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Toxicity effects of neem oil with *Prosopis juliflora* (Leguminoseae) extract against Khapra beetle *Trogoderma granarium* (Everts.)

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Abstract
The present study was conducted to test the toxicity of neem oil with *Prosopis juliflora* as a botanical synergist. Different concentrations of botanical synergist were used with neem oil and incubated for 1hrs at 150rpm and at 40°C. After incubation, toxicity experiments were set up against third instar starving larva of Khapra beetle *Trogoderma granarium* (Everts.) for 24hrs and 48hrs. Results showed that increase in the concentration of botanical synergist significantly increases the toxicity of neem oil. 70% and 50% increases in the percentage mortality of the test organism was observed after 24hrs and 48hrs respectively. Significant decrease in the LD_{50} was also observed with the increase in the concentration of botanical synergist after 48hrs i.e. 14.71 $mg.kg^{-1}$ with 0g and 8.65 $mg.kg^{-1}$, 8.65 $mg.kg^{-1}$ and 7.65 $mg.kg^{-1}$ with 2g, 6g and 10g respectively. These results suggest that toxicity of neem oil increases with increase in the concentration of the botanical synergist. Thus, we conclude that *Prosopis juliflora* used in the present work enhance the toxicity and bioavailability of azadirachtin in neem oil against the target pest and can be used as an efficient substitute for chemical synergist. Hence, this synergistic approach provides an ecofriendly and economically efficient formulation of neem oil for stored grain pest management.

Keywords: neem oil, azadirachtin, *prosopis juliflora*, toxicity effect, *trogoderma granarium*

1. Introduction
The prevalent usage of chemical and synthetic pesticides create various problems including destruction of natural enemies followed by outbreak of secondary pest, insect resistance development along with resurgence of treated primary insect. This may also cause a serious environmental contamination i.e. soil pollution, air pollution and water pollution. Such harmful and detrimental effect on environment possess the development of new strategies that could eliminate or reduce the usage of chemical and synthetic pesticides.

One of these strategies includes the replacement of synthetic pesticide with botanical (Mahmoud *et al.* 2014) [9]. These pesticides has sort of properties including toxicity, repellency, anti-feedant and growth regulatory actions against different pests. Plant origin pesticides have been used so far but only neem providing a highly effective, nontoxic and eco-friendly means of controlling agricultural as well as stored grain pest which impose losses in agriculture production (Sharma, 2008) [16]. Products of neem tree, *Azadirachtin Indica* (A. Juss.) (Meliaceae) has been reported to contain a range of chemical compounds which responsible for its irreplaceable nature. Extracts from the seeds and kernels have been reported for their adverse effect on biology of many agricultural pests (Das *et al.* 2010; Naveena *et al.* 2010; Wondafrash *et al.* 2012) [4, 10, 21]. 'Azadirachtin' active compound in neem has been reported for their varied insecticidal activity i.e. oviposition deterrent, anti-feedant, growth retardant, moulting inhibitor, sterilant etc. (Prakash and Rao 1997) [12].

There is a problem with the stability of botanical pesticide. Various chemicals had been used as a synergist to increase the stability and effectiveness of botanical pesticide. Khalequzzaman and Khanom (2006) [7] studied the synergistic effect of cypermethrin in combination with leaf and seed extract of neem oil on *Tribolium castenum* (Herbst). They observed synergistic increase in LD_{50} value after 48hrs. Constanski *et al.* (2016) [3] studied the synergistic effect of inert powder i.e. Bentonite, Kaoline and Diatomaceous earth in association with neem oil to control *Spodoptera eridania* and *Spodoptera frugiperda*. They observed that 10% of Bentonite gave 100% mortality for both specie while 10% of kaoline gave 78.5% and 98.6% mortality with both the test specie respectively. Radhika and Sahayaraj (2014) [13] studied the synergistic effect of monocrotophos with neem oil formulation on 3rd instar larvae of *Spodoptera litura* (Fab.) (Lepidoptera: Noctuidae). They observed 100% mortality after eighth day of incubation period.

Studies on combined insecticidal effects of neem and botanical extracts are rarely addressed in the country. Our preliminary works proved that there were synergistic effects of some selected botanical plant extract on neem which increase its toxicity against insect pest. Extensive experimental studies were conducted to identify botanical synergist which can stabilize azadirachtin, active component in neem oil keeping the final compound as biodegradable. By performing these studies, *Prosopis juliflora* which is abundantly available on waste land throughout India was identified and found that combination of this plant with neem oil stabilize the azadirachtin content with in the neem oil. Accordingly, this study intended to evaluate the combined effects of *Prosopis juliflora* extract as a synergist with

azadirachtin in neem oil, as insecticides against the 3rd instar larvae of *Trogoderma granarium*.

Khapra beetle *Trogoderma granarium* (Everts.) is the most abundant and injurious pest of stored grains throughout the world (Semple 1986; Arnaud *et al.* 2002) [15, 1]. It is believed that this pest is originated in India. Development and infestation rates of each stag like egg, pupae and adult depending upon temperature (20ºC), light, moisture, season and host species. This stored grain pest may have nine or more generations per year (Ramzan and Chahal 1986) [14]. In case of survivability larvae survive a month to several year under diapauses condition. The larvae consumed an average of 3-12mg of food during their development, with female eating about double the amount as compared to male (Sohi 1947; Karnavar 1973) [18, 6]. The infestation of Khapra beetle is more in constant darkness. However, constant light accelerate development but reduced oviposition (Sohi 1986) [19]. Several methods are being used to overcome the problem. Many researchers emphasized on the need for searching safe and natural management methods as an alternative to pesticides. Considering the increasing concern about health and the environment the present study was designed to increase the stability and bioefficacy of neem as an insecticide against stored grain pest and develop a stable and ecofriendly formulation of neem oil by using botanical synergist.

2. Materials and Methods

2.1 Preparation of botanical synergist extract in neem oil

Botanical plant, *Prosopis juliflora* (Leguminoseae) used as synergist were collected from IPFT farm, Gurgaon. Collected samples were shade dried at room temp (28ºC± 2ºC) and powder using electric mixer. To study the synergistic effect of *Prosopis juliflora* on the toxicity of azadirachtin present in neem oil, five different concentrations i.e. 0g, 2g, 6g and 10g were taken in a 250ml Erlenmeyer flask and mixed with 100 ml of pure neem oil. Each flask was incubated at 40ºC at 200rpm in an incubator shaker for 1hr. After incubation period, neem oil from each flask were filtered through wattman filter paper in a fresh 250ml Erlenmeyer flask.

2.2 Enrichment of botanical synergist extract in neem oil with Azadirachtin

100g technical grade azadirachtin were added in each 250 ml Erlenmeyer flask having filtered neem oil and botanical extract and again incubated at 40˚C for 24hr at 200rpm in an incubator shaker. After incubation, these samples were collected and stored in freeze temperature at 4ºC. Different concentrations (ppm) i.e. 50, 100, 150, 200, 250 and 300 of each enriched sample were prepared with the solvent acetone and kept in screw-capped bottles with rubber seals.

2.3 Toxicity bioassay test

The culture of *Trogoderma granarium* has been taken up in the IPFT, Gurgoan laboratory. The 3rd instar larvae were segregated from a culture of insect preserved in the laboratory to be used for the bioassay tests. Each 1gm of clean and sound wheat seed were kept in petridish (60 mm × 15 mm) and treated with each prepared concentration of different extract prepared for the experiment. Wet filter paper was kept inside each petridish to maintain appropriate moisture. After drying of seed within five minutes, 10 larvae of *Trogoderma granarium* were put in each petridish and then covered with muslin cloth. Assessments of petridish were recorded periodically after 24hrs and 48hrs of the initiation of experiment. Percentage mortality was calculated to observe the toxicity effect of neem oil extract with botanical synergist. The experiment was replicated six times and each replicate consisting of response of 10 larvae.

2.4 Statistical analysis

Significant differences between the mean percentage mortalities between different concentration of botanical synergist and test sample were analysed by two way analysis of variance (ANOVA) and means were separated using Tukey's test (Sigma Statv13.5). Dose response mortality data were analysed using Probit analysis (stat plus v5) to calculate LC_{50} values.

3. Results

3.1 Toxicity bioassay test

The collected sample of neem oil with different concentrations of botanical synergist were used for toxicity bioassay experiments, which were set up against starving larvae of *Trogoderma granarium* and observation were made upto 24hrs and 48hrs (Fig.1). After 24hrs, neem oil with 0g of botanical synergist showed 10% mortality in 200ppm, 250ppm and 300ppm. Lower concentration i.e. 50ppm, 100ppm and 150ppm do not show mortality in the test larvae. Percentage mortality in neem oil with 2g of botanical synergist were observed to be significantly increased with increase in the test concentration i.e. 20% with 50ppm, 40% with 100ppm, 60% with 150 and 200 ppm and finally 80% with 250 and 300 ppm. Similarly, neem oil with 6g and 10g of botanical synergist showed significant increase in the percentage mortality with respect to the 2g of botanical synergist concentration as well as with the increase in the test concentration (Fig. 1). Finally 100% mortality were observed in 300ppm of neem oil with 10g of botanical synergist.

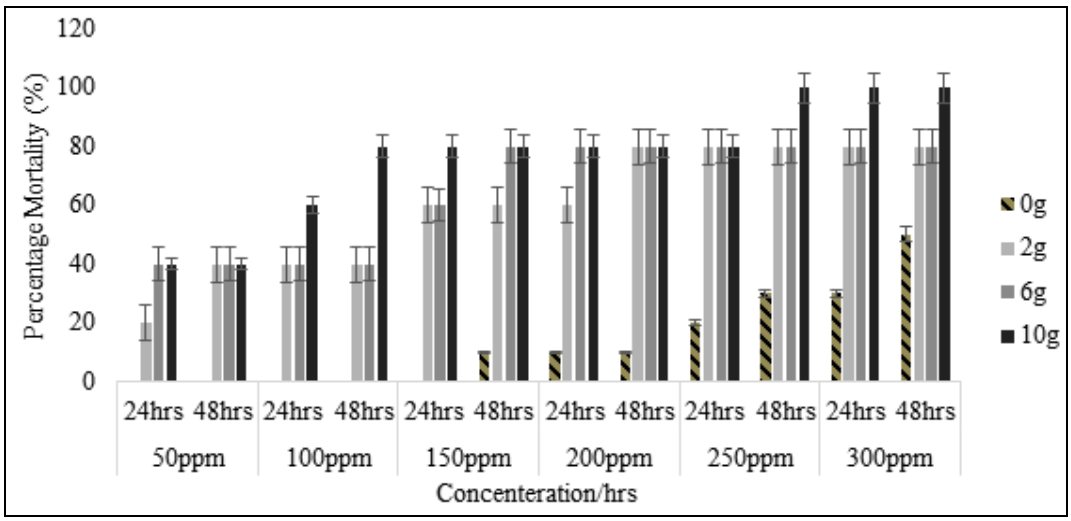

Fig 1: Percentage mortality (mean ± S.E) in the 3rd instar larvae of *Trogoderma granarium* (Everts.) after 24hrs and 48hrs with the different test concentration against neem oil having *Prosopis juliflora* as a botanical synergist.

After 48hrs of observation period, percentage mortality were showed similar trend as after 24hrs with increase in the concentration of botanical synergist but significantly higher with respect to the 24hrs (Fig. 1). Percentage mortality were also observed at the lower concentration of test samples. Neem oil with 0g of botanical synergist showed 50% mortality but at higher test concentration i.e. 300ppm. 100% mortality were observed with 10g of botanical synergist at 250 and 300ppm of test sample concentration.

LD_{50} were also calculated using probit analysis (Sigma stat v5). Significant decrease were observed in all the test concentrations with increase in the concentration of botanical synergist (Fig.2).

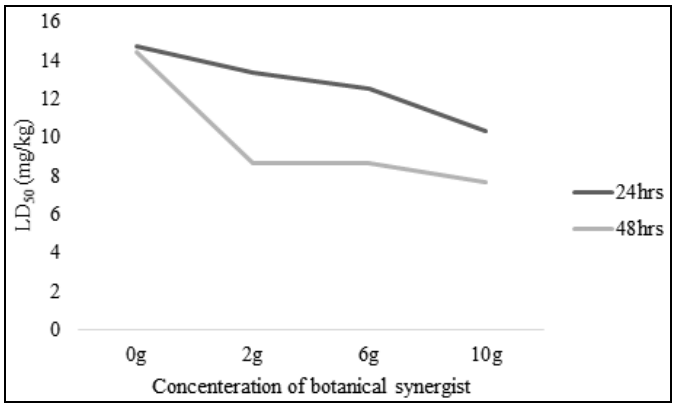

Fig 2: LD_{50} (mg/kg) of neem oil having *Prosopis juliflora* extract as a botanical synergist against 3rd instar larvae of *Trogoderma granarium* (Everts.) after 24hrs and 48hrs.

After 24hrs, neem oil with 0g of botanical synergist showed 14.71 $mg.kg^{-1}$, 13.33 $mg.kg^{-1}$ with 2g, 12.53 $mg.kg^{-1}$ with 6g and 10.30 $mg.kg^{-1}$ with 10g. While after 48hrs further decrease is LD_{50} were observed in neem oil with 2g, 6g and 10g of botanical synergist i.e 8.65 $mg.kg^{-1}$, 8.65 $mg.kg^{-1}$ and 7.65 $mg.kg^{-1}$ respectively while neem oil without any botanical synergist do not show any significant decrease in LD_{50} i.e 14.46 $mg.kg^{-1}$ and 14.71 $mg.kg^{-1}$ after 24hrs and 48hrs respectively.

4. Discussion

In the present work we applied an approach to increase the insecticidal activity of neem oil using *Prosopis juliflora* as a botanical synergist against the 3rd instar larvae of stored grains pest *Trogoderma granarium* (Everts.) L (Mahmoud *et al.* 2015). Observation were made using different concentration of botanical synergist extract in neem oil. Result showed that insecticidal activity of neem oil significantly ($P>0.001$) increased with the concentration of botanical synergist. Significant difference were observed among the different concentrations of botanical synergist as well as the test samples applied on test organisms. Neem seed oil extract having 10g of botanical synergist reflects the best mortality result as compared with 0g (Fig. 1).

Now a days, botanical pesticide from *Azadirachta indica* become very much prevalent because of their biodegradability, least persistence, least toxic to non-target organism, easily availability and economic (Debashri *et al.* 2012) [5]. But there is a drawback with their efficacy. They are least stable as compared to chemical based pesticide (Baki *et al.* 2005) [2]. Lots of work has been done to increase the stability of botanicals pesticides (Radhika and Sahayaraj 2014) [13]. Synergism between botanical and chemical insecticide against insect pest is well documented (Khalequzzaman and Khanom 2006; Radhika and Sahayaraj 2014; Constanski *et al.* 2016) [7, 13, 3]. Although the synergistic formulation of the chemicals generally exhibit faster response but there is a considerable concern over last few years about the toxicity of chemical synergist (shrivastav *et al.* 2011) [11]. They are well known for their toxic effect. However, botanicals play an important role as synergist in insect pest management both economically and ecologically (Baki *et al.* 2005) [2].

In this study, we used *Prosopis juliflora* as a botanical synergist with neem oil to increase its toxicity effect. Observation showed that, at lower concentration percentage mortality of neem oil against *Trogoderma granarium* was increase upto 40% while at the higher concentration 70% after 24hrs and 50% after 48hrs were observed without any toxic effect of botanical synergist (Fig 1). 100% mortality even after

48hrs at higher test concentration signifies that botanical synergist increase the stability and bioavailability of azadirachtin content in neem oil to the target insect pest. This might be due to the presence of glycosidic compounds present in *Prosopis juliflora* which increase the toxicity of neem oil without having their own toxic effect on test insect (Singh *et al.* 2012; Kumar *et al.* 2013; Wondafrash *et al.* 2012) [17, 8, 21]. This work is in treaty with our earlier work. We increase the efficacy of neem oil based cream against *Aedes* mosquitoes by 45% and also stabilize its active ingredient. Similarly, azadirachtin in neem kernel powder (NKP) using *Prosopis juliflora* as a botanical synergist were also showed similar results (Patanjali *et al.* 2011) [11]. Various concentrations of botanical synergist and NKP were used and out of these, 1:1 ratio of NKP and botanical synergist gave 100% mortality. The result showed that, botanical synergist enhances the insecticidal and larvicidal effect of NKP against stored grain pest.

5. Conclusion

Thus, the present study was accomplished to scientifically evaluate the activity of *Prosopis juliflora* as a botanical synergist. We applied a synergistic approach to increase the toxicity effect of neem oil against insect pest. Synergistic combination showed 100% mortality when compared with their individual at higher concentration of botanical synergist. Accession of neem product with *Prosopis juliflora* reduce the dose and application, often with increase in its efficacy and less disturbance to non-target insect. Thus, the present study suggest that *Prosopis juliflora* used as a botanical synergist significantly increased the toxicity of neem oil and provides an ecofriendly and economically efficient formulation of neem oil for the insect pest management without any toxic effect on plants as well as on farmers.

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8. Conflict of Interest

There is not any financial conflict of interest.

9. References

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