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In vitro studies on the insecticidal activity of nimbolide against fall armyworm (*Spodoptera frugiperda*)

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Abstract
The use of organosynthetic pesticides in pest management results in a number of issues that includes the loss of natural enemy populations and development of the resistance in the pest. To overcome this issue in pest management specific pesticides or biopesticides are generally recommended. In this study, we isolated nimbolide, a tetranortriterpenoid biomolecule from *Azadirachta indica* leaves and tested *in vitro* for its insecticidal efficacy against *Spodoptera frugiperda*. Insecticidal activity exerted by nimbolide and neem leaf extract was experimented in dose dependent manner and the results indicated that the maximum mortality of 93.33 and 90.00 per cent was observed at 10000 ppm on sixth day of treatment with nimbolide and neem leaf extract. Nimbolide also causes growth abnormalities in larvae as well as prolonging their growth when compared with the neem leaf extract. The present study clearly indicated nimbolide has insecticidal activity and it can be utilized for the development of nimbolide-based biopesticides.

Keywords: nimbolide, *Spodoptera frugiperda, Azadirachta indica,* biopesticides

1. Introduction
The fall armyworm (FAW), *Spodoptera frugiperda* (Lepidoptera: Noctuidae) is a migrating polyphagous pest that is native to America's sub-tropical and tropical regions and is now spread globally (Ashley *et al*., 1988; Goergen *et al*., 2016; Nagoshi *et al*., 2008) [2, 8, 16]. It is one of the most economically damaging insect pests, infesting maize, cotton, rice, soybean, sorghum, and vegetables. Over-infestation, along with substantial economic loss, has led to an increased dependency on insecticide spraying. As a result of the widespread and indiscriminate use of insecticides, fall armyworm (FAW) populations have developed resistance to a variety of insecticide classes, including benzoylureas, organophosphates, pyrethroids, and carbamates (Chen *et al*., 2019; Diez and Omoto, 2001; Yu, 1991, 1992 and Yu *et al*., 2003; Carvalho *et al*., 2013) [5, 7, 27, 28, 29, 4]. The continued use of these pesticides has a negative impact on soil health (Tripathi *et al*., 2020) [26], human health (Grewal *et al*., 2017) [10], and environment and ecosystem (Mahmood *et al*., 2016) [15]. Botanical pesticides (botanicals) derived from plants are utilized as a substantial alternative to synthetic pesticides in Integrated Pest Management (IPM). These are secondary metabolites (phytochemicals) found in nature that have anti-feeding, anti-microbial, insecticidal, and repelling properties (Grdisa and Grsic, 2013) [9].

Neem (*Azadirachta indica*) is the most common herb utilized as one of the source of botanical insecticide. *Azadirachta indica*, a member of the Meliaceae family, offers a wide range of therapeutic qualities (Bhowmik *et al*., 2010) [3]. The therapeutic qualities are due to the presence of phytochemicals that includes diterpenoids, triterpenoids, tetranortriterpenoid, steroids, flavonoids, coumarins, hydrocarbons, fatty acids that have been found in different parts of this tree (Koul *et al*. 1989; Tan *et al*., 2011) [12, 24].Nimbolide is one of the most important limonoids compound that had its occurrence in the leaves of *A. indica*. Nimbolide (5, 7, 4'-trihydroxy-3', 5'-diprenylflavanone) is a tetranortriterpenoid molecule with a limonoid skeleton, an unsaturated ketone, and a lactone ring (Anitha *et al*., 2006) [1].

2. Materials and Methods
2.1 Plant material and insect culture
Neem (*Azadirachta indica*) leaves were harvested from healthy trees at Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu (11°00'41.8"N76°56'11.5"E). Leaves were dried for 6-7 days and pulverised into fine powder. For *in vitro* insecticidal investigation, *Spodoptera frugiperda* culture was reared in Insect Bioassay Laboratory at Department of

Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu.

2.2 Nimbolide isolation
Nimbolide was isolated by adapting the procedure given by Tong *et al*., (2020) [25]. Initial extraction consists of 450 g of neem leaf powder steeped in 1500 ml of methanol for three days and concentrated *in vacuo* to yield dark green oil. It is subjected to column chromatography for further separation. Nimbolide rich fractions that are eluted at the tail end of blue fraction of first column chromatography were further purified by subjecting it to second column chromatography. Here, the brown fractions were concentrated to get a brown colour solid which was triturated with dichloromethane and hexane to yield white powder of nimbolide (800mg).

2.3 *In-vitro* insecticidal assay
2.3.1 Neem crude sample preparation
100 g of neem leaf powder was steeped in 300 ml of methanol for three days. The filtered methanol was then evaporated *in vacuo* after passing through a celite column to remove the contaminants present in the extract. The dark green residue was diluted with methanol to provide concentrations of 1000, 2500, 5000, 7500, and 10000 ppm.

2.3.2 Nimbolide sample preparation
100 mg of pure nimbolide was dissolved in 10 ml of methanol to make nimbolide stock. The working concentration was diluted with methanol from the stock solution to obtain concentrations of 1000, 2500, 5000, 7500, and 10000 ppm.

2.4 Antifeedant activity
The leaf disc method (Singh and Pant, 1980) [22] was used to test the antifeedant properties of neem leaf extract and nimbolide against *S. frugiperda* at various concentrations. Fresh and tender maize leaf (1 cm^2 x 1 cm^2) was prepared and placed in a Petri dish (15 x 90 mm) containing moist Whatman filter paper no 1. The leaf disc was covered with different concentrations of compounds (ten µl each side) and left to dry in the open air. Leaves that were treated with methanol and water were used as negative control. Each treatment consisted of thirty neonate larvae with three replications. For six days, the experiment was conducted in a controlled environment with a temperature of 26 ± 2 °C and a relative humidity of 60%. The larval mortality and development were recorded up to 6 days and the larval mortality was expressed in percentage.

3. Statistical analysis
The bioassay was carried out in three-replications in completely randomized design. The impact of several treatments on insect mortality was studied using one-way analysis of variance (ANOVA). Duncan's multiple range test (DMRT) was used performed in Statistical Package for Social Sciences (SPSS version 16.0. Chicago, SPSS Inc, USA)to compare the treatment means at a 5% significance level.

4. Results
4.1 Nimbolide isolation
About 800mg of nimbolide was isolated from the methanolic extract of *A. indica* leaves. The isolated nimbolide was confirmed by ^1H NMR and ^{13}C NMR spectrophotometry. The spectral results are in accordance with spectral details that are reported previously (Kigodi *et al*., 1989; Dhanya *et al*., 2015) [11, 6].

Nimbolide*:*^{13}C NMR (125 MHz, $CDCl_3$) δC: 200.6 (CO), 174.8 (COO), 173.0 (COO), 149.6 (CH), 144.8 (C), 143.2 (CH), 138.9 (CH), 136.4 (C), 131.0 (CH), 126.5 (C), 110.3 (CH), 88.5 (CH), 82.9 (CH), 73.4 (CH), 51.8 (OCH_3), 50.3 (C), 49.5 (CH), 47.7 (CH), 45.3 (C), 43.7 (C), 41.2 (CH2), 41.1 (CH), 32.1 (CH_2), 18.5 (CH_3), 17.2 (CH_3), 15.2 (CH_3), 12.9 (CH_3) ; ^1H NMR (400 MHz, $CDCl^3$) δH: 7.32 (t, *J* = 1.6 Hz, 1H), 7.26 (d, *J* = 9.6 Hz, 1H), 7.22 (s, 1H), 6.25 (s, 1H), 5.93 (d, *J* = 9.6 Hz, 1H), 5.53 (m, 1H), 4.62 (dd, *J* = 3.6 Hz, 12.4 Hz, 1H), 4.27 (d, *J* = 3.6 Hz, 1H), 3.66 (d, *J* = 8.4 Hz, 1H), 3.54 (s, 1H), 3.25 (dd, *J* = 5.2 Hz, 16 Hz, 1H), 3.19 (d, *J* = 12.8 Hz, 1H), 2.73 (t, *J* = 5.6 Hz, 1H), 2.39 (dd, *J* = 5.6 Hz, 16 Hz, 1H), 2.22 (dd, *J* = 6.8 Hz, 12.4 Hz, 1H), 2.10 (m, 1H), 1.70 (s, 3H), 1.47 (s, 3H), 1.37 (s, 3H), 1.22 (s, 3H).

4.2 *In vitro* insecticidal activity of nimbolide
S. frugiperda susceptibility to nimbolide was determined using leaf disc bioassay. The mortality of the larvae was measured up to 6 days. When compared to water and methanol controls, the crude leaf extract and nimbolide had significant fatal effects on *S. frugiperda* on 5 days after treatment. At 10000 ppm, both neem leaf extract and nimbolide had the maximum larvae mortality of 90.00 percent and 93.33 percent, respectively, whereas both the negative control had 3.33 percent larval mortality (Table 1.). The larvae fed on nimbolide showed slower growth than those fed on neem leaf extract-treated leaf discs. Despite the fact that feeding damages were seen in both treated and untreated leaves, the treated leaves had less foliar damage than the untreated control leaves (Fig1a, 1b).

Table 1: Effect of neem leaf extract and nimbolide against neonates of *S. frugiperda*

Sl. No.	Compounds/treatments	Larval mortality (%)
1	Neem leaf extract @ 1000 ppm	60.00 ± 0.00 (50.76)[e]
2	Neem leaf extract @ 2500 ppm	63.33 ± 5.77 (52.73)[e]
3	Neem leaf extract @ 5000 ppm	66.66 ± 5.77 (54.73)[de]
4	Neem leaf extract @ 7500 ppm	80.00 ± 0.00 (63.43)[bcd]
5	Neem leaf extract @ 10000 ppm	90.00 ± 0.00 (71.56)[ab]
6	Nimbolide @ 1000 ppm	60.00 ± 0.00 (50.76)[e]
7	Nimbolide @ 2500 ppm	63.33 ± 5.77 (52.73)[e]
8	Nimbolide @5000 ppm	70.00 ± 0.00 (56.78)[cde]
9	Nimbolide @7500 ppm	83.33 ± 5.77 (65.90)[bc]
10	Nimbolide @10000 ppm	93.33 ± 5.77 (75.03)[a]
11	Water*	3.33 ± 5.77 (10.51)[f]
12	Methanol*	3.33 ± 5.77 (10.51)[f]
SED		4.5994
CD (0.05)		9.4927
CV (%)		11.08

* Negative control

Data represented as mean ± SD and Values followed by the same letter in a column are not significantly different at 5%

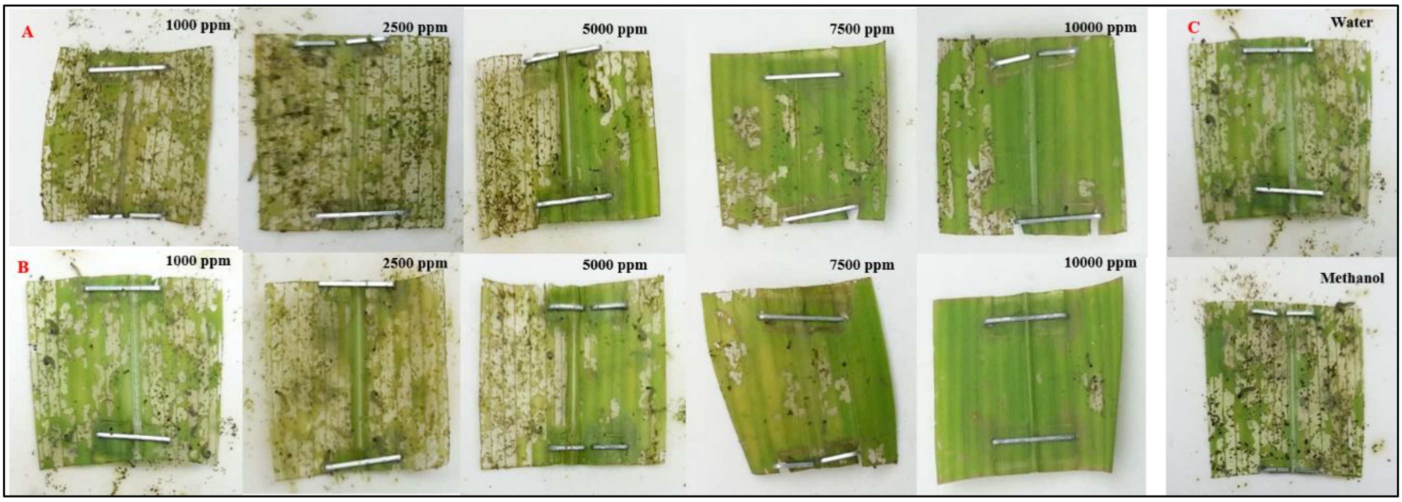

Fig 1a: Feeding activity of *S. frugiperda* on maize leaf A) Neem leaf extract B) Nimbolide C) Negative control – water and methanol

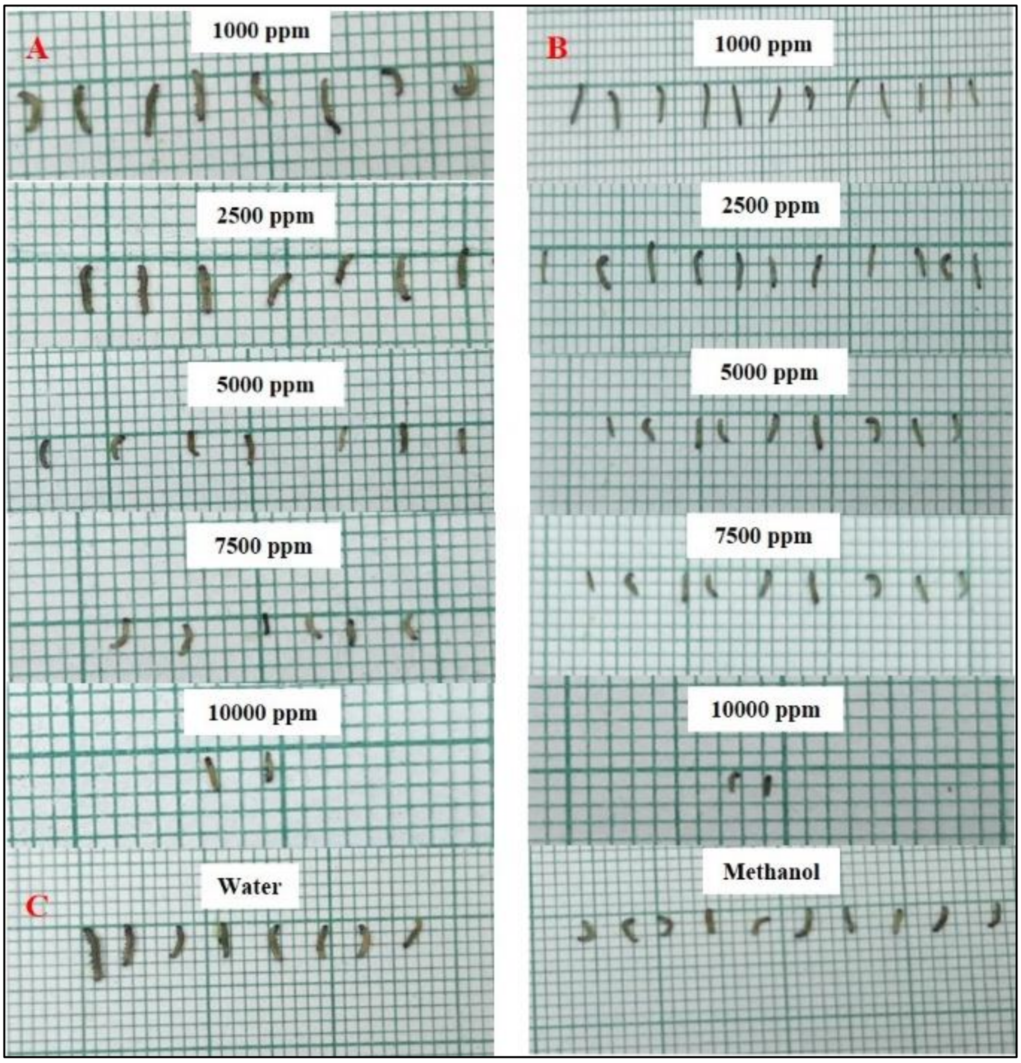

Fig 1b: Length of *S. frugiperda* larvae recorded on sixth day at different concentrations. A) Neem leaf extract B) Nimbolide C) Negative control – water and methanol

5. Discussion

Although synthetic pesticides are efficient in controlling FAW, a major worry about their usage is the increased danger to human health due to a lack of sufficient safety procedures (Rwomushana *et al*., 2018) [19]. Another issue is the development of resistance to key types of synthetic pesticides in this pest's native locations (Yu. 1991) [27]. This suggests the role of biological pesticides for controlling FAW. Conferring to previously reported studies, neem seed and leaf extract causes growth deformities and has antifeedant action against insect pests (Nisbet, 2000; Saidi *et al*., 2018) [17, 20]. Silva and his coworkers in 2015 [21] observed significant FAW larval mortality using an *A. indica* seed cake extract. Azadirachtin, a major terpenoid compound present in kernels of *A. indica* showed toxicity against *S. frugiperda* (Lin *et al*., 2021; Qin *et al*., 2020) [14, 18]. Antifeedant activity of *Spodoptera litura* larvae against nimbolide was confirmed by siva *et al*., 2017 [23].As there were no reports on the application of nimbolide against *Spodoptera frugiperda*, this was the first study to investigate the bioactivity of nimbolide against *S. frugiperda*. The present study was conducted against S. frugiperda by increase in dosage of nimbolide from 1000 to 10000 ppm. At the tested lower concentrations of 1000 and 2500 ppm both neem leaf extract and nimbolide showed 60.00 and 63.33 per cent mortality respectively. Highest larval mortality of 93.33 per cent was observed at 10000 ppm. Similarly, 90.00 per cent mortality was recorded in neem leaf extract at 10000 ppm. The bioactivity of nimbolide is increased as the concentration is increased. Hence, the activity of nimbolide is dose dependent. In this insecticidal assay, it was observed that nimbolide treatment resulted in considerable larval mortality when compared to neem leaf extract. According to the above findings, the nimbolide could be one of the key components in the development of bio insecticides to combat *S. frugiperda*. Larvae treated with nimbolide showed underdeveloped growth as in accordance with Liang *et al.,* 2003 [13], where the larval growth was prolonged due to the application of neem-based insecticides. This research showed that the insecticidal plants may be used as part of an integrated pest management approach for the discovery of nimbolide based biopesticides.

6. Conclusion

From the present study, it was observed that application of the nimbolide was effective and significantly increased FAW larval mortality, reduced leaf damage compared to the untreated control. Moreover, the larvae fed of nimbolide coated maize leaves showed stunted growth and abnormalities. Future studies will focus on how to use this biomolecule in formulating bio pesticide and using it at field level for controlling of *S. frugiperda*.

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