]. This active compound affects on 400 types of insects. It is a healthy insecticide because of its minimal toxic off-target effect in the nature. It also plays an important role in pest-combined management (IPM[2]).

Physiological effects of Azadirachtin include its anti-nutritional effects which inhibit growth, reproduction and cellular processes. On the other hand, it increases mortality and also causes abnormal peeling in all tested species. The latter conditions are mainly due to the endocrine and growth inhibitory effect of Azadirachtin. Azadirachtin prevents cell division and protein synthesis by entering the cells. These effects also play a role in paralysis of the muscles and death of the cells. For example, in digestive system it inhibits the enzymes secretion. ED50[3] is 1 mg/g in many tested species of the insects. Compounds such as Quercetin and Nimbostrol along with a number of Lemonoid (Nimbin and its derivatives) have been identified in leaves of Neem tree [6]. Neem leaf contains 1.7% protein, 22.9% carbohydrate and various minerals. The active compounds of the Neem trees include Nimbin, Nimbidin and Nimbinon. All of these compounds are derived from tetracyclic terpene. Bitter Lemons, like Azadirachtin, Malononitrile and Solanine are other compounds of Neem. Strong anti-nutritional effects and the ability to disrupt the growth and reproduction of insects are proven by this compound, although its biochemical effect at the cellular level is still not well known. So far, this material has been tested on about 400 species of insects [7]. Spawning, anti-nutrition and repellency properties of Neem extract has been evaluated when it was mixed with the insect's diet and sprayed on plant

[2]Integrated pests management.
[3]Median effective dose.

organs [8]. Among the Iranian researchers, Urumchi and Laura [9] tested the effect of aqueous extract and three commercial formulations of the Neem on Alfalfa Weevil (*Hypera postica*). They have seen anti-nutritional, repellent and lethal effects for these compounds. Bayat Asadi and Pourghaz [10] described the useful effect of the Neem extract compared to other chemical compounds. They compare the effect of few herbal extracts on cottonseed white flies (*Bemisia tabaci*). Sadeqi have reviewed and compared sensitivity of white flies to aqueous extract of the seeds, Neem oil, Decamethrin insecticides (5.2% emulsion), Pirimiphos-methyl (50% emulsion), Oxidemeton-methyl (25% emulsion), Etofenproxi (20% emulsion) and Thiomton (25% emulsion). He believes that the mature insect is the most sensitive to the insecticide while spawn is the most resistant. His studies showed that 5% Neem oil had a strong repellency effect on the mature insects compared to the control.

Pradhan *et al.* [11] for the first time showed that the plants treated with blue suspensions of Neem seeds were immune to locusts. Batterworth and Morgan [12] separated Azadirachtin from Neem and prove its anti-nutritional effects at a concentration of less than 0.004 ppm on locusts. Hyde *et al.* [13] observed that by increasing the concentration of the Neem oil sprayed on plant, the number of two species of these insect decreases when the impact of Neem oil was assessed on three species of *Cicadella viridis*. Blaney *et al.* [14] have tested anti-nutritional effects of 40 herbal compounds including Azadirachtin and its derivatives on four species of butterfly. They found that Azadirachtin and Dihydro Azadirachtin had a greater insecticidal effect than the other compounds. Investigations of Wheathersbee and Tang [15] showed that anti-nutritional activity of Neem compounds occurs when using in high concentrations in many insects. In low concentrations, growth disturbances are the most commonly observed effect. Armes *et al.* reported that the toxicity of these compounds is very low when contacts with insect's skin, while it has a significant toxic effect on digestive system. This effect has been notable especially in the larvae of the noctuidae family. Sinha [16] explained that Neem seed extract could cause a 40% reduction in the boll worm population on bean plant. Spawning, anti-nutritional and repellent properties of Neem extract have been evaluated on the insects when Neem extract was mixed with diet and sprayed on plant. In this study, the repellent effect of the Neem grown in southern Iran (Bandarabas) was studied.

2. Materials and Methods

2.1. Preparation of Leaves of Neem Plant, Drying and Powdering of Leaves

The leaves of the Neem plant were collected from Bandar Abbas city. It was spread as a thin layer following confirmation by an expert in Department of Agriculture at Isfahan University of Technology. It was then dried in a shade at 25˚C - 28˚C and was later grinded by the MX.340N blender (Japan). Finally, the fine powder was ready to extract.

2.2. Extraction of Neem Plant

50 gr of the provided powder was poured in a 1 liter bottle. 70% alcohol was used as solvent for extraction of the powder to prepare hydro-alcoholic extract. [17] Thus, 300 ml of 70% ethanol was added to the solution and let it soak for three days. The latter was repeated trice. In total, the powder was completely soaked in 900 ml of 70% alcohol to obtain the extract. The bottle was then put in the Rotary IKARV 10 (Germany) to let the solvent to completely evaporate. A mass of sludge was obtained in the end. The total amount of sludge was about 100 ml. This was considered as Neem stock solution.

2.3. Manufacturing and Spray Preparation

The final concentrations of 5%, 10% and 20% of the Neem were prepared. To prepare the spray solution, the following procedure was performed: Since the plant extract has some oil, to prepare different concentrations, it was diluted with water. So we need materials that act as an emulsion and completely make homogeneous solution. For emulsifying the oily solution in water, Hydrogenated Castor Oil (MERK, Germany) and Polysorbate 80 or Tween (CRODA, Singapore) were added. 5 gr of each of the above compounds was added into a beaker. Then 5, 10 and 20 gm of the herbal extracts were added for concentrations of 5%, 10% and 20%. Using distilled water, the total volume came to 100 ml. The solution was slowly stirred over flame to achieve a homogeneous solution. The solution was then poured into spray containers.

2.4. Mittler & Dadd Method

The anti-nutritional effect of Neem plant was investigated on cabbage aphid (*Brevicoryne brassicae*) [18]. Twelve glass tubes were placed in 4 groups (using 5%, 10% and 20% concentrations of the Neem solution in addition to control group) with three replicates in each group. Filter papers were cut to the size of each tube, soaked in Bromocresol solution (for staining) and let them dry. The filter papers were then inserted into glass tubes—in a way that completely covers the inner wall of the tube. The head of each tube was covered by parafilm after marking the tubes. 0.2 ml of a mixed solution of Neutral Red, Sucrose and Neem Extract (5%, 10% and 20%) was poured over the parafilm. Then head of the tube was covered with another layer of parafilm. In this way the insect was able to land its own crater in the lower layer of para film and feed on food. 5% sucrose was used as control. For reassurance about the correctness of the test, three replicates were assigned for each tube, and then 10 aphids inserted in each tube by brush, slowly. The tubes were covered with cork which had small holes to ensure sufficient aeration of the aphids. Filtered papers were stained with Bromocresol Green to detect spots from aphid feces (**Figure 1**). Feces were appeared as blue spots on yellow filter paper. Then the number of stains was counted 24 and 48 hours after feeding the aphids. The results were analyzed with SPSS software. $P < 0.001$ was considered statistically significant. The Poisson regression model was

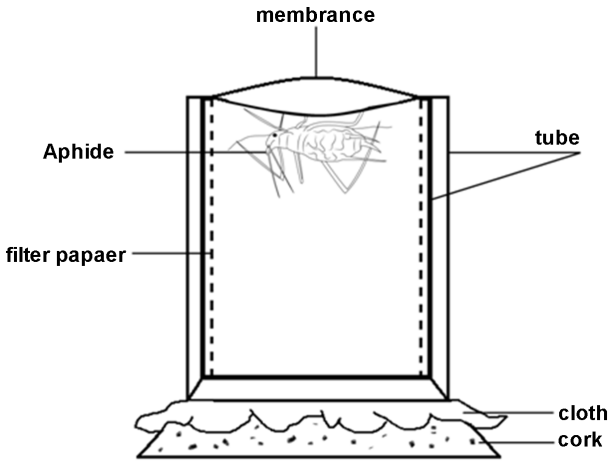

Figure 1. The schematic figure shows the Mittler & Dadd methodology.

used to investigate the effect of concentration and also the formulation type on the number of observed spots on the filter paper.

3. Results

This research was conducted to investigate the anti-nutritional effect of Neem plant based on Mittler & Dadd method on the *Brevicoryne brassicae* cabbage aphid by comparing the number of stains remained from the aphids after 24 and 48 hours. According to the results shown in Table 1, insect nutrition reduced after 24 hours, compared to control group.

Regression Model for Modeling of Stains

Figure 2 shows the average number of stains using three Neem extract concentrations (5%, 10% and 20%) over a period of 24 and 48 hours after feeding. It was identified that by increasing Neem concentration, the number of stains from the feces of aphids significantly decreases. In this regression model, the value of the Deviance Index is equal to 31.848 with 35 degrees of freedom. In Poisson regression model, the ratio of this index to the degree of freedom is expected to be 1. Deviation from 1 is a sign of deviation from the main hypothesis of the Poisson regression model and is more or less dispersion in the response variable. The value of Deviance/DF in this regression model is 0.910 which confirm the correctness of the initial assumption and the fitness of the model to the data.

According to Table 2, the effect of concentration and type of formulation is significant. The significance of the time difference coefficient showed that, after 24 hours, the average number of spots significantly reduced. According to the estimate obtained in the half formulation, the average number of stains in the first 24 hours was $e^{2.07}$, which reduced to $e^{0.504}$ (65% reduction) in the second 24 hours. The following formula was used to predict the number of stains based on concentration (Con), which is shown in the above table.

Table 1. Comparison of the number of stains from cabbage aphid after 24 and 48 hours feeding based on the Milter & Dadd method.

Control			20%			10%			5%			Concentration
Repeat			Repeat			Repeat			Repeat			Time/hour
3	2	1	3	2	1	3	2	1	3	2	1	
7	6	8	0	3	3	2	5	3	5	3	6	24
10	9	11	0	0	0	0	2	1	2	0	4	48

Table 2. Milter & Dadd statistical analysis.

Sig.	Degree of freedom	The second exponentiation value of the Wald[4] statistics	Standard error	Estimation	
0.001	1	241.813	0.133	2.07	**Width from origin**
0.001	1	13.771	0.136	**0.504**	**Time difference***
0.032	1	4.589	0.113	0.241	**Formulation**
0.001	1	58.637	0.012	−0.094	**Concentration**

*Average difference of spots after 48 hours with 24 hours.

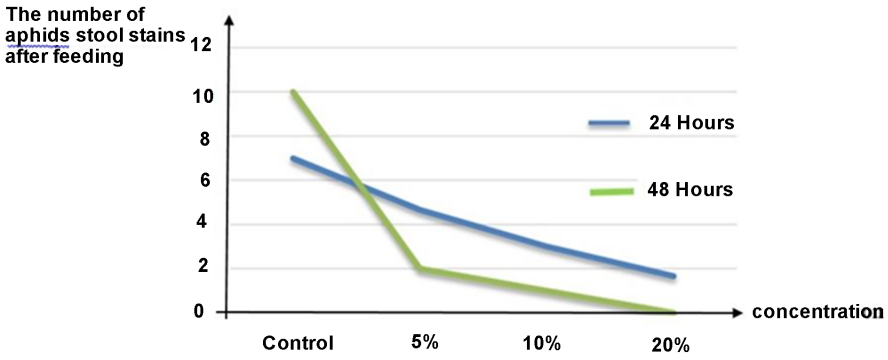

Figure 2. The number of aphid feces stains after 24 and 48 hours feeding with 5%, 10% and 20% Neem extract.

$$\log(\text{Average number of spots}) = (\text{Constant}) \cdot \text{Width from origin} + a_1 x(\text{Time}) + a_2 + a_3 x(\text{Concentration})$$

$$\ln(\mu) = 2.070 + 0.504 \times (\text{Time}) + 0.241 - 0.094 \times Con$$

In the above regression model "Time" is a variable that accept two values, zero and one. The value of zero is for a period of 24 hours and the value of one is for 48 hours. Thus, the value of 0.504 was applied when a prediction for 48 hours was required. **Figure 3** shows percentage reduction in the number of stains for

[4] $\text{Wald statistics} = \dfrac{\text{Estimate}}{\text{Standard error}}$

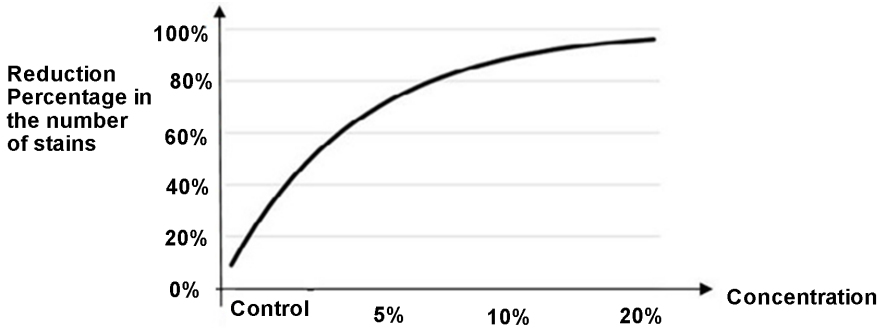

Figure 3. Percentage of the stains in correlation with different concentrations of Neem extract.

different concentrations of Neem formulation in 24-hour period, which is derived from the above equation.

The analysis suggests that increasing the concentration of the spray solution reduces the number of spots.

4. Discussion

The emergence of harmless compounds for the environment, including nutrition inhibitory compounds has created a new perspective on pest control. Using this material can be helpful in controlling arthropod pests, monitoring pest's population, disrupting the pest reproductive behavior, fighting with pests and contributing in human healthcare. Today, human effort has focused on the production and use of safe and low-risk compounds due to the increasing use of chemical compounds in controlling insects and the negative consequences of these compounds in the environment. Using herbal constituents is one of these methods. The Neem extract, and especially its main ingredient, "Azadirachtin" is one of the strongest natural substances that negatively control insects and has been used against many pests in medicine and agriculture. The observations of Guerrini *et al.* in 1998 on the flea of dogs and cats showed that the Azadirachtin could create as good protection as a mixture of citronella and Deet. The Milter & Dadd experiment was tried to compare anti-nutritional effects of Neem herb on cabbage aphid after 24 and 48 hours feeding. The test was carried out with the ability to feed the insect from the treated diet with a mixture of sugar and color. If the insect did not feed, by assuming the same nutritional value for control group, they concluded that it was a repellent compound. The latter was found by our experiment with Neem extract. The insects in the test group fed a mixture of Neem extract. According to the results shown in Table 1, we observed a significant decrease in the number of spots from aphid feces in different concentrations compared to control group. This represents anti-nutritional effects of this plant. The experiments showed the interesting effect of the Neem extract as a repellent on Anopheles mosquitoes. More comprehensive studies are required to study the longevity, strength and effectiveness of Neem oil on insects. One of the disadvantages of Neem compounds is lack of a quick fatal effect. This slow-acting effect may have a negative impact on farmers for using the compound, because

they usually seek for compounds that are rapidly and evidently induce a desired effect. Using a side-compound in a synergistic mixture may satisfy the farmers by immediate impact. Another solution is to provide enough instructions for the farmers to encourage them to use the compounds with less environmental hazardous impact. This study showed that the aphids are sensitive to the Neem compounds. Further research is advisable to investigate the effect of the Neem in farm environment. Therefore, developing and applying new plans to use Neem solutions against pests are required so that it will suit its place as an effective and environment-friendly repellent in the near future.

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