

Nanoemulsion Formulation of Cold Pressed *Neem* Oil As a Repellent Against *Anopheles Stephensi*

Mohammadreza Taheri

Baqiyatallah University of Medical Sciences

Maryam Iman (✉ iman1359@yahoo.com)

Baqiyatallah University of Medical Sciences <https://orcid.org/0000-0002-2753-6089>

Mehdi Khoobdel

Baqiyatallah University of Medical Sciences

Omid Dehghan

Baqiyatallah University of medical Sciences

Seyed Mohammad Zarei

Baqiyatallah University of Medical Sciences

Research

Keywords: Neem oil, N,N-Diethyl-m-Toluamide, Nanoemulsion, *Anopheles Stephensi*, Mosquito repellent, Pseudo Ternary Phase Diagram

Posted Date: December 29th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-134842/v1>

License:  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: To formulate a nanoemulsion (NE) from cold pressed neem oil and to compare its repellent activity with normal cold pressed oil and N,N-Diethyl-meta-toluamide (DEET) as a standard chemical compound and to improve efficacy of repellency against *Anopheles stephensi*, through NE formulations.

Methods: In this study, complete protection time (CPT) and failure time of samples were evaluated on human subjects using the arm in cage assay, and their values were determined against *An. stephensi*. The droplet size of the NE were measured with dynamic light scattering. Pseudo ternary phase diagrams were constructed consisting of an emulsion system of an active ingredient (neem oil), surfactant (Tween 80), and water. Two formulations were selected from the isotropic regions and showed particle sizes of 12.3 and 93.9 nm in diameter.

Results: The results (expressed in minutes as mean \pm SD) showed that CPTs of neem oil NE in tween 80, and DEET 20% were 240 \pm 39 min and 360 \pm 24 respectively. Dose values for Neem oil in formulation and for DEET were 77 $\mu\text{g}/\text{cm}^2$ and 308 $\mu\text{g}/\text{cm}^2$, respectively. Nano neem oil in tween 80 provided significantly longer protection than normal cold pressed neem oil ($P<0.01$). The prepared NEs showed high kinetic stability for weeks, without phase separation, sedimentation or creaming. Nevertheless, their droplet size increased slightly over time. Compared to non-formulated neem oil, the NE significantly increased the Repellency of the tested species.

Conclusions: NE of neem oil significantly increases the CPT and reduces needed dose values. Hence, neem oil NE can be a good alternative to DEET and other chemical repellents.

Background

Malaria affects about 36% of the world's population, most often children under the age of five years (1). There are more than 200 million cases and 0.4 million deaths every year because of malaria, which is one of the deadliest mosquito-borne illnesses (2). The recent detection of the South and Central Asian malaria vector, *Anopheles stephensi*, in the Horn of Africa has raised concerns about its potential impact on malaria transmission (3). N,N-diethyl-m-toluamide (DEET) is known as a "gold standard" in the repellents experiments due to providing long-term protection but continuous use of synthetic chemicals to control malaria-transmitting mosquitoes has resisted these mosquitoes (4). Furthermore, numerous studies on DEET have shown its high detrimental effects (skin toxicity, seizure, or acute manic syndrome) and displeasure among customers (5). Therefore using the safe natural formulations provides more pleasure among customers. However, more attempts are needed to increase the efficiency of natural products. Some natural products have significant repellent activity against *Anopheles* spp. mosquitoes. There is a public tendency to use of natural products as repellents as these are commonly perceived as "safe" in comparison to long-established synthetic repellents. Therefore, many recent studies have dedicated their attention to find new natural repellents and some of them has achieved acceptable repellent activities, but a few of them have been developed to date. (6). Among natural compounds, oils are the most

promising cases (7). Also, it has been shown that smoking of oils or other dry parts of some plants like *citronella*, *menta piperita*, *Azadirachta indica* (neem) and eucalyptus have repellent activity (8,9). Cold pressed neem oil, which is a product of neem fruits, presents many biological activities such as insecticide, nematocide, fungicide, etc. (10). Despite the contradictory results regarding the repellent effect of neem products, and being not approved by US EPA for use as a topical insect repellent, they are widely advertised as natural alternatives to DEET. While neem oil provides some protection against nuisance biting mosquitoes, due to the lack of trustworthy studies, it is not currently advisable as an effective repellent for use by travelers to endemic areas (11). In a field trial of neem oil (20%) against *An. arabiensis*, Mean percentage repellency and complete protection time (CPT) has been claimed to be 71% and 3 h, respectively (12). However, use of undiluted neem oil can cause skin irritation. Although neem oil has a sharp odor like garlic but its efficiency decline very fast after using on the skin. This may be resulted from several reasons like fast vaporization, skin adsorption, and/or decomposition (13).

In general, it is clear that the repellent effect of natural oils is less than synthetic substances such as DEET, but their effectiveness can be increased by modification. The natural oils are insoluble in water in required concentration for their repellent action (Most repellents are active in concentration above 10%) (14). Most classic formulations are solutions and lotions. Spray solutions contain a large amount of alcohol can cause irritation and dryness when applying to the skin. In addition, alcohol can promote rapid evaporation of the active essentials (15). Lotions are classic emulsions containing unstable nano droplets that need to be shaken before use. Nanoemulsions (NEs) are more stable than classic emulsions. They present an excellent sensory and aesthetic appearance, without leaving the skin white coloured. NE preparation of natural oils is a promising technology for mosquito repellents (16,17). Formulation of natural oils in nano scales will made some advantages including slow releasing and increasing lifetime(18,19) that can cover the disadvantages of fast release and decay of neem oil (20). Emulsifying the natural oils in water using proper surfactants is a popular method to produce a water in oil or oil in water NE. Both low-energy and high-energy methods can do this task. Low-energy methods includes slow mixing of components together(21) while the others include high pressure homogenization, microfluidization, sonication, method in jet disperser, and high-amplitude ultrasonic method (22). As the natural extracts contain a range of different chemical structures, the nanoemulsifying of them is more difficult than synthesized or pure chemicals (23). The present work shows how nanoemulsifying of neem oil enhances the repellency effect rather than unprocessed neem oil against the *An. Stephensi*.

Methods

Cold press prepared neem oil was obtained from Falcons essential oils (Bangalore, India). DEET with CAS NUMBER 3-62-134 and a density of 0.99 g/mL, ethanol, and i-propanol, were procured from Merck Chemicals Inc. (Germany). Tween 80 and Tween 20 were purchased from Loba Chemie (Mumbai, India). Mature female mosquitoes were obtained from the insectarium of mosquito nursery (Baqiyatallah University of Medical Sciences) where they have been raised at $(27 \pm 3)^\circ \text{C}$, a relative humidity of $(80 \pm 10)\%$, and a photoperiod of 16 h light: 8 h dark.

Construction of pseudo-ternary phase diagram

Neem oil and a non-ionic surfactant (Tween 80) were mixed in ratios (w/w) of 10 : 0, 9 : 1, 8 : 2, 7 : 3, 6 : 4, 5 : 5, 4 : 6, 3 : 7, 2 : 8, 1 : 9, and 0 : 10. The prepared compositions were stirred for 1 min to obtain equilibrium. Afterward, Milli-Q water (5% (w/w)) was added by titrating the mixtures of neem oil and surfactant until a 95% water content was achieved in the emulsion system. The process was done in a water bath adjusted on 60 °C while the components weights were controlled by an analytical balance (Raga, Tehran, Iran). All of the components were sealed and homogenized by magnetic stirrer 2000 rpm. Afterward, the emulsions were centrifuged (Labline, Gujarat, India) at 3000 rpm at 60 °C for 30 min. The samples were visually investigated to determine their phase transition on the basis of clarity, stability, and transparency. The phase domains of the components were determined to be isotropic (transparent and one-phase) or anisotropic (cloudy or two-phase). Then, the samples were sonicated (Soltec, Milan, Italy) for 15 min at 60 °C operating at 40 KHz to obtain NEs. The phase diagrams were constructed using a ProSim software, version 1.0 (UK).

Measuring droplet size of NEs

Droplet size of the NEs were measured by a particle size analyzer device of Scatteroscope (Young Lin Instrument Co., Anyang, Korea) with a measurement range of (8 – 6 500) nm, and a zeta potential measurement range of (20–200) mV in the Central Laboratory of Tabriz University. The zeta potential for the droplet stability were performed by dynamic light scattering (DLS) using Horiba zeta analyzer (Japan) for the most potent 5:45:50 formulation.

Repellency tests

This research employed male volunteers aged 26–32 years (29 years on average) to determine the protection time and failure time. The volunteers were recommended to avoid the use of perfume, colognes, chewing gum, cigarettes, caffeinated materials (e.g. tea and coffee) as well as hair gel, fragrant soap, and redolent chocolate 12 h prior to and during the test according to WHO 2009 guidelines. It should be noted that before the test, informed consent was obtained from all volunteers. This research was approved by the Ethics Committee of Baqiyatallah University of Medical Sciences, Tehran, Iran (Approval ID: IR. BMSU. REC. 1397.072).

Skin allergy test of human volunteers

The volunteers arms were first disinfected using 72% alcohol. For skin allergy test, a circle with a surface area of 6.6 cm² was drawn at the upper arm of each volunteer using a standard model. Subsequently, 50 µL of Sample and DEET were spread on the drawn circle by a sampler. Then, the test candidate was advised not to contact the test area with water for 2 days. After 3 days, no symptoms of skin allergy (such as burning, itching, inflammation, and skin redness) were detected in all the volunteers.

Important conditions for mosquitoes used in the repellency test

Adult female, non-blood fed, and nulliparous mosquitoes of 7–8 days old were kept in cages [(35 × 35 × 35) cm]. The 10% sugar solution was picked up from the cage, 12 hour before starting the experiments. Then, in the event of sitting or fleshing the snout or biting without blood feeding, the presence of 10–20 mosquitoes on the forearm of the volunteer for 30 sec showed the suitability of mosquitoes to start the test.

Estimation of protection and failure time

The protection and failure times were estimated for samples and DEET 20%. The volunteer's hands were then impregnated with the repellants (2.0 mL) from the elbows to the wrists by a sampler. The volunteer's hands were covered by latex gloves to prevent mosquitoes from biting in the area below the wrists and fingers not impregnated with the repellents. After 5 min of hand impregnation with the repellents, the volunteer placed his forearm in a cage containing about 200 blood-deprived mosquitoes for 3 min. Any biting, probing, and sitting of mosquitoes on the skin were recorded during the above 3 min. Thereafter, the volunteers were kept without any activity and contact of impregnated parts with various surfaces for 30 min. The 3-min test and 30-min rest periods continued until two bites occurred in a 3-min test or two bites in two common 3-min tests at 30 min intervals. If the bite was not confirmed within subsequent 3 min after a bite in a 3-min test, the test continued until a bite was confirmed. To determine the failure times of the substances, the test confirmation did not stop after receiving a bite and continued until the 10th bite. After each test, the mosquitoes used in the previous test were discarded and not used for subsequent tests. To determine the complete protection time (CPT) and failure time (FT), each repellent was used for four volunteers with three replications.

Statistical analysis

Values of protection and failure time were expressed as mean ± standard deviation (SD) using Kaplan-Meier equations; also, means of them were compared by the ANOVA, Tukey test. The 1% level was employed in tests of significance.

HPLC Analysis

Neem oil was analyzed by HPLC-PDA using High Performance Liquid Chromatography (HPLC) system fitted with a photodiode array (PDA, at 210–400 nm) and a liquid chromatography quaternary pump. A Eurospher C-18 column (250 mm × 4.6 mm, 5 μm, 100A) (Knauer, Germany, Berlin) was used. The mobile phase was acetonitrile/water (60:40 v/v) at a flow rate of 0.50 mL min⁻¹, and the runtime was 40 min with a sample volume injection of 20 μL.

Mass spectrometry (MS)

Liquid chromatography–mass spectrometry (LC–MS) consisted of Agilent HPLC–DAD system (1200 series), a Rheodyne injector model 7725i with a 100 μL loop with Phinigan LCQ mass spectrometer. DAD and MS data was collected and processed using Agilent instrument online and Xcalibur respectively.

LC–MS Condition

The mobile phase used was acetonitrile and water (0.1% formic acid) in the ratio of 60:40 v/v. Flow was kept at 0.5 mL/min. A PerfectSil ODS C18 5 μm (250 mm \times 4.6 mm) column was used. The LC effluent passed through a UV detector at 217 and 254 nm and the mass spectrometer. Mass spectra were acquired by a FinniganTM LCQTM DECA ion trap instrument. An ionization device was used for sample analysis (sheath gas: 80 mL/min, auxiliary gas: 20 mL/min, spray voltage: 5 kV, capillary temperature: 300 $^{\circ}\text{C}$, capillary voltage: 46 kV, and tube lens: - 60 kV). The Xcalibur 2.0 SR2 software (copyright Thermo Electron Corporation 1998–2006) was used.

Results And Discussion

HPLC and LC-MS Analysis

The chromatogram in 215 nm shows components of used neem oil. Further MS analysis of chromatogram show the azadirachtin H (7.5 min), azadirachtin D (8.0 min), azadirachtin A (8.9 min), azadirachtin B (9.9 min), deacetylnimbin (13.1), nimbin (15.9 min), deacetylsalannin (17.0), and salannin (22.4 min) as the major components of neem oil.

Table 1
Mass data of the limonoids from neem oil by online-HPLC-DAD-MS

No.	RT (min)	Exact mass (m/z)	Observed [$M + H$] ⁺ (m/z)	Formula	Identification
2	7.5	662.3	662	$\text{C}_{33}\text{H}_{42}\text{O}_{14}$	Azadirachtin H
3	8.0	676.3	676	$\text{C}_{34}\text{H}_{44}\text{O}_{14}$	Azadirachtin D
4	8.5	720.3	721	$\text{C}_{35}\text{H}_{44}\text{O}_{16}$	Azadirachtin A
5	9.9	662.3	663	$\text{C}_{33}\text{H}_{42}\text{O}_{14}$	Azadirachtin B
6	13.1	498.3	499	$\text{C}_{28}\text{H}_{34}\text{O}_8$	Deacetylnimbin
7	15.9	540.3	541	$\text{C}_{30}\text{H}_{36}\text{O}_9$	Nimbin
8	17.0	554.3	555	$\text{C}_{32}\text{H}_{42}\text{O}_8$	Deacetylsalannin
9	22.4	596.3	597	$\text{C}_{34}\text{H}_{44}\text{O}_9$	Salannin

Pseudo ternary phase digram

According to the phase diagram, it can be seen that the higher amount of surfactant, makes more single-phase areas, and with increasing amounts of oil, more two-phase areas would be made. The ratios O50:T50, O10:T90 and T100 are ratios in which adding water does not cause loss of single-phase state.

Other ratios of neem oil and tween 80 will finally lose their monophasic state with the gradual addition of water.

As the pseudo-ternary-phase diagrams show, with increasing the amount of surfactant, more single-phase areas are created, while with increasing the amount of oil, two-phase areas increase. The single-phase regions, except for the ratios of 50T: 50O, 90T: 10O, are two or more phasic after increasing the amount of water.

Droplet size of NE

The smallest nanoparticles are related to the ratio of 90% of surfactant with a size of 12.6 nm, and in contrast, the largest particle size are made in the ratio of 90 to 10 oils to surfactant in the range of 93.9 nm to 600 nm.

Graphs of particle size to oil to surfactant ratio and vice versa show that particle size is inversely related to oil to surfactant ratio, so that with increasing surfactant ratio, particle size decreases, which can be seen exponentially in the relevant diagrams.

CPT

The effectiveness of each formulation was evaluated up to 10 landings/bites. In these evaluations, it was observed that 5% neem oil in i-propanol does not have a high ability to repel *An. stephensi* mosquitoes (30 ± 20 minutes). In addition, a 5% solution of neem oil in coconut oil, which is recommended as a suitable repellent, also showed low effect (60 ± 21 minutes). CPT in 20% DEET solution was up to 360 ± 24 minutes.

Comparison of two emulsions prepared from neem oil in twin 80 and water with ratios of 50:45:5 and 50:40:10, respectively, reveals that the first emulsion with only 5% neem oil was superior to the second emulsion with 10% neem oil. It is noticeable in repelling *Anopheles* mosquitoes (240 ± 39 minutes vs. 60 ± 22 minutes). Although the amount of neem oil in the second emulsion is twice as much, but the smaller particles of the first emulsion have made its effect longer. These data clearly show the superiority of a NE repellent over a ME repellent.

Another interesting point is that when the CPT compares the two emulsions of neem oil in tween and water in ratios of 50:45:5, the first being tween 80 and the second tween 20, it still indicates the superiority of the emulsion prepared with twin 80 (39 ± 240 minutes vs. 15 ± 90 minutes). This is probably due to the higher viscosity of the Tween 80 emulsion, which prevents the rapid release of neem oil due to its higher surface tension. The solution of Tween 80 in water with a ratio of 50:45 does not show any special insect repellent properties (30 ± 12 minutes). Therefore, the effect of emulsion base materials on insect repellency is eliminated. Significant differences of protection time and failure time between repellents were observed by ANOVA (Games-Howel), $P < 0.05$.

FT

The FTs were 90, 120, and 90 minutes for 5% neem oil in isopropanol, the 5% neem oil solution in coconut oil, and the tween 80 in water (50:45) solution, respectively. FT was 450 min for DEET 20% in ethanol, and for neem oil in twin 80 and water at 50: 45: 5 and 50:40:10, 330 and 180 minutes, respectively.

When FT compares two emulsions of neem oil in twin and water in 50: 45: 5 ratios, the first being twin 80 and the second twin 20, it still indicates the superiority of the emulsion prepared with twin 80 (330 minutes vs. 120 Minutes).

In this study, protection time of 6 h and failure time of 7.30 h were obtained for DEET 20%. Tavasoli *et al.* 2011^[23] reported a protection time of 6.23 h and a failure time of 7.30 h, which are almost the same as those in here. Fradin and Day^[30] reported that protection time, failure time, and DEET depend on the concentration, formulation, and the tested mosquito species and can vary in different conditions.

Of 33 *Anopheles* species known from Iran, seven species play an important role in malaria transmission in Iran, of which *An. stephensi* is one of the most important species. The main method to control mosquitoes is the use of synthetic insecticides that have harmful effects on human and animal health and the environment. Therefore, the use of natural products, including natural oils, is of paramount importance as being environmentally compatible and degradable. The results of this study showed that nanoformulated neem oil increased its protection time from 0.5 h to 4 h and the failure time from 1.5 h to 5.5 h. In a similar study, a NE was prepared from the essential oil of citronella plant and the repellent effect of this NE essential oil was compared with the normal status. The results showed that addition of glycerol improved the physical appearance and stability of the NE, and increased the protection time of NE product, which is completely in line with those of this research. Another study by Nuchuchua *et al.* (24) showed that preparation of NE from citronella, hairy basil, and vetiver could enhance the protection time of these essential oils up to 4.7 h. Nuchuchua *et al.* (25) reported that the droplet size of NE and the composition of repellents play an important role in determining the protection time, and that a smaller droplet size of NE improves physical stability, and improves protection time and the efficacy of the compounds due to forming an integrated coating on the human skin.

Conclusions

The NE formulations of neem oil containing Tween 80 were successfully created via the two step low-energy and high-energy method. All of the formulations provided a nano particle-size, with the smallest size being 12.6 nm. The ratio of 5:45:50 with the smallest particle size was found to be most effective. Overall, the results of this study show that preparation of a proper NE from neem oil made it a natural potent repellent.

Abbreviations

NE: nanoemulsion; DEET: N,N-Diethyl-meta-toluamide; CPT: complete protection time; DLS: dynamic light scattering, FT: failure time; SD: standard deviation; HPLC: High Performance Liquid Chromatography,

PDA: photodiode array, MS: Mass spectrometry, LC–MS: Liquid chromatography–mass spectrometry.

Declarations

Ethics approval and consent to participate

Ethical confirmation was obtained from Baqiyatallah University of Medical sciences ethics committee with the reference Number of: IR.BMSU.REC.1397.072.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was funded personally by contributing researchers without any financial support from any organization.

Authors' contributions

Maryam Iman developed the research idea and participated in the study design and fieldwork, conducted study practice and prepared data interpretation and finalized draft of this manuscript. Mohammadreza Taheri and Omid Dehghan conducted the laboratory activities and did writing primary manuscript of this article. Mehdi Khoobdel and Seyed Mohammad Zarei assisted in the study design and supervised fieldwork activities and revised primary manuscript before finalization. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

References

1. Roberts D, Glenda M. Risk factors of malaria in children under the age of five years old in Uganda. *Malaria journal*. 2016; 15(1): p. 1-11.

2. Malaria. [Online].; 2020 [cited 2020 11 17. Available from: <https://www.who.int/news-room/fact-sheets/detail/malaria#:~:text=Disease%20burden,416%20000%20deaths%20in%202017>.

3. Balkew M, Mumba P, Dengela D, Yohannes G, Getachew D, Yared S, et al. Geographical distribution of *Anopheles stephensi* in eastern Ethiopia. *Parasites & Vectors*. 2020; 13(1): p. 35-42.

4. Soko W, Chimbari MJ, Mukaratirwa S. Insecticide resistance in malaria-transmitting mosquitoes in Zimbabwe: a review. *Infectious diseases of poverty*. 2015; 4(1): p. 46.

5. Logan JG, Stanczyk NM, Hassanali A, Kemei J, Santana AE, Ribeiro KA, et al. Arm-in-cage testing of natural human-derived mosquito repellents. *Malaria journal*. 2010; 9(1): p. 239-249.

6. Shukla D, Wijayapala S, Vankar PS. Effective mosquito repellent from plant based formulation. *International Journal of Mosquito Research*. 2018; 5(1): p. 19-24.

7. Patel RV, Shafer KM, Patel P, Garmaza A, Wiangkham K, Franks RB, et al. EPA-registered repellents for mosquitoes transmitting emerging viral disease. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy*. 2016; 36(12): p. 1272-1280.

8. Trivedi A, Rai P, Kumar J, Trivedi CA. Formulation of low smoke herbal mosquito repellent sticks by using different essential oils. *The Pharma Innovation Journal*. 2018; 7(4): p. 173-175.

9. Murugan K, Pitchai GJ, Madhiyazhagan P, Nataraj T, Nareshkumar A, Hwang JS, et al. Larvicidal, repellent and smoke toxicity effect of neem products against malarial vector, *Anopheles stephensi*. *Int J Pure Appl Zool*. 2014; 2(2): p. 71-83.

10. Uchegbu M, Okoli I, Esonu B, Iloeje M. The growing importance of neem (*Azadirachta indica* A. Juss) in agriculture, industry, medicine and Environment: A review. *Research Journal of Medicinal Plant*. 2011; 5(3): p. 230-245.

11. Khater HF, Selim AM, Abouelella GA, Abouelella NA, Murugan K, Vaz NP, et al. Commercial mosquito repellents and their safety concerns. *Malaria*. 2019; p. 1-26.

12. Amer A, Heinz M. Repellency effect of forty-one essential oils against *Aedes*, *Anopheles*, and *Culex* mosquitoes. *Parasitology research*. 2006; 99(4): p. 478.

13. Abiy E, Gebre-Michael T, Balkew M, Medhin G. Repellent efficacy of DEET, MyggA, neem (*Azadirachta indica*) oil and chinaberry (*Melia azedarach*) oil against *Anopheles arabiensis*, the principal malaria vector in Ethiopia. *Malaria Journal*. 2015; 14(187): p. 187.

14. Tavares M, da Silva MRM, de Siqueira LBDO, Rodrigues RAS, Bodjolle-d'Almeida L, Dos Santos EP, et al. Trends in insect repellent formulations: A review. *International journal of pharmaceuticals*. 2018; 539(1-2): p. 190-209.

15. Mamood SNH, Hidayatulfathi O, Budin SB, Rohi GA, Zulfakar MH. The formulation of the essential oil of *Piper aduncum* Linnaeus (Piperales: Piperaceae) increases its efficacy as an insect repellent. *Bulletin of entomological research*. 2017; 107(1): p. 49-57.

16. de Campos VE, Ricci-Júnior E, Mansur CR. Nanoemulsions as delivery systems for lipophilic drugs. *Journal of nanoscience and nanotechnology*. 2012; 12(3): p. 2881-2890.

17. Anjali CH, Sharma Y, Mukherjee A, Chandrasekaran N. Neem oil (*Azadirachta indica*) nanoemulsion—a potent larvicidal agent against *Culex quinquefasciatus*. *Pest management science*. 2012; 68(2): p. 158-163.

18. Campos EV, de Oliveira JL, Pascoli M, de Lima R, Fraceto LF. Neem oil and crop protection: from now to the future. *Frontiers in plant science*. 2016; 7: p. 1494.
19. Ghayempour S, Montazer M. Micro/nanoencapsulation of essential oils and fragrances: Focus on perfumed, antimicrobial, mosquito-repellent and medical textiles. *Journal of microencapsulation*. 2016; 33(6): p. 497-510.
20. Nazeer AA, Rajan HV, Vijaykumar SD, Saravanan M. Evaluation of larvicidal and repellent activity of nanocrystal emulsion synthesized from *F. glomerata* and neem oil against mosquitoes. *Journal of Cluster Science*. 2019; 30(6): p. 1649-1661.
21. Solans C, Isabel S. Nano-emulsions: formation by low-energy methods. *Current opinion in colloid & interface science*. 2012; 17(5): p. 246-254.
22. Jasmina H, Džana O, Alisa E, Edina V, Ognjenka R. Preparation of nanoemulsions by high-energy and lowenergy emulsification methods. In *CMBEBIH 2017*. 2017; Springer(Singapore): p. 317-322.
23. Dammak I, Sobral PJDA, Aquino A, Neves MAD, Conte-Junior CA. Nanoemulsions: Using emulsifiers from natural sources replacing synthetic ones—A review. *Comprehensive Reviews in Food Science and Food Safety*. 2020; 19(5): p. 2721-2746.
24. Sakulku U, Nuchuchua O, Uawongyart N, Puttipipatkachorn S, Soottitantawat , A Ruktanonchai U. Characterization and mosquito repellent activity of citronella oil nanoemulsion. *International journal of pharmaceutics*. 2009; 327(1-2): p. 105-111.
25. Nuchuchua O, Sakulku U, Uawongyart N, Puttipipatkachorn S, Soottitantawat A, Ruktanonchai U. In vitro characterization and mosquito (*Aedes aegypti*) repellent activity of essential-oils-loaded nanoemulsions. *Aaps Pharmscitech*. 2009; 10(4): p. 1234.

Figures

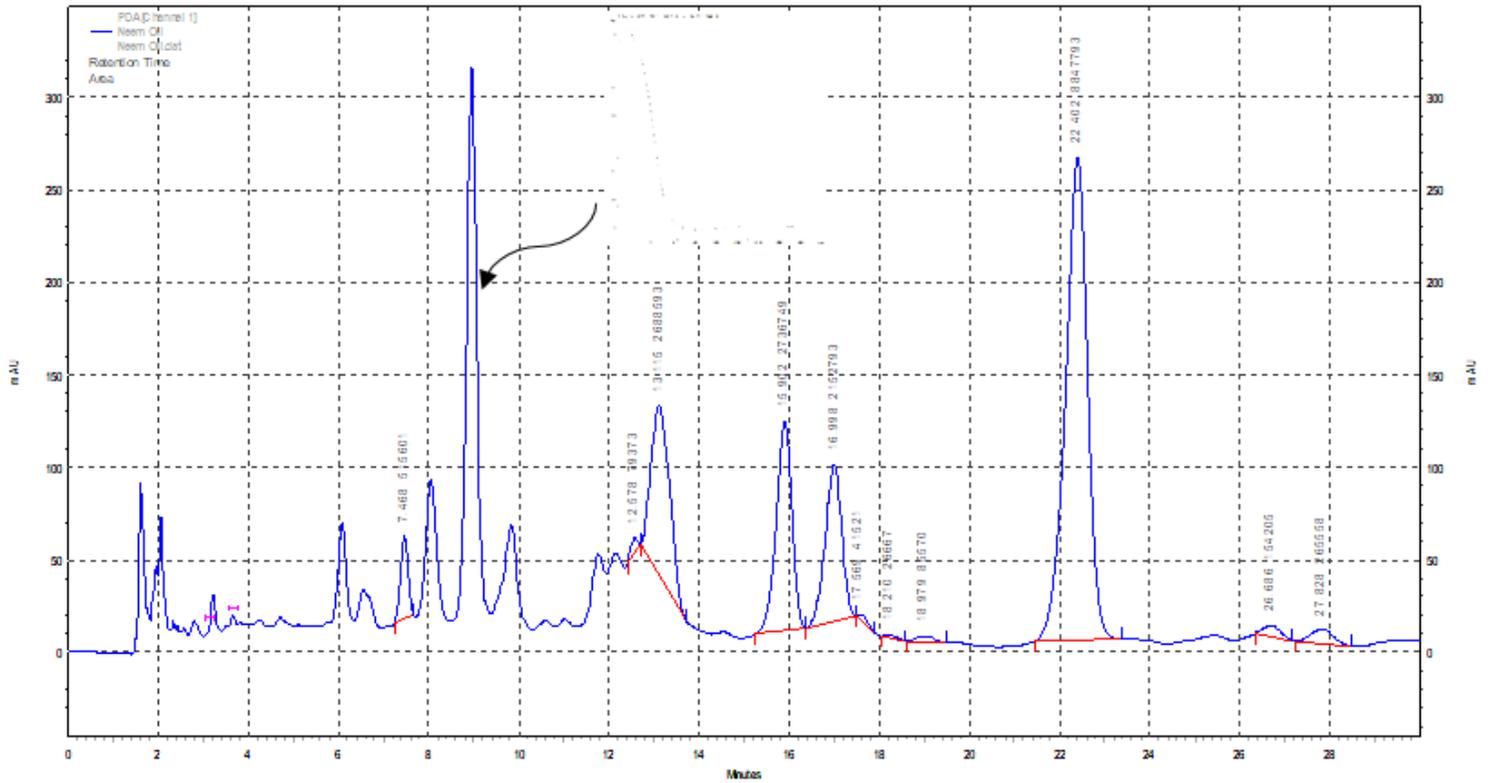


Figure 1

HPLC-DAD chromatogram of neem oil recorded at 215 nm in water/acetonitrile (40:60) as mobile phase, with flow rate 1mL/min

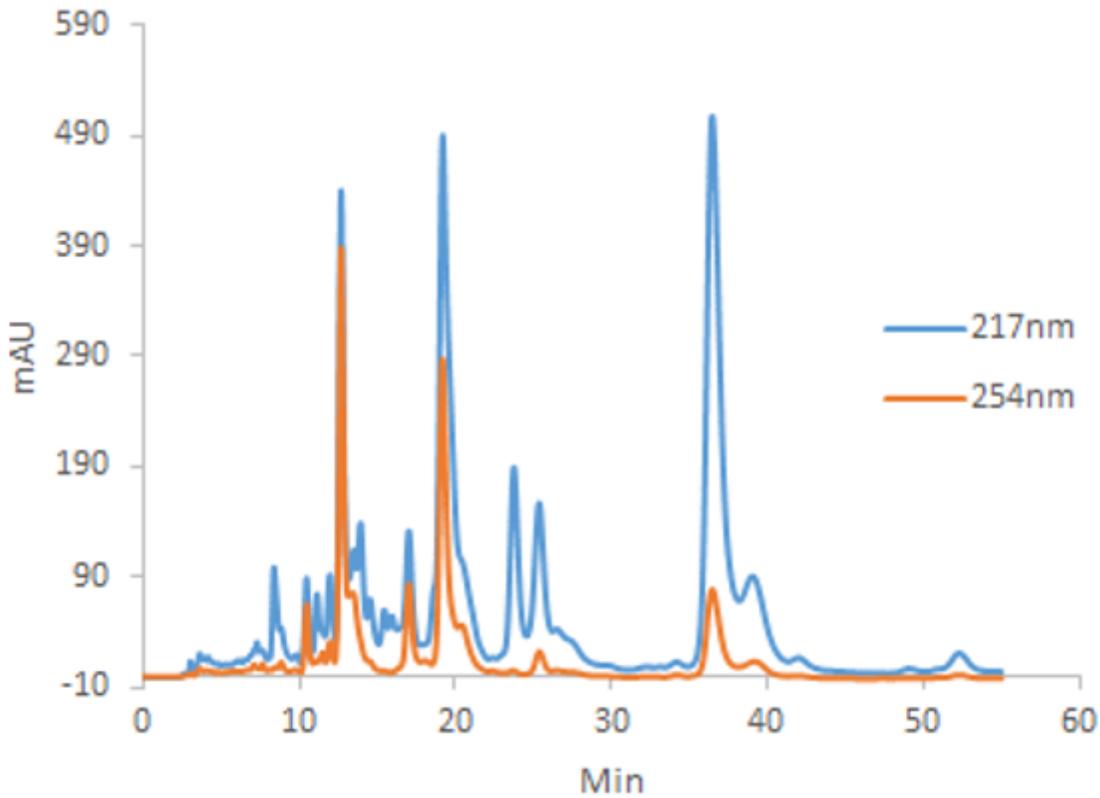


Figure 2

HPLC-DAD-MS chromatogram of neem oil recorded at 217 and 254 nm in water/acetonitrile (40:60) as mobile phase, with flow rate 1 mL/min

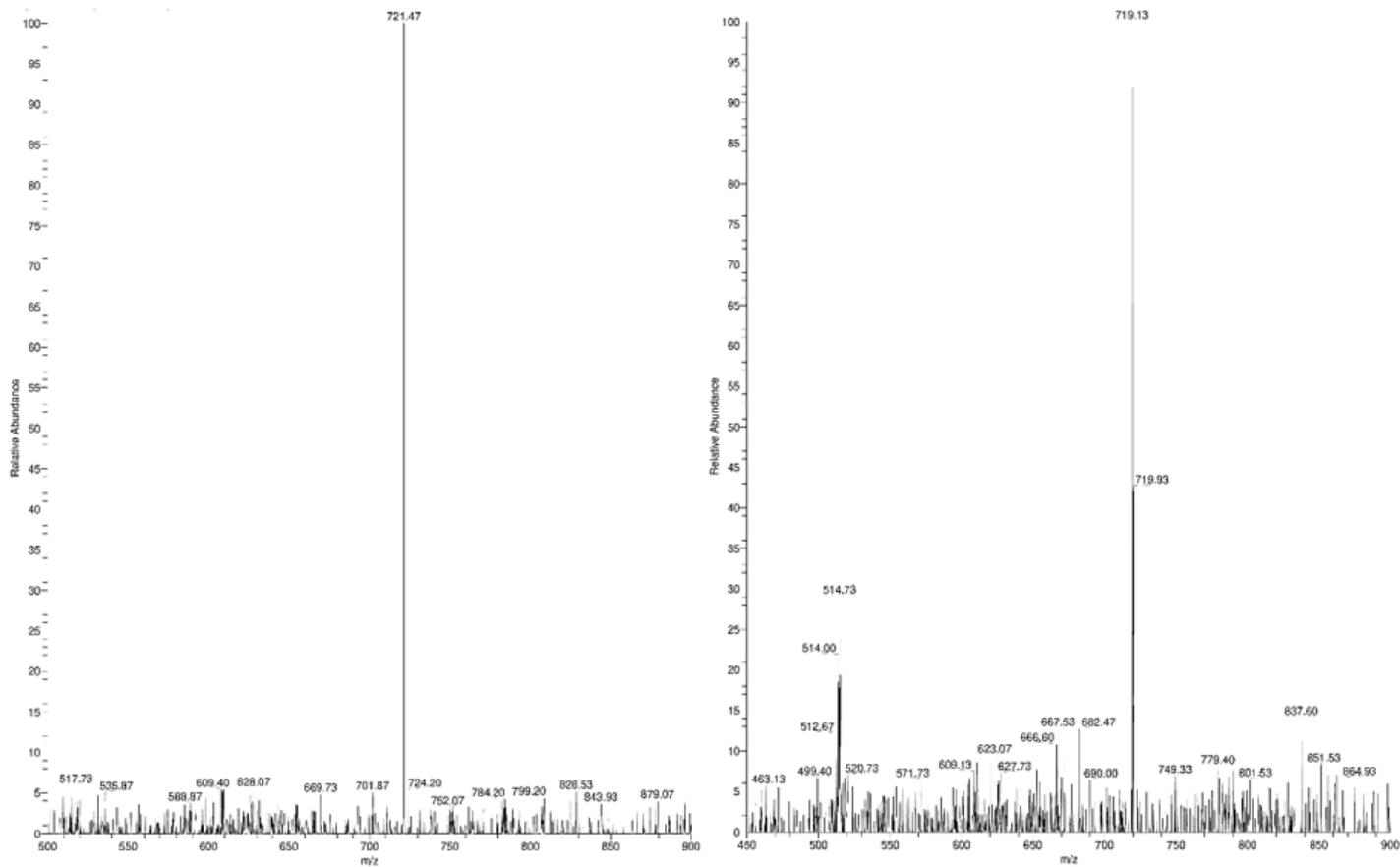


Figure 3

MS of Azadirachtin A at positive (right) and negative (left) modes in water/acetonitrile (40:60) as mobile phase, with flow rate 0.5 mL/min (RT=19.2 min)

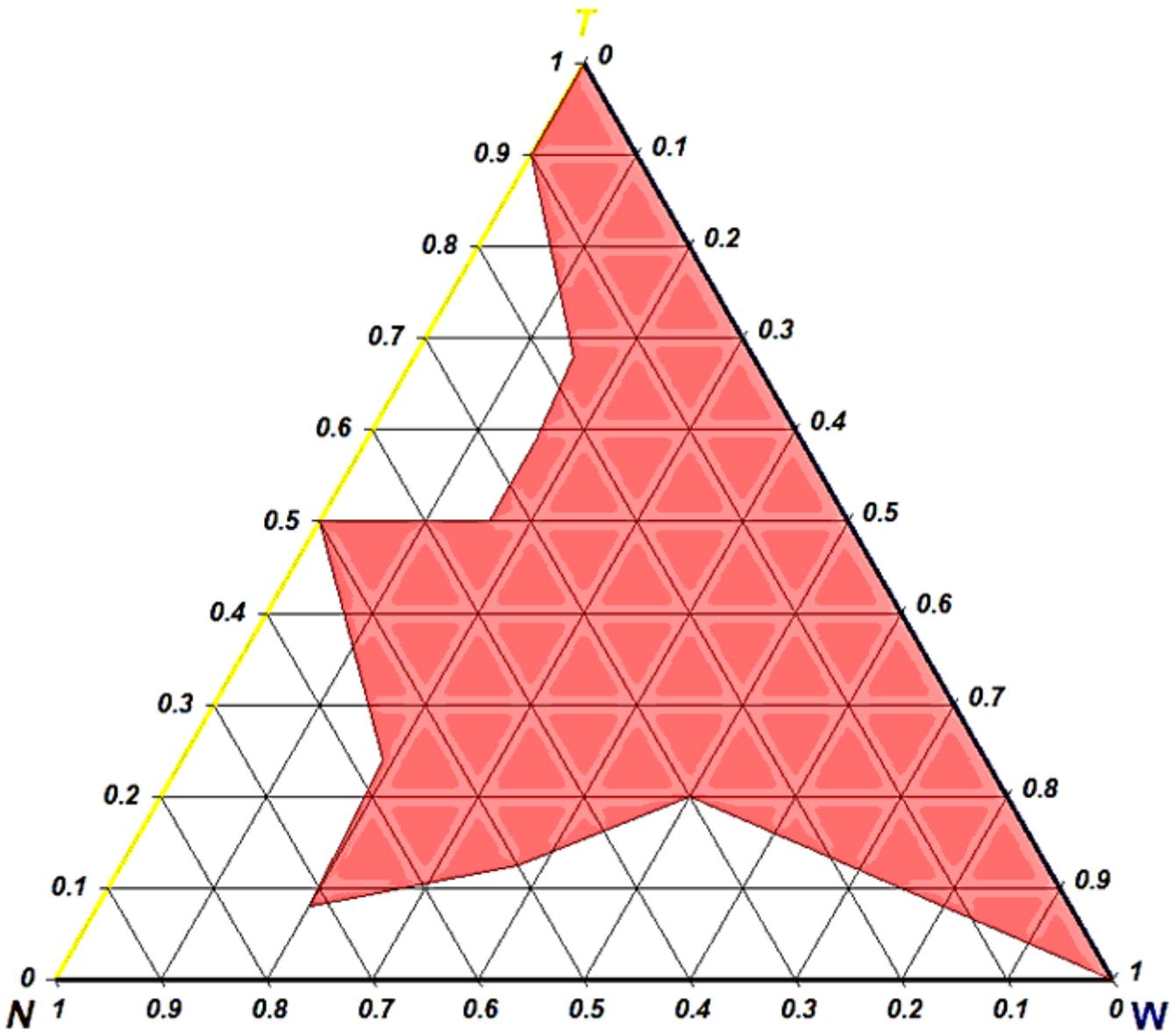


Figure 4

Drawn diagram for neem oil emulsion in tween 80 and water. The red region is mono-phase

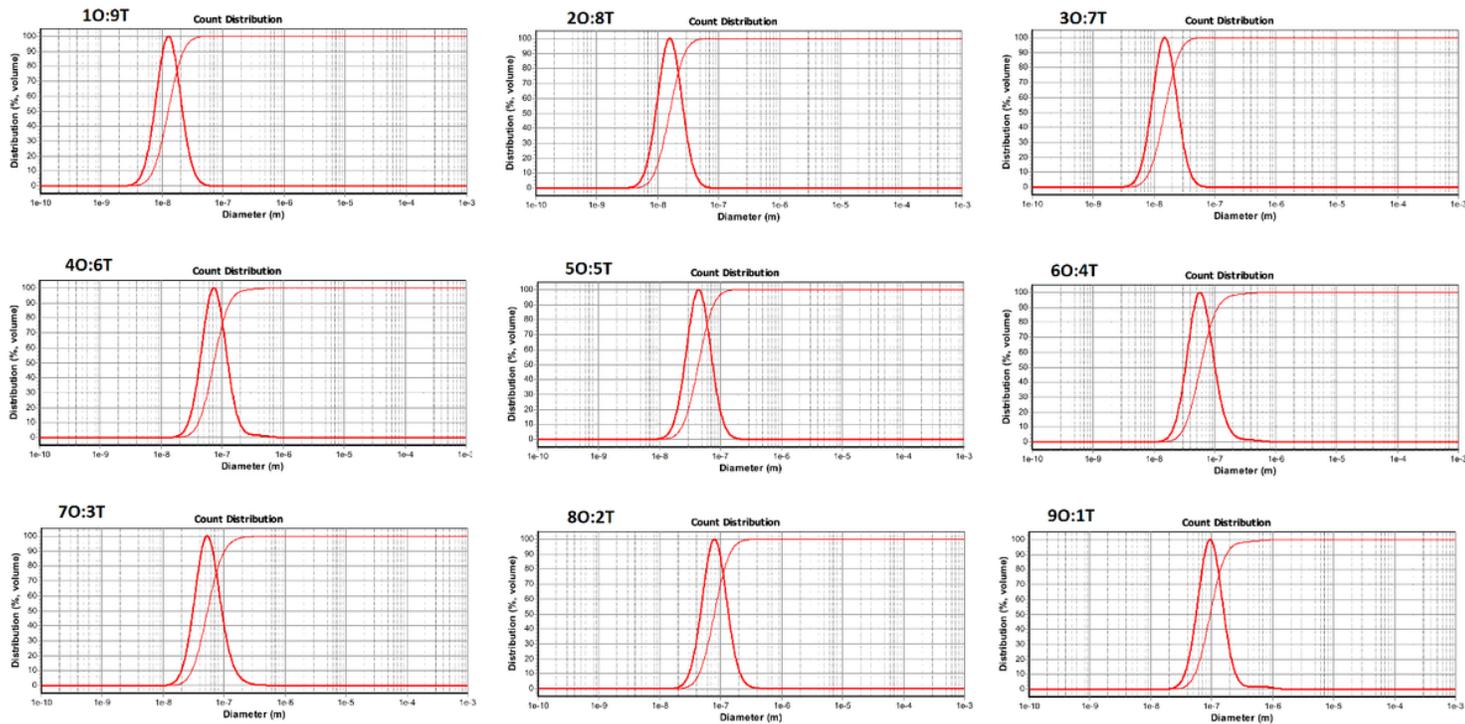


Figure 5

Results of particle size analysis related to different ratios of surfactant Tween 80 (T) and neem oil (O) in water prepared at 60 ° C

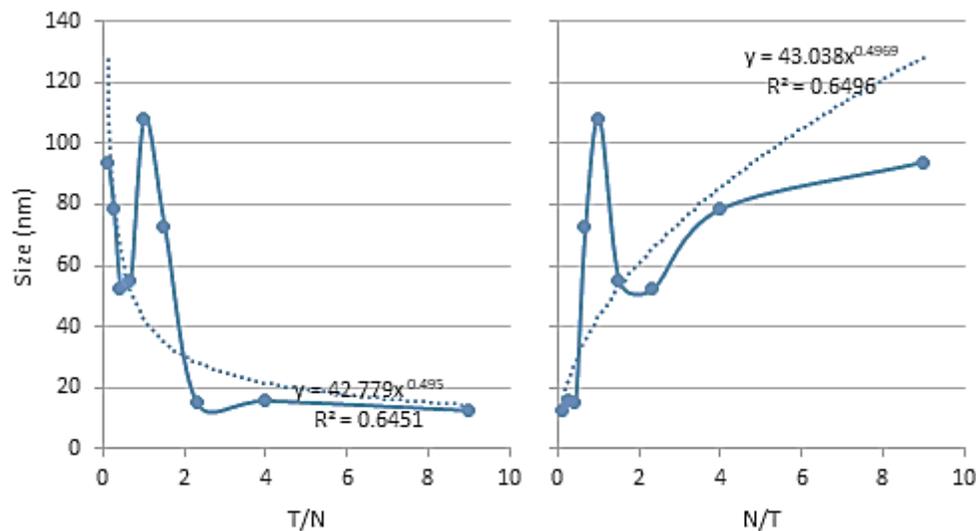


Figure 6

Particle size plot emulsions in terms of the ratio of Tween 80 (T) to neem oil (N) in water prepared at 50 ° C