Bioengineering of Neem Colloidal Nano-Emulsion Formulation With Adjuvant for Better Surface Adhesion and Long Term Activity in Insect Control

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Abstract

Although safe and eco-friendly botanical pesticides have been intensively promoted to combat pest attacks in agriculture, but their stability and activities remain an issue for their wide acceptability as sustained and effective approaches. The purpose of this work was to develop stable neem oil based nano-emulsion (NE) formulation with enhanced activity employing suitable bio-inspired adjuvant. So, Neem NEs (with and without) natural adjuvant (lemongrass oil and Prosopis juliflora) were prepared and different parameters dictating kinetic stability, acidity/alkalinity, viscosity, droplet size, zeta potential, surface tension, stability and compatibility were monitored using Viscometer, Zetasizer, Surface Tensiometer, High Performance Liquid Chromatography (HPLC) and Fourier Transform Infrared Spectroscopy (FTIR). Nano-emulsion biosynthesis optimization studies suggested that slightly acidic (5.9-6.5) NE is kinetically stable with no phase separation; creaming or crystallization may be due to botanical adjuvant (lemongrass oil). Findings proved that Prosopis juliflora, acted as bio-polymeric adjuvant to stabilize NE by increasing Brownian motion and weakening the attractive forces with smaller droplets (25-50nm), low zeta potential (-30mV) and poly-dispersive index (<0.3). Botanical adjuvant based NE with optimum viscosity (98.8cPs) can give long term storage stability and improved adhesiveness and wetting with reduced surface tension and contact angle. FT-IR analysis assured azadirachtin's stability and compatibility with adjuvant. With negligible degradation (1.42%) and higher half-life (t$_{1/2}$) of 492.95 days, natural adjuvant based NE is substantially stable formulation may be due to presence of glycosidic and phenolics compounds. Neem 20NE (with adjuvant) remarkably exhibited insecticidal activity (91.24%) against whitefly (Bemisia tabaci G.) in brinjal (Solanum melongena) as evidenced by in-vivo assay. Results thus obtained suggest, this bio-pesticide formulation may be used as safer alternative to chemical pesticides to minimize pesticide residue problems and natural adjuvant as key input in stability and efficacy enhancement of pesticides for crop protection in organic agriculture and Integrated Pest Management also.

Introduction

Eggplant (Solanum melongena L.) is one of the important vegetable crop in South and South-East Asia with 37% of the total cultivated area, and 53.31 million tons production (worldwide (FAOSTAT, 2016). Major insect pests of eggplant are shoot and fruit borer (Leucinodesorbonalis Lepidoptera: Pyralidae), leafhopper (Amrascabигuttulabiguttula, Hemiptera, Cicadelloidea), whitefly (Bemiciatabaci, Hemiptera: Aleyrodidae), aphid (Aphis gossypii, Homoptera: Aphididae), thrips (Thripstabaci, Thysanoptera: Thripidae), etc. Sucking pests cause considerable loss of crop and also serve as vector for the transmission of various pathogens. Therefore, these pests damage the crop directly or indirectly in terms of yield and quality. Among the sucking pests, whiteflies are major destructive pests of brinjal and responsible for 70-92% brinjal crop loss.

Indiscriminate use of pesticides usage leads to pest resistances and pest resurgence problems in eggplants. Therefore, in view of these adverse effects caused by chemical pesticides there is a need to search for novel pest management strategies for long term activity and safety toward users and environment. Numerous botanical formulations have been developed with good plant defense mechanisms against pest attack. Most commonly used botanical formulations are based on plant essential oils, plant extracts, or secondary metabolites of plants. Among botanical pesticides, neem has been the most effective botanical since last 30 years. The main active ingredients in neem oil are Azadiractin A, Azadirachtin B, Salanine, Nimbin, etc. Azadirachtin has various biological properties like anti-feedant, growth inhibition, oviposition deterrent, etc with
potentiality to kill various insect species\textsuperscript{17}. Neem contains many constituents that possess varying degrees of stability and biological activity as a result very few neem formulations have been commercialized\textsuperscript{18}. So, there is an urgent need to find out effective adjutants to stabilize the active ingredients of most valuable botanical pesticides obtained from neem to give desired bio-efficacy for longer duration.

Hence in the present study attempts will make to enhance the stability and efficacy of neem-based Nanoemulsion. Thus, botanical adjuvant may give good stability of active ingredients in NE formulation. The stabilized neem NE formulation with botanical adjuvant in a kinetically stabilized the system and may also give good bio-efficacy against agricultural pests.

\section*{Results And Discussion}

\subsection*{Nanoemulsion formulation process optimization}

The oil-in-water nanoemulsion was formulated using suitable inert ingredients through low-energy emulsification approach. It is a spontaneous, simple and cost-effective process of formulating oil into nanoemulsion\textsuperscript{18}. This method can be accomplished by the aqueous phase or oil phase titration process. The NE (O/W) was prepared using neem oil, lemongrass oil, surfactants, botanical adjuvant, anti-freezing agent and water (Fig. 7). The concentration of the oil phase and surfactants were varied during the preparation and ternary phase diagram based on three components: surfactant, water and oil were generated\textsuperscript{19}. Atlox 4916 and Nonylphenol ethoxylates were selected for being non-ionic surfactants and hence least affected by pH and ionic strength\textsuperscript{20}. The 1:5 ratio of the NE was found to be stable with droplet size (25-50nm) and polydispersity index (<0.301) (Fig. 1) which is indicative of the system\textsuperscript{21}. Selection of optimal adjuvant blend is a key to stable nanoemulsion due to strong repulsive force that averts flocculation and coalescence between the nanodroplets\textsuperscript{22}. Lemongrass oil (\textit{Cymbopogon citratus}) and \textit{Prosopis juliflora} extracts were found to be suitable adjuvant for the formulation of stable NE since these are less affected by pH, and are considered to be nontoxic and biocompatible\textsuperscript{23}. The stability of the NE can persist over longer period of time due to the presence of the stabilizing adjuvant that inhibits the coalescence of the nanodroplets\textsuperscript{24,25}.

\subsection*{Effect of lemongrass oil on kinetic stability}

Stability is one of the most important parameters in nanoemulsion system because of their small droplet size and large surface area. The small droplet size of nanoemulsions provides stability against sedimentation or creaming because of the Brownian motion and consequently the diffusion rate are higher than the sedimentation rate induced by the gravity force. A stability test of nanoemulsions was conducted by varying the storage time (0-14days) or temperatures (4-45\textdegree C). The study was accomplished by observing the sample appearance or measuring their physicochemical properties such as zeta potential or particle size at predetermined interval time and the samples without any major changes in their appearance likes phase separation, creaming, flocculation, coalescence and sedimentation was considered as a stable system.

In the present study, no phase separation, creaming or crystallization were observed in neem oil nano-emulsion with botanical adjuvant after storage for 14 days at low (0\textdegree C) and high temperature (45\textdegree C). The results indicate that neem nano-emulsion is kinetically stable formulation during storage for extended period of time\textsuperscript{23}. Generally nanoemulsion formation requires external energy to increase Gibbs free energy (\Delta G) in nano droplets
for extended stability. Suitable adjuvant system is also necessary for improved kinetic stability of nano-emulsion. Lemongrass oil has been previously reported as stabilizer and co-surfactant for neem oil microemulsion²⁰.

**Role of Prosopis juliflora on droplet size, poly dispersive index (PDI) and zeta potential**

Droplet size and zeta potential measurements are the general requirements to test the nano-emulsion stability²⁴. The most common destabilization phenomenon like flocculation, creaming, and coalescence etc. are directly related to droplet size²⁵,²⁶. The small size of nanoemulsion is desirable in achieving optimal efficiency. A complete picture of droplet size population and distribution in Neem NE formulations were obtained by analysis of data generated by dynamic-light-scattering (DLS). The average droplet size was found in the range of 25-50nm without any major changes in droplet size during storage. Nano-emulsions with smaller the droplet size will increase the Brownian motion and weaken the attractive forces in nano-emulsion systems to produce stable formulation²⁷. During storage conditions, smaller droplets generally converted into larger droplet size by Ostwald ripening process which can be inhibited by selective surface active agents or adjuvant. Droplet growth rate is explained by the Lifshitz-Slezov and Wagner equation:-

\[
\omega = \frac{d\tau}{dt} = \frac{8 \gamma V_m D}{9 \eta r^2} \tag{1}
\]

In neem nano-emulsion, average droplet radius (\(r\)) during storage time (\(t\)) are reduced due to low interfacial tension (\(\gamma\)) generated by surface active agents or adjuvant and stabilizers which ultimately reduced the molecular volume of neem oil (\(V_m\)) at absolute temperature (\(T\)). Occurrence of Ostwald ripening was avoided by increasing the elasticity of droplet¹⁰³ and the addition of natural adjuvant which reduced interfacial free energy forming a mechanical barrier against coalescence. *Prosopis juliflora*, may be acted as bio-polymeric adjuvant due to presence of cellulose fiber²⁸,²⁹ in this formulation.

Polydispersity values below 0.2 indicate uniformity among oil droplet sizes or monomodal distributions and therefore better stability, whereas values close to 1 indicate a heterogeneous or multimodal distribution²⁹. In the present study, values were closer to 0.3 indicating mono-dispersive nature of developed nano-emulsion with good stability during storage. The selection of PDI value which is less than 0.5 is acceptable for agricultural use and is considered as a good uniformity of the droplet diameter.

Zeta potential values provide the information about the homogenous behavior of the nano-emulsion. Particles with zeta potential more positive than + 30 mV or more negative than -30 mV are usually considered to be stable, since electrical charge of droplets is strong enough to assume that repulsive forces between droplets are predominant in the nanoemulsion⁶⁰. The surface of NE was negatively charged with an average zeta potential (-30 and -40mV) which indicates that the formulation is stable. A negative zeta potential value induces repulsive forces that are greater than the attraction forces among droplets, thus averting the coagulation and coalescence to occur in disperse emulsion. Zeta potential quantifies surface charge over the emulsion droplet indicating the physical stability of nano-formulations³⁰. Zeta potential gives the electrophoresis mobility data related to the stability of NE formulation. Higher value indicates good stability of nano-formulation due to higher repulsion between the droplets to prevent coagulation and flocculation during storage³¹. Droplet size, PDI and
zeta potential signify clear, uniform and homogenous micelle based neem nanoemulsion formulation with long term stability.

**Botanical adjuvant on acidity/alkalinity (pH)**

The surface properties around the droplet determine the pH value as an indicator of nanoemulsion stability. The pH of the formulations was found in the slightly acidic range (5.9-6.5). However, the formulations become almost neutral at application concentration (0.1–1%). Previous studies have reported that high alkalinity or acidity of the nano-formulations leads to degradation of neem ingredient which in turn, reduces the bio-efficacy of the formulation\(^{32}\). In another study it was reported that pH aggregates and destabilize the nano-emulsion during storage\(^{33}\). In the present study, it was observed that botanical adjuvant stabilizes the pH to slightly acidic (5.9-6.5) to reduce degradation (1.42%) of neem during storage at low, ambient and high temperatures.

**Viscosity**

Neem NE without botanical adjuvant was found to be less viscous (88.7cPs) compared to Neem NE with botanical adjuvant (98.8cPs). Optimum viscosity of a formulation is required for long term storage without sedimentation, complete transfer from container to spray tank, homogeneous dispersion for spray solution and adhesion and reduction of run-off from target surfaces\(^{34}\). Optimum viscosity also reduces the rate of aggregation in nano-emulsion during storage\(^{35}\). The viscosity value may be affected by the nature of surfactants, organic phase components and oil viscosity. Pesticide nanoemulsion produces low viscosity as it is categorized as O/W type with high water loading. However, the viscosity of nanoemulsion can be altered by surfactant concentration\(^{35}\). Results showed that Neem NE with botanical adjuvant can give long term stability during storage and improved adhesion on applied surfaces for good bio-efficacy.

**Surface tension**

Low surface tension (23.4mNm\(^{-1}\)) in neem NE (with adjuvant) provides better spreading of formulation on brinjal leaves with good bio-efficacy (91.24%) compared to neem NE (without adjuvant) (33.6 mNm\(^{-1}\)). Finer droplet size allows the spreading of droplet uniformly on the plant leaf surface\(^{36}\). Lower surface tension improves wetting, spreading and penetration into the applied surface of plants\(^{37}\). Wetting is the important parameter which directly linked with the contact angle and surface tension. Retention and contact angle of leaves are measured to relate the affinity of the pesticide liquid towards the leaf surfaces. Contact angle is the quantitative measurement of surface adhesion of liquid on solid surface\(^{38}\). Contact angle measurement is linked to Young’s equation:

\[
SA = SL + LA \cos \theta \tag{2}
\]

Where:

SA= Surface tension between solid and air  
SL= Surface tension between solid and liquid  
LA= Surface tension between liquid and air
Cos Θ = Contact angle

The efficiency of neem nanoemulsion was enhanced by increasing the adhesion work of nanoemulsion towards the leaves. Neem nano-emulsion reduces the surface tension and contact angle due to spreading of oil droplets over leaf surface (Fig. 2). It worth noted that the contact angle of nanoemulsion decreased as the increasing *P. juliflora* content, showing that neem oil has low interfacial tension which effectively allowing neem oil diffusion in the plant surface. Adjuvant along with water based neem nano-emulsion binds firmly over the leaf surface with grip induced by sticky adjuvant (Fig. 3). Neem nanoemulsion with *P. juliflora* conferred further lowering of surface tension may be due to the presence of cellulose polymer. So, the addition of botanical adjuvant improves wetting and dispersion of NE.

**Effect of botanical adjuvant on active ingredient (azadirachtin) compatibility**

FT-IR spectroscopy is a valuable technique for the identification of any functional group among various bioactive constituents. FTIR spectroscopy of Neem nanoemulsion, neem oil and adjuvant was done and the obtained data were compared to identify the possible interaction of various functional groups that were involved in the synthesis (Fig. 4). Natural adjuvant *P. juliflora* showed broadband at 3314.96 cm\(^{-1}\) due to hydroxyl groups of phenolic constituents, band at 1613 cm\(^{-1}\) due to aromatic C=C and similar peaks have been previously reported by Kumara et al. The study explained that functional groups were corresponds to flavonoid compounds. In view of previous studies, hydroxyl and aromatic peaks may be due to the flavonoid constituents. Phenolic and alkene derivatives in *P. juliflora* extract may enhance the storage stability of azadirachtin.

Neem nano-emulsion shows the corresponding peaks of neem oil, azadirachtin constituent corresponding peaks at 1745.72 cm\(^{-1}\) and at 2848.17 cm\(^{-1}\) without any chemical modification. FT-IR data results concluded that azadirachtin active constituents were stable and compatible with botanical adjuvant in nano-emulsion formulation system.

**Effect of natural adjuvant on active ingredient (azadirachtin) storage stability**

In water-based formulations, azadirachtin content is generally degraded by hydrolysis reactions (Fig. 6) during storage to reduce the bio-efficacy. So suitable stabilizer is required to protect the azadirachtin from undesirable degradation. Analytical chromatogram of neem NE formulation obtained by HPLC analysis revealed the degradation pattern of azadirachtin (Fig. 5). Azadirachtin showed linear detector response (Fig. 8) over the concentration range (0.05-1.0 mg L\(^{-1}\)) tested, with correlation coefficient linear functions > 0.999 and regression equations, y = 93322x - 51.38. In the present study, *P. juliflora* acts as a stabilizing agent in neem NE formulation as results indicate that azadirachtin content remained stable with negligible degradation (1.42%) compared to NE formulation without *P. juliflora* (15.26%). This decrease might be due to the presence of glycosidic and phenolics compound in botanical adjuvant.

Half-life (t\(^{1/2}\)) of azadirachtin in neem oil with and without of botanicals was observed and it ranged from 45.84 to 492.95 days (Table 1). The lowest t\(^{1/2}\) value was obtained without botanicals and increased manifolds with the addition of botanical synergist. An increase in the half-life of azadirachtin again signifies its stability due to the presence of botanical adjuvant.
Azadirachtin is the secondary metabolite of neem, but high rate of degradation confines its efficient usage in controlling insects\(^\text{42}\). Farmers are not benefitted from neem products due to its low stability as reported in various research findings\(^\text{15}\). Some stabilizers have been used to reduce degradation of azadirachtin\(^\text{45, 46}\). All these stabilizers are good and efficient but due to their chemical nature and limited availability are less popular in farmers. Whereas, in the present study, naturally occurring botanical adjuvant was used to enhance the stability of azadirachtin in neem oil which is quite safe for plants as well as for human health also.

**In-vitro insecticidal activity of Neem nanoemulsion against whitefly**

The results of *in-vitro* bioassay of the selected formulations against whitefly were presented in **Table 2**. Mortality (%) of whitefly was increased significantly \((p < 0.05)\) with the increment of dose and time of exposure. Neem 20 NE (with adjuvant) exhibited the best insecticidal activity against whitefly with LC\(_{50}\) value of 2.05 mLL\(^{-1}\). Moreover, Neem 20 NE showed good mortality (56.1%) of target pest after 5 days of treatment at 8 mLL\(^{-1}\) (**Table 2**) which was quite better than crude neem oil at 1% dose (Pissinati and Ventura, 2015). Neem 20NE formulation (with adjuvant) demonstrating the strongest insecticidal activity with the lowest LC\(_{50}\) value of 2.05 mLL\(^{-1}\) which indicates that *Prosopis juliflora* extract acted as a promising bio-efficacy enhancer in neem nano emulsion.

**Table 2. In vitro insecticidal activity of neem NEs against whitefly.**
<table>
<thead>
<tr>
<th>Insecticide Formulation</th>
<th>Treatment/ Doses</th>
<th>Mortality (%) at hours of application</th>
<th>Lethal Concentration (mL\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24hrs</td>
<td>48hrs</td>
</tr>
<tr>
<td>Neem 20NE</td>
<td>1 mL\textsuperscript{-1} (T\textsubscript{1})</td>
<td>6.12</td>
<td>12.78</td>
</tr>
<tr>
<td></td>
<td>2 mL\textsuperscript{-1} (T\textsubscript{2})</td>
<td>18.88</td>
<td>30.55</td>
</tr>
<tr>
<td></td>
<td>4 mL\textsuperscript{-1} (T\textsubscript{3})</td>
<td>28.88</td>
<td>37.78</td>
</tr>
<tr>
<td></td>
<td>8 mL\textsuperscript{-1} (T\textsubscript{4})</td>
<td>36.67</td>
<td>42.22</td>
</tr>
<tr>
<td>Neem 20 NE+adjuvant</td>
<td>1 mL\textsuperscript{-1} (T\textsubscript{5})</td>
<td>12.78</td>
<td>20.55</td>
</tr>
<tr>
<td></td>
<td>2 mL\textsuperscript{-1} (T\textsubscript{6})</td>
<td>25.55</td>
<td>37.78</td>
</tr>
<tr>
<td></td>
<td>4 mL\textsuperscript{-1} (T\textsubscript{7})</td>
<td>34.45</td>
<td>47.78</td>
</tr>
<tr>
<td></td>
<td>1 mL\textsuperscript{-1} (T\textsubscript{8})</td>
<td>54.45</td>
<td>63.88</td>
</tr>
<tr>
<td>Cypermethrin (10% EC)</td>
<td>1 mL\textsuperscript{-1} (T\textsubscript{9})</td>
<td>72.78</td>
<td>82.22</td>
</tr>
<tr>
<td>Control (Water)</td>
<td>1 mL\textsuperscript{-1} (T\textsubscript{10})</td>
<td>0.55</td>
<td>2.22</td>
</tr>
<tr>
<td>S.Em±</td>
<td>-</td>
<td>2.63</td>
<td>2.15</td>
</tr>
<tr>
<td>CD (p=0.05)</td>
<td>-</td>
<td>7.82</td>
<td>6.4</td>
</tr>
<tr>
<td>CV %</td>
<td>-</td>
<td>14.98</td>
<td>10.17</td>
</tr>
</tbody>
</table>

**In-vivo insecticidal activity enhancement of neem NE against whitefly on brinjal**

Neem oil has been widely reported as potent bio-pesticide due to anti-feedant, repellent, and growth-inhibiting properties\textsuperscript{47}. Neem oil comprises various azadirachtin analogs that offer various insecticidal properties against different agricultural as well as household insect pests\textsuperscript{48}. Azadirachtin imparts chemical defense against pest attack for efficient pest management strategies without hampering non-target organisms. Neem oil in comparison to synthetic pesticides has been extensively studied for the control of whiteflies which showed equivalent control as synthetic pesticides\textsuperscript{49}.

Nano-emulsion is a new formulation technology for improving the bio-efficacy of neem oil for long term pest control\textsuperscript{50}. Biological activity of neem NE can be improved further by addition of adjuvant or other inert ingredients\textsuperscript{51}. The effect of adjuvant on neem nano formulation was evaluated on whitefly population and percent reduction was compared with control replication wise to nullify the impact of different factors in the field such as the presence of natural enemies, free movement of insects and conventionally used synthetic...
insecticide Cypermethrin 10% EC also (Table 3). Pre-treatment (PT) population of target pest was homogeneous before application and increased steadily over the period of observation till 14 days. Among formulations developed, Neem 20NE with adjuvant recorded significantly \((p < 0.05)\) low population of \(B. \text{tabaci}\) from 1 to 14 days after treatment @ 1000 mLha\(^{-1}\).

In the present study, botanical adjuvant was used as possible bio-efficacy inducer against whiteflies of brinjal. Neem 20 NE (without adjuvant) reduced population (43.30%) which was further enhanced to 91.24% by incorporating botanical adjuvant and equivalent to Cypermethrin 10% EC (95.23%). Inclusion of adjuvant (\(P. \text{juliflora}\)) efficiently increased the biological effectiveness of neem nano-emulsion. Insecticidal activity of n-hexane extract of \(P. \text{juliflora}\) seed oil against termite (\(Odontotermes \text{obesus}\)) and cockroach (\(blattella \text{germanica}\)); antimicrobial activity of \(P. \text{juliflora}\) seed pods aqueous extract against some common pathogens was reported earlier. Moreover, in presence of \(P. \text{juliflora}\), neem oil nano-formulation showed possible synergistic activity (110.71%).

### Table 3: Effect of botanical and synthetic formulations on whitefly infestation in brinjal crop

<table>
<thead>
<tr>
<th>Insecticide Formulation</th>
<th>Treatment/Doses (%</th>
<th>Mean no. of whiteflies* per 3 leaves per plant at pre-treatment (PT) and different days (D) after treatments</th>
<th>% reduction of whitefly population±</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neem 20 NE</td>
<td>1000 mLha(^{-1}) ((T_1))</td>
<td>PT: 1.10 (1.26) 1D: 1.06 (1.25) 3D: 1.01 (1.23) 5D: 0.91 (1.19) 7D: 0.86 (1.17) 10D: 1.27 (1.33) 14D: 1.47 (1.40)</td>
<td>43.30 ((S))</td>
</tr>
<tr>
<td>Neem 20 NE with botanical adjuvant</td>
<td>1000 mLha(^{-1}) ((T_2))</td>
<td>PT: 1.06 (1.25) 1D: 0.33 (0.91) 3D: 0.21 (0.84) 5D: 0.18 (0.82) 7D: 0.15 (0.80) 10D: 0.12 (0.79) 14D: 0.23 (0.85)</td>
<td>91.24 ((S))</td>
</tr>
<tr>
<td>Cypermethrin (10 EC)</td>
<td>500 mLha(^{-1}) ((T_3))</td>
<td>PT: 0.98 (1.22) 1D: 0.40 (0.95) 3D: 0.29 (0.89) 5D: 0.27 (0.88) 7D: 0.07 (0.76) 10D: 0.06 (0.75) 14D: 0.12 (0.79)</td>
<td>95.23 ((S))</td>
</tr>
<tr>
<td>Control (Water)</td>
<td>500 Lha(^{-1}) ((T_4))</td>
<td>PT: 1.14 (1.28) 1D: 1.23 (1.31) 3D: 1.26 (1.32) 5D: 1.52 (1.42) 7D: 1.95 (1.56) 10D: 2.28 (1.66) 14D: 2.59 (1.75)</td>
<td>0 ((S))</td>
</tr>
<tr>
<td>S.Em+</td>
<td>-</td>
<td>PT: 0.05 1D: 0.06 3D: 0.06 5D: 0.06 7D: 0.05 10D: 0.05 14D: 0.05</td>
<td>- ((S))</td>
</tr>
<tr>
<td>CD (p=0.05)</td>
<td>-</td>
<td>PT: 0.15 1D: 0.16 3D: 0.17 5D: 0.16 7D: 0.15 10D: 0.15 14D: 0.16</td>
<td>- ((S))</td>
</tr>
<tr>
<td>CV %</td>
<td>-</td>
<td>PT: 6.94 1D: 8.09 3D: 8.31 5D: 8.21 7D: 7.38 10D: 7.65 14D: 7.53</td>
<td>- ((S))</td>
</tr>
</tbody>
</table>

*Values in parentheses are square-root transformed; PT: Pre-treatment; ± Compared to untreated (control) 14 days after application.

Tukey’s Honest Significant Difference (HSD) Test or Post-hoc analysis was performed to evaluate how Neem 20 NE (\(T_1\)), Neem 20NE+adjuvant(\(T_2\)), Cypermethrin 10% EC (\(T_3\)) and untreated control (\(T_4\)) treatments affect the behaviour of percent reduction of whiteflies at different days interval (Table 4). From this study, it was found...
that these four categories of treatments differ in the way they reacted towards whitefly population management. Test results indicated that Neem 20NE and Neem 20NE+adjuvant yielded significant differences (0.5203) on the reduction (%) of whiteflies. No significant difference accumulated for Cypermethrin (10% EC). It means that botanical adjuvant had strong influence on percent reduction of whiteflies.

**Table 4:** Tukey’s Honest Significant Difference (HSD) Test of botanical and synthetic formulation’s effects on whiteflies

<table>
<thead>
<tr>
<th>Group (A)</th>
<th>Group (B)</th>
<th>Mean Difference</th>
<th>Std. Error</th>
<th>Sig.</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lower Bound</td>
</tr>
<tr>
<td>Neem 20 NE</td>
<td>Neem 20 NE with botanical adjuvant</td>
<td>1.2400</td>
<td>0.05</td>
<td>0.5203</td>
<td>-3.7302</td>
</tr>
<tr>
<td>Neem 20 NE</td>
<td>Cypermethrin (10 EC)</td>
<td>-1.3500</td>
<td>0.06</td>
<td>0.0502</td>
<td>-4.1031</td>
</tr>
<tr>
<td>Neem 20 NE</td>
<td>Control (Water)</td>
<td>-1.1200</td>
<td>0.06</td>
<td>0.3903</td>
<td>-0.8089</td>
</tr>
<tr>
<td>Neem 20 NE with botanical adjuvant</td>
<td>Cypermethrin (10 EC)</td>
<td>-0.1100</td>
<td>0.06</td>
<td>0.0996</td>
<td>-3.2159</td>
</tr>
<tr>
<td>Neem 20 NE with botanical adjuvant</td>
<td>Control (Water)</td>
<td>-2.3600</td>
<td>0.05</td>
<td>0.0558</td>
<td>-0.0458</td>
</tr>
<tr>
<td>Cypermethrin (10 EC)</td>
<td>Control (Water)</td>
<td>-2.4700</td>
<td>0.05</td>
<td>0.0773</td>
<td>-0.2069</td>
</tr>
</tbody>
</table>

*The mean difference is significant at the 0.05 level.

**Methods**

**Materials**

Neem oil (azadirachtin content: 2000mgL⁻¹) purchased from Gujarat Chemicals, Gujrat, India. For botanical adjuvant, Kekar pods [(*Prosopis juliflora*, Kingdom: Plantae, Order: Fabales, Family: Fabaceae, Subfamily: Caesalpinioideae, Genus: Prosopis, Species:P. juliflora) collected from local forest nearest to the Institute. Two nonionic surfactants [Nonylphenol ethoxylate is nonionic in nature with high HLB (13); neither ionizes in water nor hydrolyzes in aqueous acid or alkaline solutions and Atlox 4916 is a high molecular weight polymeric emulsifier with low HLB (6) to provide excellent stability] were procured from Croda Surfactant Ltd, Mumbai, India. Lemongrass oil was obtained from Gorgia Chemicals, Delhi, India. Acetonitrile (HPLC grade) purchased from Merck, India. Azadirachtin standard (95% purity) procured from Sigma Aldrich, France. All the formulations were prepared using double distilled water.
**Preparation of botanical adjuvant aqueous extracts**

Mature (yellow) *Prosopis juliflora* dried pods were collected from the local forest, India. Pods were washed thoroughly to remove undesirable dust and other impurities under gentle flow of tap water and dried under shade at room temperature. After complete drying, pods were ground to powder using a common grinder (Bajaj, Bravo DLx 500) at 1200 rpm for 10 minutes. Grinded powder was sieved by BS (British Standard) sieve (200 mesh = 74 µm pore size) to get the finest particles. *Prosopis juliflora* powder sample (2 g) was taken in 100mL beaker containing 50 mL double distilled water. The beaker was placed over the orbital shaker and incubated at speed of 150 rpm for 24hrs. The extract was filtered through muslin cloth and filtrate was centrifuged at 4000 rpm for 15 minutes. Supernatant was filtered through Whatman filter paper (No.1) and used as aqueous dispersion medium for nano-emulsion formulation.

**Preparation of neem NE formulation**

Nonionic surfactants were blended (10mL) in 3:2 ratios with glass rod in 100mL beaker and prepared blend was added to aqueous extract (30mL) of botanical adjuvant (*P. juliflora*) under slow mixing at 200 rpm and 35-40°C to produce homogenous and transparent solution. Co-surfactant (glycerol, 10mL) as anti-freezing agent was added to this subsequently obtained solution under slow mixing at 200 rpm and 35-40°C to stabilize the formulation. Finally 50mL of lemongrass:neem oil (6:4, v/v) ratios was added drop wise under constant stirring at 800 rpm at 35-40°C to biosynthesize neem oil nano-emulsion formulation.

**Physico-chemical properties characterization of neem Nano-emulsion (NE)**

Studies on physico-chemical attributes like kinetic stability, droplet size, pH, viscosity, surface tension, FTIR, active ingredients, etc. were evaluated in triplicates following the protocol laid down in Collaborative International Pesticides Analytical Council (CIPAC) and Indian Standard (IS) specifications (BIS 1997) using different instruments as follows.

**Kinetic stability assessment of developed nano-formulations**

Kinetic stability was evaluated as per the previously discussed method by Teo et, al. in 2017. For testing the thermodynamic stability, neem oil nano-emulsion was stored at various temperatures (4°C, 30°C and 45°C) for 48 hrs. Neem formulations were centrifuged at 3600 rpm for 30 minutes. Kinetic stability neem NE was also checked by preserving at room temperature for 180 days.

**Droplet size distribution, polydispersity index (PDI) measurement of Neem NEs**

Dynamic light scattering (DLS) measurement of dispersion of NEs was performed as described previously using Zetasizer (NanoS90, Malvern Instruments, UK) to ascertain the aggregation and size distribution of the dispersed nano droplets in the formulations. The formulation was diluted with distilled water (1:20) prior analysis to minimize the multiple scattering effects caused due to the viscosity of formulated product. Diluted formulations were homogenized using sonicator and thereafter, 10 mL was poured in measuring chamber equipped with a detector sensor to measure Brownian motions of droplets at ambient temperature and average size and distributions were analyzed.

**Zeta potential analysis of Neem NEs**
Zeta potential was also measured by Malvern Zetasizer (Malvern, UK and samples were prepared by the method as described by Ch et.al, 2012). The samples were dispersed in 10 mM aqueous sodium chloride (NaCl) solution. All the measurements was performed in triplicates.

**Acidity/alkalinity (pH) determination of Neem NEs**

The pH value of NEs was measured using a pH meter by immersing the electrode into 10% aqueous solution of formulation at 25 ± 1 °C after calibration of pH meter using buffer solutions viz., pH 7, 4 and 9.2 (CIPAC MT 75.3, 2000). All measurements were triplicated.

**Rheology studies of Neem NEs**

The dynamic viscosity measurements of Neem NEs (with and without botanical adjuvant) were conducted using Brookfield viscometer at 25 °C. The analyzed data were examined to observe the viscosity behavior. All the viscosity measurement was done in triplicates at room temperature.

**Measurement of surface tension of Neem NEs**

In the present study, surface tension of developed products was measured by Surface Tensiometer (Model: DST-30) by Elico Marketing Ltd in triplicates at ambient temperature and atmospheric pressure. The needle and the dispensing system were regularly rinsed with distilled water to prevent residue from prior experiments to impact measurements.

**FTIR analysis for functional group characterization of Neem NEs**

Fourier-transform infrared spectroscopy (FTIR) (PerkinElmer Spectrum) was used to study the chemical interactions between neem oil and neem oil Nano-emulsion with adjuvant by comparing their spectral absorptions. The transmittance was measured against the wave number between 500 and 4000 cm⁻¹.

**Chemical characterization of active ingredient by HPLC in Neem NEs**

Chemical constitutes were characterized by HPLC technique. Active ingredient was quantified in neem NE formulations (with and without adjuvant) as per BIS method 14299: 1995 with following operating conditions (Table 5).

**Table 5: HPLC operating conditions**
<table>
<thead>
<tr>
<th>Model</th>
<th>Perkin Elmer Series 200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detector</td>
<td>Photo Diode Array (PDA)</td>
</tr>
<tr>
<td>Column</td>
<td>BDS Hypersil 250 x 4.6 mm (RP C18)</td>
</tr>
<tr>
<td>Pump</td>
<td>Isocratic</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>Acetonitrile: water (35:65, v/v)</td>
</tr>
<tr>
<td>Wavelength</td>
<td>214 nm</td>
</tr>
<tr>
<td>Flow</td>
<td>1.2 mLmin⁻¹</td>
</tr>
</tbody>
</table>

A stock solution (50 mgL⁻¹) of Azadirachtin (analytical grade) used as an external standard and the retention time was recorded in HPLC. Neem NE formulations were diluted with HPLC grade acetonitrile (1:200v/v) and centrifuged at 8000 rpm for 5 min. Supernatant was filtered through 0.2 μm Nylon 66 membrane filter paper with the help of syringe. Filtrate (25mL) of each cleaned up formulations was injected to HPLC. Azadirachtin was identified by comparing the retention time of formulation peaks with that of standard and quantified (mgL⁻¹) as follows.

\[
\text{Residues in mgL}^{-1} = \frac{A_1 \times V \times C \times R_f}{A_2 \times W \times V_2}
\]

Where,

- \(A_1\) = Area of sample in chromatogram
- \(A_2\) = Area of standard in chromatogram
- \(V_1\) = Total volume of sample in mL
- \(V_2\) = Injected volume in μL
- \(C\) = Concentration of analytical standard in (mgL⁻¹)
- \(W\) = Weight of the sample in g
- \(R_f\) = Recovery factor

**In-vitro insecticidal activity of Neem nanoemulsion against whitefly**

*In-vitro* evaluation of insecticidal activity of the developed neem nano-emulsion against whitefly (*Bemisia tabaci* G.) of brinjal (*Pusa Purple Long* cultivar) was conducted using the modified leaf dip bioassay technique as described in prior research. Freshly reared and mature (apterous adult) whiteflies were collected from polyhouse (27 ± 2°C and 70–80% relative humidity (RH)), under a diurnal day/night cycle of 16/8 h of Agricultural Research Farm of BCKV, Kalyani, Nadia, West Bengal (India). Associated species of whitefly was
identified and confirmed by Entomologists using identification keys and relevant literature. Experiment was conducted with ten ($T_1$-$T_{10}$) treatments and three replications of Neem 20NE and Neem 20NE (with natural adjuvant). Entire brinjal leaves were dipped (30 secs) in emulsions of neem nano formulation with final concentrations in distilled water and air dried at room temperature ($27 \pm 2^\circ$ C). Treated leaves were placed over in separate containers and whiteflies obtained from polyhouse were released (60) and secured by small piece of cotton cloths using rubber bands under controlled laboratory conditions ($27 \pm 2^\circ$C and 70–80% relative humidity (RH)), under a diurnal day/night cycle of 16/8 h). Containers without formulation served as the negative control and chemical insecticide Cypermethrin (10% EC) as standard check for comparison. The mortality (%) of whiteflies was recorded at 24, 48 72 and 96 hrs of intervals and inhibition levels [mortality (%)] were calculated using the formula:

$$\frac{(x - y)}{x} \times 100 \text{-------------------(3)}$$

where

$x$ = whitefly population in the negative control,

$y$ = whitefly population in the presence of the tested formulation.

$LC_{50}$ and $LC_{90}$ values (Table 2) of different formulations were calculated by Probit analysis (Finney, 1971).

**In-vivo insecticidal activity of neem oil nano-emulsion against whitefly on brinjal**

A field experiment was conducted at the experimental farm of District Seed Farm (AB - Block Farm), Directorate of Research BCKV, Kalyani, West Bengal, from August to October 2019, with an aim to test the efficacy of the developed formulations against whitefly [(*Bemisia tabaci* Gennadius,1889) Kingdom:Animalia, Phylum:Arthropoda, Class:Insecta, Order:Hemiptera, Family:Aleyrodidae, Genus:Bemisia, Species:B. tabaci]. A randomized block design (RBD) was used with three replications for each treatment and plot (5x4 m²) were separated by ridges. The brinjal variety, *Pusa Purple Long*, was transplanted with recommended package of practices. Developed formulations were applied at 1000 mLha⁻¹ of neem 20NE ($T_1$) neem 20NE+adjuvant ($T_2$) and commercially available formulation of Cypermethrin 10EC at 500 mLha⁻¹ ($T_3$) in separate plots after observing the whitefly population above ETL (Economic Threshold Level). Three different plots were maintained as control ($T_4$) with absolutely no application of pesticides (only water was sprayed at 400 Lha⁻¹). Each treatment was replicated thrice to minimize pest population data count error. Two round sprayings were executed by high volume knapsack sprayer at 14 days interval based on pest severity and resurgence possibility. Observations of total number (adult +pupae) of whiteflies were recorded very carefully to avoid the flying of adults from three leaves per plant selected from top, middle and bottom per plot early in the morning. Before spray, first count (pre-treatment, PT) of insect population was noted followed by post-treatment counts on 1, 3, 5, 7, 10, and 14 days after last application.

**Statistical analysis**

Statistical analysis was carried out by performing necessary transformation representing standard error of the mean (SEM), co-efficient of variation (CV), and critical difference (CD) at 5% level of significance using (ANOVA) with SPSS® software version 16.0 (SPSS Inc., Chicago, IL). Data of insect population recorded after
each round of spray were pooled and analyzed. Pre-application data were used to work out % reduction of population as per Henderson-Tilton formula. Square root transformation was adopted for analysis of insect population data. Probit analysis was used to work out LD$_{50/90}$. Post-hoc analyses were done by the Tukey test (Zar 2010). Significant differences among the extracts in each assay were recorded when 95% confidence intervals (CI) did not overlap. LD$_{50}$ values of the tested formulations and confidence limits were calculated for whitefly with log dosage–mortality probit regression equations.

**Conclusion**

Nano-emulsion formulation of neem oil with botanical adjuvant having standardized quality parameters was developed. Droplets of developed NE are in nano-range (25-50 nm) with zeta potential (-30mV). FTIR analysis assured the compatibility among the inert ingredients, botanical adjuvant with neem oil. Azadirachtin was substantially stable (1.42% degradation) in neem formulation with botanical adjuvant. Formulation produced comparable reduction of whitefly (91.24%) with synthetic pesticide (95.23%). Thus, neem oil with botanical adjuvant could be a good alternative to conventional pesticide formulations and may play a significant role in Integrated Pest Management (IPM) and organic cultivation.

**Declarations**

**Ethical approval.**

This manuscript does not contain any studies involving human participants or animals.

**References**


**Figures**

**Figure 1**

Representative DLS spectrum of Neem NE (without adjuvant) (A) Neem NE (with adjuvant) (B) showing hydrodynamic radius and Polydispersity index (PDI)
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Figure 1

Representative DLS spectrum of Neem NE (without adjuvant) (A) Neem NE (with adjuvant) (B) showing hydrodynamic radius and Polydispersity index (PDI)

\[ y = 21.74x - 14.6 \]
\[ R^2 = 0.986 \]

Without Adjuvant

\[ y = 20.31x - 21.26 \]
\[ R^2 = 0.989 \]

Figure 2

Variation of contact angle with surface tension variation
Figure 2

Variation of contact angle with surface tension variation
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Variation of contact angle with surface tension variation
Figure 3

Behaviour of contact angle and surface adhesion properties without and with adjuvant based neem NE formulation
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Behaviour of contact angle and surface adhesion properties without and with adjuvant based neem NE formulation
Figure 4

FT-IR spectra of (a) neem oil, (b) adjuvant and (c) neem nano-emulsion (NE) with bio-adjuvant
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FT-IR spectra of (a) neem oil, (b) adjuvant and (c) neem nano-emulsion (NE) with bio-adjuvant
Figure 5

HPLC chromatogram showing peaks of Neem NE with synergist (A) and Neem NE without adjuvant (B)
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Figure 6

Hydrolytically unstable products of azadirachtin with hydrolysis sites (red color arrows)
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Figure 7

Diagrammatic representation of neem oil nano-emulsion preparation
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Diagrammatic representation of neem oil nano-emulsion preparation

Figure 8

Area vs. Concentration (ppm)

\[ y = 93322x - 51.38 \]

\[ R^2 = 0.999 \]
Detector linearity for Azadirachtin in HPLC

Figure 8

Detector linearity for Azadirachtin in HPLC

\[ y = 93322x - 51.38 \]
\[ R^2 = 0.999 \]