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

Nusrat Iqbal (✉ nusratsiddiq20@gmail.com)

Institute Of Pesticides Formulation Technology

Dipak Hazra

Department of Agricultural Chemicals, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur

Aloke Purkait

Department of Soil Science and Agricultural Chemistry, Palli-Siksha Bhavana (Institute of Agriculture), Visva - Bharati, Sriniketan

Natish Kumar

Institute Of Pesticides Formulation Technology

Jitendra Kumar

Institute Of Pesticides Formulation Technology

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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 Asia¹ 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 (*Leucinodes orbonalis* Lepidoptera: Pyralidae), leafhopper (*Amrasca biguttulabiguttula*, Hemiptera, Cicadelloidea), whitefly (*Bemisia tabaci*, Hemiptera: Aleyrodidae), aphid (*Aphis gossypii*, Homoptera: Aphididae), thrips (*Thrips tabaci*, Thysanoptera: Thripidae), etc². Sucking pests cause considerable loss of crop and also serve as vector for transmission of various pathogens³. Therefore, these pests damage the crop directly or indirectly in terms of yield and quality^{4,5}. Among the sucking pests, whiteflies are major destructive pests of brinjal^{6,7} and responsible for 70-92% brinjal crop loss⁸.

Indiscriminate use of pesticides usage leads to pest resistances and pest resurgence problems in eggplants¹⁰. 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 safeness toward users and environment¹¹. Numerous botanical chemical formulations have been developed with good plant defense mechanisms against pest attack^{12,13}. Most commonly used botanical formulations are based on plant essential oils, plant extracts, or secondary-metabolites of plants^{14,12,15}. Among botanical pesticides, neem has been the most effective botanical since last 30 years¹⁶. The main active ingredients in neem oil are Azadirachtin 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 species¹⁷. Neem contains many constituents that possess varying degrees of stability and biological activity as a result very few neem formulations have been commercialized¹⁸. So, there is an urgent need to find out effective adjuvants 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 Nano-emulsion. 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.

Results And Discussion

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 nanoemulsion¹⁸. 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 generated¹⁹. Atlox 4916 and Nonylphenol ethoxylates were selected for being non-ionic surfactants and hence least affected by pH and ionic strength²⁰. 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 system²¹. Selection of optimal adjuvant blend is a key to stable nanoemulsion due to strong repulsive force that averts flocculation and coalescence between the nanodroplets²². Lemongrass oil (*Cymbopogon citratus*) and *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 biocompatible²³. 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.^{24,25}

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°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 like 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°C) and high temperature (45°C). The results indicate that neem nano-emulsion is kinetically stable formulation during storage for extended period of time²³. Generally nanoemulsion formation requires external energy to increase Gibbs free energy (ΔG) 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 size^{25, 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:-

$$\omega = \frac{dr^3}{dt} = \frac{8}{9RT} C \gamma V_m D \text{-----(1)}$$

In neem nano-emulsion, average droplet radius (r) during storage time (t) are reduced due to low interfacial tension (Y) 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 fiber^{28, 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 formulation³². In another study it was reported that pH aggregates and destabilize the nano-emulsion during storage³³. 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 surfaces³⁴. Optimum viscosity also reduces the rate of aggregation in nano-emulsion during storage³⁵. 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 concentration³⁵. 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 surface³⁶. Lower surface tension improves wetting, spreading and penetration into the applied surface of plants³⁷. 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 surface³⁸. Contact angle measurement is linked to Young's equation;

$$SA = SL + LA \cos\theta \text{-----}(2)$$

Where:

SA= Surface tension between solid and air

SL= Surface tension between solid and liquid

LA= Surface tension between liquid and air

$\cos \Theta$ = 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 surface¹⁰⁴. 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 constituents³⁹. 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 3314.96cm^{-1} due to hydroxyl groups of phenolic constituents, band at 1613cm^{-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 compounds⁴⁰. In view of previous studies, hydroxyl and aromatic peaks may be due to the flavounoid constituents. Phenolic and alkene derivatives in *P. juliflora* extract may enhance the storage stability of azadirachtin⁴¹.

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 ($0.05\text{-}1.0\text{mgL}^{-1}$) 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.

Table 1: Degradation dynamics of azadirachtin in neem NE formulations during storage.

Duration of storage (Days)	Degradation of azadirachtin	
	Neem NE without adjuvant	Neem NE with adjuvant
	Mean Degradation %	Mean Degradation %
	$\pm$	$\pm$
	Standard Deviation	Standard Deviation
1	-	-
4	2.0 $\pm$ 0.1	0.07 $\pm$ 0.01
10	7.1 $\pm$ 0.1	0.09 $\pm$ 0.01
14	15.26 $\pm$ 0.15	1.42 $\pm$ 0.07
Half-life (Days)	45.84	492.95

Azadirachtin is the secondary metabolite of neem, but high rate of degradation confines its efficient usage in controlling insects⁴². Farmers are not benefitted from neem products due to its low stability as reported in various research findings¹⁵. Some stabilizers have been used to reduce degradation of azadirachtin^{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.

Insecticide Formulation	Treatment/Doses	Mortality (%) at hours of application				Lethal Concentration (mLL ⁻¹)	
		24hrs	48 hrs	72hrs	96hrs	LC ₅₀	LC ₉₀
Neem 20NE	1 mLL ⁻¹ (T ₁)	6.12	12.78	16.67	18.88	4.3	56.4
	2 mLL ⁻¹ (T ₂)	18.88	30.55	38.33	41.12		
	4 mLL ⁻¹ (T ₃)	28.88	37.78	47.22	55.00		
	8 mLL ⁻¹ (T ₄)	36.67	42.22	48.88	56.12		
Neem 20 NE+adjuvant	1 mLL ⁻¹ (T ₅)	12.78	20.55	23.33	26.67	2.05	12.33
	2 mLL ⁻¹ (T ₆)	25.55	37.78	50.55	57.78		
	4 mLL ⁻¹ (T ₇)	34.45	47.78	56.12	63.33		
	1 mLL ⁻¹ (T ₈)	54.45	63.88	65.55	84.45		
Cypermethrin (10% EC)	1 mLL ⁻¹ (T ₉)	72.78	82.22	96.12	98.33		
Control (Water)	1 mLL ⁻¹ (T ₁₀)	0.55	2.22	4.45	3.88		
S.Em±	-	2.63	2.15	2.15	1.58		
CD (p=0.05)		7.82	6.4	6.39	4.68		
CV %	-	14.98	10.17	8.93	5.99		

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 properties⁴⁷. Neem oil comprises various azadirachtin analogs that offer various insecticidal properties against different agricultural as well as household insect pests⁴⁸. 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 pesticides⁴⁹.

Nano-emulsion is a new formulation technology for improving the bio-efficacy of neem oil for long term pest control⁵⁰. Biological activity of neem NE can be improved further by addition of adjuvant or other inert ingredients⁵¹. 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. 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. juliflora*) efficiently increased the biological effectiveness of neem nano-emulsion. Insecticidal activity of n-hexane extract of *P. Juliflora* seed oil against termite (*Odontotermes obesus*) and cockroach (*blattella germanica*); antimicrobial activity of *P. juliflora* seed pods aqueous extract against some common pathogens ⁶¹ was reported earlier. Moreover, in presence of *P. 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

Insecticide Formulation	Treatment/Doses (%)	Mean no. of whiteflies* per 3 leaves per plant at pre-treatment (PT) and different days (D) after treatments							% reduction of whitefly population [±]
		PT	1D	3D	5D	7D	10D	14D	
Neem 20 NE	1000 mLha ⁻¹ (T ₁)	1.10 (1.26)	1.06 (1.25)	1.01 (1.23)	0.91 (1.19)	0.86 (1.17)	1.27 (1.33)	1.47 (1.40)	43.30
Neem 20 NE with botanical adjuvant	1000 mLha ⁻¹ (T ₂)	1.06 (1.25)	0.33 (0.91)	0.21 (0.84)	0.18 (0.82)	0.15 (0.80)	0.12 (0.79)	0.23 (0.85)	91.24
Cypermethrin (10 EC)	500 mLha ⁻¹ (T ₃)	0.98 (1.22)	0.40 (0.95)	0.29 (0.89)	0.27 (0.88)	0.07 (0.76)	0.06 (0.75)	0.12 (0.79)	95.23
Control (Water)	500Lha ⁻¹ (T ₄)	1.14 (1.28)	1.23 (1.31)	1.26 (1.32)	1.52 (1.42)	1.95 (1.56)	2.28 (1.66)	2.59 (1.75)	0
S.Em±	-	0.05	0.06	0.06	0.06	0.05	0.05	0.05	-
CD (p=0.05)	-	0.15 (S)	0.16	0.17	0.16	0.15	0.15	0.16	-
CV %	-	6.94	8.09	8.31	8.21	7.38	7.65	7.53	-

*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 (T₁), Neem 20NE+adjuvant(T₂), Cypermethrin 10% EC (T₃) and untreated control (T₄) 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

Group (A)	Group (B)	Mean Difference	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Neem 20 NE	Neem 20 NE with botanical adjuvant	1.2400	0.05	0.5203	-3.7302	1.2502
Neem 20 NE	Cypermethrin (10 EC)	-1.3500	0.06	0.0502	-4.1031	1.4031
Neem 20 NE	Control (Water)	-1.1200	0.06	0.3903	-0.8089	3.0489
Neem 20 NE with botanical adjuvant	Cypermethrin (10 EC)	-0.1100	0.06	0.0996	-3.2159	2.9959
Neem 20 NE with botanical adjuvant	Control (Water)	-2.3600	0.05	0.0558	-0.0458	2.9959
Cypermethrin (10 EC)	Control (Water)	-2.4700	0.05	0.0773	-0.2069	5.1469

*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 2017⁵². 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 previously¹⁵ 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 product⁵⁵. 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, 2012⁵³. The samples were dispersed in 10 mM aqueous sodium chloride (NaCl) solution⁵³. 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

Model	Perkin Elmer Series 200
Detector	Photo Diode Array (PDA)
Column	BDS Hypersil 250 x 4.6 mm (RP C ₁₈)
Pump	Isocratic
Mobile Phase	Acetonitrile: water (35:65, v/v)
Wavelength	214 nm
Flow	1.2 mLmin ⁻¹

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 0.2µm 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.

$$\text{Residues in mgL}^{-1} = \frac{A_1 \times V_1 \times C}{A_2 \times W \times V_2} \times R_f \text{-----(2)}$$

Where,

A₁ = Area of sample in chromatogram

A₂ = Area of standard in chromatogram

V₁ = Total volume of sample in mL

V₂ = 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 ⁶². 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 ($27 \pm 2^\circ \text{C}$). 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 ($27 \pm 2^\circ \text{C}$ 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:

$$[(x - y)/x] \times 100 \text{—————(3)}$$

where

x = whitefly population in the negative control,

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

LC₅₀ and LC₉₀ 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^R 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. LD₅₀ 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

1. Sulaiman, M.N.N. et.al., Genetic Variability of Eggplant Germplasm Evaluated under Open Field and Glasshouse Cropping Conditions. *Agronomy*. **10**, 436-439 (2020).
2. Dutta, N.K. et.al., Effect of insecticides on population reduction of sucking insects and lady bird beetle in eggplant field. *J. Agr. Res.* **42**, 35-42 (2017).
3. Zogli, P., Pingault, L., Grover, S., & Louis, J. Ento(o)mics: the intersection of “omic” approaches to decipher plant defense against sap-sucking insect pests. *Opin. Plant Biol.* **56**, 153–161 (2020).
4. Mpumi, N., Machunda, R. S., Mtei, K. M. & Ndakidemi, P. A. Selected Insect Pests of Economic Importance to Brassica oleracea, Their Control Strategies and the Potential Threat to Environmental Pollution in Africa. *Sustainability*. **12**, 3824. doi:10.3390/su12093824 (2020).
5. Yarahmadi, F., Rajabpur, A. & Shabazi, A. Investigations on toxic effects of some insecticides on population of Aphis gossypii Glover and its parasitoids Hibiscusrosa chinensis in Ahwaz’s groon landscape. Proceedings of the 1st Congress of Modern Agricultural Sciences and Technology, Zanjan, Iran, 2011.
6. Ghosal, A. & Chatterjee, M.L., Bioefficacy of imidacloprid 17.8 SL against whitefly, Bemisia tabaci (Gennadius) in brinjal. *Plant Protect Sci.* **5**, 37-41 (2012).
7. Kumar, S., Sachan, S.K., Kumar, V. & Gautam, MP., Abundance of insect pests associated with brinjal (Solanum melongena L.) crop. *J. Entomol. Zool.* **7**, 1014-1017 (2019).

8. Omprakash, S. & Raju, S.V.S., A brief Review on abundance and management of major insect pests of brinjal (*Solanum melongena* L.). *Int J Appl Biol Pharm.* **5**, 228-234 (2014).
9. Meftaul, I.M., Venkateswarlu, , Dharmarajan, R., Annamalai, P. & Megharaj, M. Pesticides in the urban environment: a potential threat that knocks at the door. *Sci. Total Environ.* **15**,134612. doi: [10.1016/j.scitotenv.2019.134612](https://doi.org/10.1016/j.scitotenv.2019.134612) (2019).
10. Gautam, M., Hafle, S., Regmi, B., Thapa, G. & Paudel, S. Management of brinjal fruit and shoot borer (*Leucinodes orbonalis* Guenee) in Nepal". *Acta Scientific Agri.* **9**.doi:10.31080/ASAG.2019.03.0632 (2019).
11. Dângelo, R.A.C, et.al, Insecticide resistance and control failure likelihood of the whitefly *Bemisia tabaci*(MEAM1; B biotype): a Neotropical scenario. *Ann Appl Biol*, **172**, 88–99 (2018).
12. Korunic, Z. & Fields, P. G. Evaluation of three new insecticide formulations based on inert dusts and botanicals against four stored-grain beetles. *Stored Prod. Res.* **88**, 1-8 (2020). 101633.doi:10.1016/j.jspr.2020.101633.
13. Purkait, A. et.al. Formulation of plant based insecticides, their bio-efficacy evaluation and chemical characterization. *Crop Prot.* **125**, 1-9 (2019).
14. Borges, D. F et.al. Formulation of botanicals for the control of plant-pathogens: A review. *Crop Protection*, **110**, 135–140(2018).
15. Sharma, R. et.al Development and stability enhancement of neem oil based microemulsion formulation using botanical synergist. *J. Mol. Liq.* **21**,234-289(2019).
16. Elanchezhyana, K. & Vinoth kumar, B. Neem: An Eco-Friendly Botanical Insecticide in Pest Management. *Insect Sci., Photon.* **116**,207-217 (2015).
17. Chaudhary, S. et.al, Progress on Azadirachta indicaBased Biopesticides in Replacing Synthetic Toxic Pesticides, *Front Plant Sci.* **8**, 610-634 (2017).
18. Chang, Y.; McLandsborough, L.; McClements, D. J. Physicochemical properties and antimicrobial efficacy of carvacrol nanoemulsions formed by spontaneous emulsification. *J. Agric. Food Chem.* **61** (37): 8906–8913 (2013).
19. Zhang, J., et.al. Physicochemical and pharmacokinetic characterization of a spray-dried malotilate emulsion. *Int. J. Pharm.* **414**,186-192 (2011).
20. Ali M.K. et.al, Seed priming by sodium nitroprusside improves salt tolerance inwheat (*Triticum aestivum*L.) by enhancing physiological andbiochemical parameters , *Plant Physiology and Biochemistry* **119**: 202-232 (2017).
21. Jafari SM , Assadpoor E , He Y , Bhandari B Encapsulation Efficiency of Food Flavours and Oils during Spray Drying *Drying Technology* **26**:816-835 (2008).
22. Sugumar S, Ghosh V, Nirmala MJ, Mukherjee A, Chandrasekaran N. Ultrasonic emulsification of eucalyptus oil nanoemulsion: antibacterial activity against *Staphylococcus aureus*and wound healing activity in Wistar rats. *Ultrason Sonochem.* **21**(3):1044–1049 (2014).
23. Rohman, A.& Man, Y.C. Fourier transform infrared (FTIR) spectroscopy for analysis of extra virgin olive oil adulterated with palm oil. *Food Res. Int.* **43**, 886–892(2010).
24. Siva Kumara, T. et.al, Isolation of chemical constituents from *Prosopis juliflora* bark and anti-inflammatory activity of its methanolic extracts. *Pharm. Res.* **2**, 551-556 (2009).

25. Kumari, A., Siddhiqa, N., Sharma, R. & Patanjali P.K., Activity enhancement of Neem (*Azadirachta indica*) based repellent cream using biodegradable botanical synergist, *J. Pharm. Res.* **9**, 18-21(2017).
26. Zuleta-Castro, C., Rios, D., Hoyos, R., & Orozco-Sánchez, F. First formulation of a botanical active substance extracted from neem cell culture for controlling the armyworm. *Agron Sustain Dev*, **37**,doi:10.1007/s13593-017-0448-4 (2017).
27. Singla, M., & Patanjali, P. K. Phase behaviour of neem oil based microemulsion formulations. *Crops Prod.* **44**, 421–426(2013).
28. Iqbal, N., Kumar, N. Agrawal, A. & Kumar, J. Development of highly stabilized neem oil microemulsion system: a green app *World J. of Pharm. Res.* **8**, 1507-1517 (2019).
29. Shao, H., Xi, Na. & Zhang, Y. Microemulsion formulation of a new biopesticide to control the diamondback moth (Lepidoptera: Plutellidae). *Sci. Rep.* **8**, 105-121 (2018).
30. Liu, H., Chen, L., & Wang. Food-Grade Nanoemulsions: Preparation, Stability and Application in Encapsulation of Bioactive Compounds. *Molecules*, **24**, 4242.doi:10.3390/molecules24234242(2019).
31. Aboofazeli, R. Nanometric-Scaled Emulsions (Nanoemulsions). *Iran J Pharm Res.* **9**,325–326(2010).
32. Gianeti, M.D. et.al, The use of nanotechnology in cosmetic formulations: The influence of vehicle in the vitamin a skin penetration. *Nanosci.* **8**,526–534 (2011).
33. Costa, M., Freiria-Gandara, J., Losada-Barreiro, S., Martins, P, F., Bravo-Diaz, C.,. Effects of droplet size on the interfacial concentrations of antioxidants in fish and olive oil-in-water emulsions and nanoemulsions and on their oxidative stability. *Jcolloid interf sci*, **562**, 352-362, (2020).
34. Payet, L. & Terentjev, E.M. Emulsification and stabilization mechanisms of O/W emulsions in the presence of chitosan. *Langmuir.* **24**, 12247-12252 (2008).
35. Saravanakumar, S.S. et.al, Characterization of a novel natural cellulosic fiber from *Prosopis juliflora* bark. *Polym.* **92**, 1928–1933 (2013).
36. Dickinson, E. Hydrocolloids as emulsifiers and emulsion stabilizers. *Food Hydrocoll.* **23**, 1473-1482 (2009).
37. Jiang, J., Oberdörster, G. & Biswas, P. Characterization of Size, Surface Charge, and Agglomeration State of Nanoparticle Dispersions for Toxicological Studies. *JNanopart Res.* **11**, 77-89 (2008).
38. Hwang, Y. et al., Stability and thermal conductivity characteristics of nanofluids, *Thermochimica Acta.* **455**, 70–74 (2007).
39. Gué, E., et.al, Evaluation of the versatile character of a nanoemulsion formulation. *Int J Pharm.* 498,49-65(2016).
40. Juttulapa, M., Piriyaarasarth, S. & Sriamornsak, P. Effect of pH on Stability of Oil-in-water Emulsions Stabilized by Pectin–Zein Complexes, *Adv. Mater. Res.* **747**, 127-130 (2013).
41. Mustafa I.F., Hussein, M.Z. Synthesis and technology of nanoemulsion-based pesticide formulation, *Nanomaterials*, **10**, 1-26 (2020).
42. Bouyer, E., et.al. Proteins, polysaccharides, and their complexes used as stabilizers for emulsions: Alternatives to synthetic surfactants in the pharmaceutical field. *Int. J. Pharm.* **436**,359-378 (2012).
43. Wang, L., Li, X., Zhang, G. , Dong, J.& Eastoe, J. Oil-in-water nanoemulsions for pesticide formulations, *Colloid Interface Sci.***314**,230 –235 (2007).

44. Tadros, T. Formation and stability of nano-emulsions. *Adv. Colloid Interface Sci.*, 108: 303-318 (2004).
45. Dauben, T. J. et.al. Quantifying the relationship between surfaces' nano-contact point density and adhesion force of *Candida albicans*. *Colloid surface b*, 111177.doi:10.1016/j.colsurfb.2020.111177(2020).
46. Rohman, A. & Man, Y.C. Fourier transform infrared (FTIR) spectroscopy for analysis of extra virgin olive oil adulterated with palm oil. *Food Res. Int.* **43**, 886–892(2010).
47. Siva Kumara, T. et.al, Isolation of chemical constituents from *Prosopis juliflora* bark and anti-inflammatory activity of its methanolic extracts. *Pharm. Res.* **2**, 551-556 (2009).
48. Kumari, A., Siddhiqa, N., Sharma, R.& Patanjali P.K., Activity enhancement of Neem (Azadirachta indica) based repellent cream using biodegradable botanical synergist, *J. Pharm. Res.* **9**, 18-21(2017).
49. Nathan, S. Physiological and biochemical effect of neem and other Meliaceae plants secondary metabolites against Lepidopteran insects. *Front Physiol.* **4**,1-17(2013).
50. Ley, S.V., Denholm, A.A.& Wood, A. The chemistry of azadirachtin, *Prod. Rep.* **10**,109-157(1993).
51. Barrek, S., Paisse, O.& Grenier-Loustalot, M.F, Analysis of neem oils by LC-MS and degradation kinetics of azadirachtin-A in a controlled environment. Characterization of degradation products by HPLC-MS-MS. *Bioanal. Chem.* **378**,753-763(2004).
52. Kumar, J. & Parmar, B. S. Stabilization of Azadirachtin A in Neem Formulations: Effect of Some Solid Carriers, Neem Oil, and Stabilizers, *J Agr Food Chem.* **47**,1735-1739 (1999).
53. Johnson, S., Dureja, & Dhingra, S. Photostabilizers for Azadirachtin-A (A Neem Based Pesticide) *J Environ Sci Health B.* **38**, 451-62(2003).
54. Schmutterer, H., Properties and potential of natural pesticides from the neem tree, Azadirachta indica. *Annu Rev Entomol.* **35**, 271-297(1990).
55. Isman, M. et.al. Developing a neem-based insecticide for Canada. *Entomol. Soc. Can.* **123**, 39–46(1991).
56. Rehman, H., Nadeem, , Ayyaz, M. & Begum, H.A. , Comparative efficacy of neem oil and lambdacyhalothrin against whitefly (*Bemisia tabaci*) and jassid (*Amrasca Devastans* Dist.) in okra field, *Russ. Agric. Sci.* **41**,138–145(2015).
57. Anjali, C.H., Sharma, Y., Mukherjee, A.& Chandrasekaran, N. Neem oil (Azadirachta indica) nanoemulsion—a potent larvicidal agent against *Culex quinquefasciatus*.,*Pest Manag Sci.* **68**,158-163(2012).
58. Green, J.M., Beestman, G.B. Recently patented and commercialized formulation and adjuvant technology. *Crop Prot.* **26**,320–327 (2007).
59. Teo, A., Lee, S. J., Goh, K. K. T., & Wolber, F. M Kinetic stability and cellular uptake of lutein in WPI-stabilised nanoemulsions and emulsions prepared by emulsification and solvent evaporation method. *Food Chem.*, **221**, 1269–1276, (2017).
60. Kurpiers, M., Wolf, J. D., Steinbring, C., Zaichik, S., & Bernkop-Schnürch, A. Zeta potential changing nanoemulsions based on phosphate moiety cleavage of a PEGylated surfactant. *Mol. Liq.*, **316**,113868.doi:10.1016/j.molliq.2020.113868 (2020).
61. Sekar, G., Sivakumar, A., Mukherjee, A. & Chandrasekaran, N. Probing the interaction of neem oil based nanoemulsion with bovine and human serum albumins using multiple spectroscopic techniques, *J. Mol. Liq.* **212**, 283-290 (2015).

62. Riquelme N, Zuniga RN, Arancibia C Physical stability of nanoemulsions with emulsifier mixtures: replacement of tween 80 with quillaja saponin. *LWT Food Sci Technol* 111:760–766 (2019).

63. Salvia-Trujillo L , Rojas-Graü MA, Soliva-Fortuny R, Martin-Belloso O. Use of antimicrobial nanoemulsions as edible coatings: Impact on safety and quality attributes of fresh-cut Fuji apples, *Postharvest Biology and Tech.* 105 (4):8-16 (2015).

64. Tajbakhsh S, Barmak A, Vakhshiteh F, Gharibi M. Invitro Antibacterial Activity of the Prosopis Juliflora Seed Pods on Some Common Pathogens. *J Clin Diagn Res.* 9(8): 13-25 (2015).

65. Sabtharishi S and Naveen N, Bioassay for monitoring insecticide toxicity in Bemisia tabaci populations. *Protocol exchange* 7(12):12-30 (2017).

Figures

Figure 1

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

Figure 1

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

Figure 1

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

Figure 2

Variation of contact angle with surface tension variation

Figure 2

Variation of contact angle with surface tension variation

Figure 2

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

Figure 3

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

Figure 3

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

Figure 4

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

Figure 4

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)

Figure 5

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

Figure 5

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

Figure 6

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

Figure 6

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

Figure 6

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

Figure 7

Diagrammatic representation of neem oil nano-emulsion preparation

Figure 7

Diagrammatic representation of neem oil nano-emulsion preparation

Figure 7

Diagrammatic representation of neem oil nano-emulsion preparation

Figure 8

Detector linearity for Azadirachtin in HPLC

Figure 8

Detector linearity for Azadirachtin in HPLC

Figure 8

Detector linearity for Azadirachtin in HPLC