



## **Larvicidal and Adulticidal Activities of Neem Oil against *Anastrepha fraterculus* (Wied.) (Tephritidae)**

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### **Authors' contributions**

*This work was carried out in collaboration among all authors. Author AR designed the study and wrote the first draft of the manuscript. Authors SBS, EMS and LRFL contributed to the maintenance of fruit-fly colonies and the development of the experiments. All authors read and approved the final manuscript.*

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### **ABSTRACT**

In the laboratory, a neem oil-based formulation was evaluated for its insecticidal potential against the South American fruit fly *Anastrepha fraterculus* (Wied.), as efficient alternative for growers harvest fruits more harmless for the human consumption. The commercial product was evaluated against eggs *in vitro*, guavas infested with eggs and young larvae, and adults. The application of 0.8% neem oil significantly reduced the larval hatching of *A. fraterculus*. Neem oil at 0.4, 0.6 and 0.8% significantly reduced the number of pupae and adults per fruit infested with eggs. Guava infested with *A. fraterculus* eggs and treated with neem oil at 0.8% exhibited a reduction of 90.0% and 92.6% of pupae and adults per fruit, respectively. The same dosage to guava infested by larvae achieved a 44.6 and 51.4% reduction of pupae and larvae, respectively. At 360 minutes after treatment, 0.8% of neem oil provided 67.9% of corrected adult mortality. This double insecticidal effect of neem oil and lack of phytotoxicity in ripe guavas at the tested concentrations demonstrate its potential in biorational management.

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## 1. INTRODUCTION

Guava (*Psidium guajava* L.) is grown successfully in a wide range of climatic and edaphic conditions [1], in rural and urban areas, or among native vegetation on disturbed lands. In 2016, the southeast region of Brazil produced 44.3% of all guava grown in the country, and the state of São Paulo alone produced 34.92%, corresponding to 146,943 tons [2].

Tephritids are pests in almost all fruit growing areas of the world [3]. Some fruit fly species (Tephritidae) are the key pests of guavas [4]. The South American fruit fly, *Anastrepha fraterculus* (Wiedemann) has been detected from Mexico to Argentina and Uruguay and represents the main pest species of its genus in Brazil [5], infesting 116 plant hosts [6]. This native species infesting many horticultural crops [6,7], especially common guava (*P. guajava*) and other Myrtaceae in Brazil [8,9].

Fruit growers have constantly applied broad-spectrum synthetic insecticides, mainly by cover spray [10]. However, in our conditions, the continuous spraying of insecticides for controlling fruit flies in guava crops has caused infestation peaks of secondary pests, such as mealybugs and psyllids, and risks spreading residues during distribution.

Avoiding the use of synthetic pesticides is a way to reduce their negative impacts on biological control organisms [11] and environmental safety [12]. Adverse side effects of synthetic insecticides have led to the investigation of alternatives such as natural plant protection agents [13]. Research on natural products for use in agriculture was sparse for many years, but the use of the natural products is increasing for eco-friendly pest management [14] and for supply fresh fruits more harmless for the human consumption.

Extracts of neem *Azadirachta indica* A. Juss. (Meliaceae) are among the four major types of botanical products used worldwide for insect control. Neem oil, obtained by cold-pressing seeds, can be effective against soft-bodied arthropods. Neem seeds contain more than a dozen azadirachtin analogues, but the major compound is azadirachtin [15]. Azadirachtin has antifeedant, insect growth regulatory (IGR), anti-ovipositional and fecundity- and fitness-reducing

properties [16]. Lepidoptera and Diptera species are the majority susceptible to neem products [17]. Depend on the fruit, the neem application on third-instar larvae and pupae of fruit flies, azadirachtin reduces the emergence of adults and the longevity [18]. In other cases, such when applied to melon fruit, neem oil reduced the infestation of *Bactrocera cucurbitae* (Coquillet) but did not affect its emergence [19].

Perceptions of pesticide efficacy were found to play a major role in the behavior of farmers towards the use of alternative compounds [20]. Despite extensive studies for controlling insects, data on the effect of neem oil in fruit flies are scarce and there is no information in the literature of the action of that compound and its effective doses on immatures of *A. fraterculus*. Here, we report the results of laboratory neem oil exposure on *A. fraterculus* eggs *in vitro*, on immature stages from infested guava and survivorship of adults, for the purposes of controlling the fruit fly infestation into fresh fruit and under the tree canopy.

## 2. MATERIALS AND METHODS

*Anastrepha fraterculus* were obtained from laboratory colonies that have been maintained at the Instituto Biológico, in Campinas, State of São Paulo (SP), Brazil, since 2002 [10]. Fitoneem (neem oil 85% w/v; Dalneem Brasil Comércio de Produtos Agropecuários Ltd.) at multiple dilutions was evaluated against immature stages and adults of *A. fraterculus*. The product is an authorized plant protection product in Brazil for controlling of whiteflies *Bemisia tabaci* Gennadius and *Bemisia argentifolii* Bellows & Perring (Aleyrodidae) in all plant hosts.

### 2.1 Effects of Neem Oil Dilutions on Eggs *In vitro*

The eggs of *A. fraterculus* were collected into perforated red PVC tubes (14.5 cm high and 5.0 cm in diameter), equipped with lids and wrapped laterally by parafilm. The tubes were filled with distilled water and installed at the base of the cage. Twenty eggs (up to 6 hours old) were dispensed into Petri dishes (3.5 cm diameter with lid) containing filter paper and respective neem concentrations. The commercial product was diluted in distilled water at 0.2, 0.4, 0.6 and 0.8% (v/v). Dead eggs were evaluated 96 hours after

treatment and compared with eggs of untreated control (water).

## 2.2 Effects of Neem Oil on Eggs and Larvae in Fruit

The ovicidal and larvicidal effects of neem oil were evaluated using infested guavas cv. Tailandesa (red pulp). The mean weights of guava infested by eggs and larvae of *A. fraterculus* were 189.8 and 167.4 g, respectively. The fruit was exposed for 24h into jail (60 × 50 × 40 cm) to ten *A. fraterculus* pregnant females (11 – 16 days old) per fruit. Fruit infested with eggs (6 – 30h old) and with the first instar larvae were exposed to neem oil at the same treatments described above (section 2.1) for eggs *in vitro*. The fruit was submerged for 30 sec in the respective neem oil solution in a glass beaker (2.0 L). After exposure, guavas were individually kept in circular plastic containers (1.00 L) with approximately 1.5 cm of vermiculite substrate. The containers were capped with voile bound with an elastic. The fruit were kept a room at 24.1°-25.7°C and 57–80% RH. We evaluated the number of recovered pupae and adults per fruit. Each fruit was considered one replication.

## 2.3 Effect of Neem Oil on Adults

*Anastrepha fraterculus* adults were exposed to 0.1, 0.2, 0.3%, 0.4%, 0.6% and 0.8% (v/v) of the commercial neem oil (the untreated control was water only). The pH of the solutions was determined with a pH meter Alphaslab (model PA 200).

Five females and five males of 9–14d old were captured from a laboratory cage in glass tubes that were plugged with cotton wool. Prior to the application, the tubes were stored in a refrigerator at approximately 1.5°C for 4 minutes. Flies were transferred immediately to the shallower half of glass Petri dishes (150 mm diameter) and submitted to 2 mL of an insecticide suspension under a Potter spray tower at 60.0kPa. After the treatment, the flies were maintained at 24.5–25.4°C and 75-80% RH, deprived of water and food.

Evaluations of cumulative mortality were conducted at 15, 30, 45, 60, 90, 120, 150, 180, 240, 360 min and 24 h after initial exposure. Irreversible knockdown, followed by the death of the adults, was the criterion to determine mortality [21]. Each Petri dish was considered one replication per treatment (in total 10 replicates).

## 2.4 Phytotoxicity Determination

The mature fruits were submerged for 30 seconds in a beaker (2,000 mL) containing neem oil solutions at 0.2, 0.4, 0.6 and 0.8%. Forty eight hours after application, the fruit surface was evaluated based on the ascendant scale of phytotoxicity (0–3), corresponding to injury rating of healthy (0), slight (1), moderate (2) and severe (3) [22,23].

## 2.5 Statistical Analysis

We used ten replicates in all tests. All data were analysed by ANOVA followed by the Tukey mean comparison test. Two-factor ANOVA was used to compare the mortality of females and males (Sisvar, version 5.6) [24] at  $P < 0.05$ .

## 3. RESULTS

### 3.1 Effects of Neem Oil Dilutions on Eggs *In vitro*

The application of neem oil diluted at 0.8% significantly reduced the larval hatching of *A. fraterculus* (16.5 percentage points), while the other concentrations were similar to the control (Table 1). The mortality in other neem oil concentrations was similar to that of the control.

**Table 1. Mean number ( $\pm$  SE) of dead eggs of *Anastrepha fraterculus*, 96 hours exposure to different concentrations of commercial neem oil *in vitro* (n=20)**

Treatment	Mean	Hatchability (%)
Neemoil 0.2%	2.9 $\pm$ 0.4a	85.5
Neemoil 0.4%	3.2 $\pm$ 0.6a	84.0
Neemoil 0.6%	3.1 $\pm$ 0.6a	84.5
Neemoil 0.8%	5.9 $\pm$ 0.6b	70.5
Control	2.6 $\pm$ 0.4a	87.0

Means followed by different letters are significantly (Tukey HSD test,  $p < 0.05$ )

### 3.2 Effects of Neem Oil on Eggs and Larvae in Fruit

There were significant differences in recovered pupae and adults from guavas infested with *A. fraterculus* eggs for all neem concentrations except 0.2% (Table 2). Neem oil at 0.4, 0.6 and 0.8% showed a similar mean number of pupae and adults. Guava infested with *A. fraterculus* eggs and treated with neem oil at 0.8% exhibited a reduction of 90.0% and 92.6% of average pupae and adults per fruit, respectively. No

statistically significant differences among emergence rates were detected.

Guava infested with *A. fraterculus* first instar larvae and treated with 0.8% neem oil showed significantly lower recovered pupae and adults per fruit, at 44.6 and 51.4% of reduction, respectively (Table 3). Neem oil at 0.6% and 0.8% dilutions allowed for the production of significantly similar quantities of adults of *A. fraterculus* per fruit, which differed statistically from the control. Neem oil affected the emergence rate in the lowest and the highest concentrations.

### 3.3 Effects of Neem Oil on Adults

The pH of 0.1, 0.2, 0.3, 0.4, 0.6 and 0.8% neem oil were 8.3, 8.1, 7.8, 7.6, 7.5 and 7.0,

respectively. Up to 120m post-exposure, all concentrations of neem oil were similar to the untreated control (Table 4). At 180 and 360 minutes, 0.8% of neem oil differed from the control, reaching 53.3 and 67.9% the corrected mortality [25], respectively. Substantial incremental mortality of adults was observed between 45 and 60 min after treatment (Fig. 1). Except for flies of untreated control (no available food), almost no incremental mortality at all concentrations of neem oil was detected between 360 min and 24 hours of exposure (Table 4). No statistical differences on mortality were detected between sexes of *A. fraterculus* exposed to all neem concentrations at 360 minutes after treatment (Table 5). The number of females of *A. fraterculus* exposed to 0.8% of neem oil differed statistically from the control, reaching 68.9% of corrected mortality.

**Table 2. Mean number of pupae and adults ( $\pm$  EP) per guava infested by *Anastrepha fraterculus* eggs after laboratory treatment with neem oil and respective emergence percentages**

Treatment	Pupae	Adults	Emergence (%)
Neemoil 0.2%	76.50 $\pm$ 15.47ab	44.60 $\pm$ 11.28ab	47.70 $\pm$ 11.21a
Neemoil 0.4%	45.00 $\pm$ 9.27bc	23.90 $\pm$ 6.04bc	46.56 $\pm$ 9.53a
Neemoil 0.6%	36.50 $\pm$ 9.39bc	20.50 $\pm$ 5.68bc	41.88 $\pm$ 9.72a
Neemoil 0.8%	11.90 $\pm$ 9.19c	4.80 $\pm$ 1.65c	23.69 $\pm$ 6.97a
Control	118.90 $\pm$ 21.77a	65.10 $\pm$ 13.23a	54.17 $\pm$ 10.34a

Means followed by different letters are significantly (Tukey HSD test,  $p < 0.05$ )

**Table 3. Mean number of pupae and adults ( $\pm$  EP) per guava infested by *Anastrepha fraterculus* first-instar larvae after laboratory treatment with neem oil and respective emergence percentages**

Treatment	Pupae	Adults	Emergence (%)
Neemoil 0.2%	110.40 $\pm$ 20.05ab	95.30 $\pm$ 17.47ab	81.62 $\pm$ 14.04a
Neemoil 0.4%	104.60 $\pm$ 19.87ab	98.50 $\pm$ 19.08ab	90.65 $\pm$ 14.96ab
Neemoil 0.6%	102.60 $\pm$ 17.40ab	89.10 $\pm$ 15.26b	86.83 $\pm$ 14.25ab
Neemoil 0.8%	81.10 $\pm$ 16.56b	68.80 $\pm$ 14.48b	80.77 $\pm$ 13.35a
Control	146.40 $\pm$ 25.40a	141.50 $\pm$ 24.54a	96.72 $\pm$ 15.70 b

Means followed by different letters are significantly (Tukey HSD test,  $p < 0.05$ )

**Table 4. Mean cumulative number of dead females and males ( $\pm$  EP) of *Anastrepha fraterculus* after exposure to different concentration of commercial neem oil under Potter tower (n = 10)**

Treatment	Time of exposure				
	60 min	120 min	180 min	360 min	24 h
Neemoil 0.1%	3.30 $\pm$ 1.14a	3.30 $\pm$ 1.14a	3.30 $\pm$ 1.14ab	3.90 $\pm$ 1.07ab	3.90 $\pm$ 1.07ab
Neemoil 0.2%	3.30 $\pm$ 1.18a	3.30 $\pm$ 1.18a	3.30 $\pm$ 1.18ab	3.40 $\pm$ 1.20ab	3.40 $\pm$ 1.26ab
Neemoil 0.3%	4.20 $\pm$ 1.14a	4.30 $\pm$ 1.18a	4.40 $\pm$ 1.22ab	4.70 $\pm$ 1.23ab	4.70 $\pm$ 1.23ab
Neemoil 0.4%	3.10 $\pm$ 0.96a	3.60 $\pm$ 0.83a	4.90 $\pm$ 1.00ab	5.40 $\pm$ 1.03ab	5.40 $\pm$ 1.03ab
Neemoil 0.6%	4.10 $\pm$ 1.39a	4.10 $\pm$ 1.34a	4.50 $\pm$ 1.38ab	5.10 $\pm$ 1.40ab	5.10 $\pm$ 1.40ab
Neemoil 0.8%	5.80 $\pm$ 1.39a	5.90 $\pm$ 1.42a	6.70 $\pm$ 1.17b	7.40 $\pm$ 0.93b	7.40 $\pm$ 0.93b
Control	1.80 $\pm$ 0.63a	1.80 $\pm$ 0.63a	1.80 $\pm$ 0.63a	1.90 $\pm$ 0.60a	1.90 $\pm$ 0.60a

Means in the same column followed by different letters are significantly different (Tukey HSD test,  $p < 0.05$ )

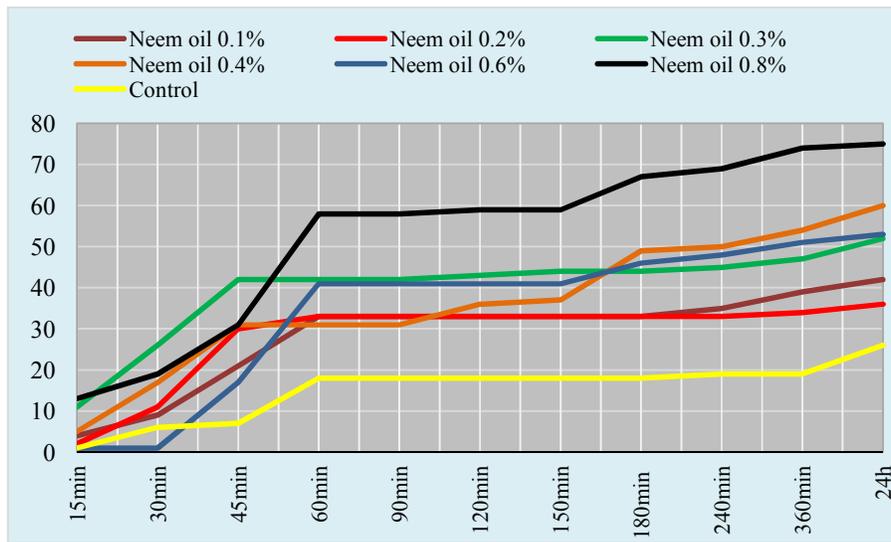


Fig. 1. Cumulative mean number of dead adults (females + males) of *Anastrepha fraterculus* at different times after laboratory neem oil treatment (n=100)

Table 5. Number of adult *A. fraterculus* dead 24 hours after exposure to different concentrations of neem oil under Potter tower (n=5)

Treatment	Female	Male
Neemoil 0.1%	1.10 ± 0.48 abA	2.80 ± 0.74 abA
Neemoil 0.2%	2.50 ± 0.83 abA	0.90 ± 0.50 aA
Neemoil 0.3%	2.60 ± 0.69 abA	2.40 ± 0.67 abA
Neemoil 0.4%	2.40 ± 0.62 abA	3.00 ± 0.54 abA
Neemoil 0.6%	2.60 ± 0.76 aA	2.50 ± 0.67 abA
Neemoil 0.8%	3.60 ± 0.54 bA	3.80 ± 0.55 bA
Control	0.50 ± 0.27 aA	1.40 ± 0.67 abA

Means followed by similar upper-case letters in the row and similar lower-case letters in the column are not statistically different using Tukey HSD test,  $p < 0.05$

### 3.4 Phytotoxicity

There were no significant symptoms of phytotoxicity among the treatments (Tukey's test;  $P < 0.05$ ). The means of the scale notes observed were 0.1, 0.2, 0.2, 0.3 and 0.0 for neem oil at 0.2, 0.4, 0.6 and 0.8%, and the control, respectively. No individual fruit scored higher than 1.

## 4. DISCUSSION

Azadirachtin is a typical representative of botanical pesticides, is biologically active against a variety of insects and has shown growth and developmental inhibition effects in the oriental fruit fly *Bactrocera dorsalis* (Hendel) [26]. In our study, 0.8% neem oil against *A. fraterculus* eggs *in vitro* induced reduction of larval eclosion and the other concentrations were not effective.

Testing lower doses of neem oil, Mahmoud & Shoeib [27] obtained *in vitro* reduction of larval hatchability of *Bactrocera zonata* (Saunders) above 18.7 ppm.

Azadirachtin did not avoid oviposition of *B. dorsalis* in guava [28]. Therefore, oviposition-deterrent is not a tactic for neem against fruit flies. In our study, neem oil above 0.4% applied onto infested guava during embryonic development reduced the pupal and adult populations of *A. fraterculus*, similar to studies in other species such as *B. cucurbitae* in melon [19]. In addition, a few hours after treatment, neem causes feeding inhibition [29].

Azadirachtin showed larvicide activity against *Ceratitis capitata* (Wiedemann) neonates *in vitro* for neonates [30] and third-instar larvae [31]. Neem oil at 0.8% seems to show a depth effect

[16,32] in tested guava, reducing the number of pupae and adults of *A. fraterculus*. Systemic and translaminar action of neem oil was reported in leaves of tomato for the effective control of *Tutaabsoluta* (Meyrick) (Lep.: Gelechiidae) in tomato [33].

However, guava infested with *A. Fraterculus* eggs and treated with neem oil at 0.8% showed a substantial reduction in pupae and adults, reaching almost double that guava exposed to larvae. Further studies should be conducted to determine the neem penetrating characteristics inside the fruit [30]. The recommended doses for whiteflies according to the manufacturer's instructions ranged from 0.75 to 1.50% v/v. The neem oil dilutions tested in the present study are within or below the product's commercial dose range, which did not cause phytotoxicity symptoms in mature guava. Care must be taken with the application of neem on young fruits (nonbagged) to reduce the risk of phytotoxicity.

In our study, the lethal and sublethal effects of neem oil occurred substantially during the first- and/or second-instar larval stages of *A. fraterculus*, providing subsequent effects on the emergence rate. Similar results are found by Zebitz [34], when treating the fourth instar larvae of *Aedes aegypti* L. with neem diluted in water, resulting in a conspicuous growth-disrupting effect during imaginal development.

Azadirachtin induces apoptosis, destroying the midgut cell structure and intestinal walls, affecting the digestion and absorption of carbohydrates, fats, proteins, vitamins, minerals, amino acids, sugars and vitamins [26]. The suppressive effect of azadirachtin on tephritid emergence appeared to be a result of the inability of treated flies to expand their ptilina and general paralysis [18].

The neem oil showed a tendency to increase the mortality of *A. Fraterculus* adults as oil concentrations increased and, at 0.8%, showed a reasonable mortality rate 24h after treatment. Our results agree with Stark et al. [18] that the susceptibility to azadirachtin was not sex-dependent, observed in *B. dorsalis* and *C. capitata*.

The use of azadirachtin seems to be a great alternative to synthetic insecticides for guava growers because of the effects on both immature and adult *A. fraterculus*. In addition, commercial

neem products are safe to mammals compared to synthetic agrochemicals [35], predators [36] and non-targeted biological systems [37]. The adoption of required neem oil doses for controlling fruit pests, depend on the further studies to ensure a minimal potential risk to *humans*, animals and the environment.

## 5. CONCLUSION

Neem oil applied on guavas infested with eggs and young larvae of *A. fraterculus* reduced the infestation, resulting in fewer pupae and adults per fruit. The adulticide effect of neem oil was more pronounced at 0.8% v/v. This double effect of neem oil results indicates a biorational pest management technology available for the fruit producers.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

It is not applicable.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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