JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

DEVELOPMENT OF NANOEMULSION AND ITS CHARACTERIZATION FOR ANTI BIOFILM AND LARVICIDAL ACTIVITY

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doi: 10.15414/jmbfs.2021.10.4.553-556

ARTICLE INFO

Received 17. 3. 2020
Revised 26. 9. 2020
Accepted 2. 10. 2020
Published 1. 2. 2021

Regular article

OPEN ACCESS

ABSTRACT

Dengue is a mosquito borne viral disease and the main root cause vector is *Aedes aegypti*. Hence in this work a rapid measure was taken to eradicate the vector using nanoemulsion. Plant essential oil based nanoemulsions was formulated by ultrasonic emulsification method. In the current study, nanoemulsion (O/W) was developed using oil extracted from the leaves of a Tulsi, Neem and Nochi. The emulsions were prepared in different ratios of oil and surfactant (Tween 80) and evaluated for their mechanical and thermal stability. After the confirmation of stable nanoemulsion formation, it was evaluated for its antibiofilm activity. Results revealed that the formulated mixed oil nanoemulsion exhibited enhanced antibiofilm activity against isolated microorganisms from contaminated wall samples. Further the biofilm forming capability of bacterial culture was indirectly assessed by staining with 1% crystal violet and measured absorbance using ethanol as a destaining solution. The mean biofilm production of S3 bacterial isolates significantly greater than that observed for S1 & S2. In addition, antibiofilm activity of the emulsion NE3 was higher against bacterial culture S3. Different concentration of mixed oil nanoemulsiom (NE3) treated group was subjected to evaluate the larvicidal activity against *Aedes aegypti* between 1 to 12 hours respectively. It was observed that an increase in the nanoemulsion concentration (NE3) to 25 ppm and 50 ppm resulted in complete loss of larval viability in 5 hr and 6 hr respectively. There is no significant difference in larval viability after 6 hr and 12 hr in all the treatment groups. Hence the above findings suggest the applicative potential of mixed nanoemulsion formulation for various applications including mosquito larval control and antibiofilm activity.

Keywords: Microbial biofilms, Nanoemulsion, Tween20/80, Microtiter plate assay, Dengue, Crystal violet assay, larvicidal activity

INTRODUCTION

Dengue is a rapidly spreading mosquito borne viral disease in the world. The various serotypes of the dengue virus are transmitted to humans by the mosquito bites of *Aedes aegypti & Culex quinquefasciatus*. These organisms complete one half of their life cycle in the gut of mosquitoes and the other in a living host and are responsible for transmitting deadly diseases like malaria, dengue, chikungunya, yellow fever, encephalitis, and filariasis **(Osanloo *et al*., 2017)**.
Repellent based essential oils for controlling mosquitoes are being developed as an alternative to chemical agents such as DEET (N,N-diethyl-meta-toluamide),kerosene fumes which leads to environmental pollution as well as health hazards like skin itching, rashes, eye irritation and neurological issues. Prolonged use of these chemicals results in damage to kidney, liver and in pregnant women, it leads to teratogenic effects in the foetus **(Anjali *et al*., 2012)**.
In the last 50 years, different groups of medicinal plants have been screened as an effective source for repellents and insecticides. Biopaint is a formulation that contains the natural essential oils to prevent the biofilm formation and also aids in the control of mosquitoes and insects. The Nano bio paint prepared from the natural oil is supposed to eradicate mosquitoes in a better way when compared to the synthetic repellent since they are nontoxic and eco-friendly **(Mohammadi *et al*., 2019)**. The production cost of nanocolloidal biopaint is less when compared to the conventional mosquito repellent paint.
Studies on plants such as *Ocimum tenuflorium (Tulsi), Azadirachta indica (Indian neem), Vitex Negundo* (Nochi leaves) shows that they have strong tendency to repel insects **(Arciola *et al*., 2005; Singh *et al*., 2019)**. The medicinal plants used in this experiment are being used traditionally by South Indians for repelling mosquitoes and other insects. Tulasi or tulsi, commonly known as holy basil is an aromatic perennial plant in the family Lamiaceae. Essential oils extracted from tulasi is mostly used for medicinal purposes and herbal cosmetics. *Azadirachta indica*, commonly known as neem, is a tree in the mahogany family Meliaceae. The bioactive compounds, Azadirachtin, Nimbin, Nimbidin and Nimbolides have antifeedant activity, ovicidal activity, fecundity suppression besides insect growth regulation and repellency against insects and pests **(Burt, 2004)**. It induces resistance due to their multiple mode of action on insects.., Neem is considered environmentally safe and its products are highly accepted by the public for crop pests control and various mosquito control programmes **(Da Costa *et al*., 2014).**
Nochi has Astringent and anti-inflammatory property. It is found to be effective against ringworm, eczema and various other skin infections, liver disorders, spleen enlargement, rheumatic pain, gout, abscess and backache **(Karunakaran *et al*., 2019)**.
Bacterial population in the environment associated with surfaces is called biofilms. It causes serious sanitary problems for both humans and animals and also in clinical and industrial settings. Biofilm producing bacteria has specific properties such as increased resistance to antibiotics, UV light and chemical biocides, greater genetic exchange, distorted biodegradability**(Famuyide *et al*., 2019)**. To overcome these issues, Biofilm inhibitors are screened from different medicinal plants and are made as a nanoemulsion formulation to control the growth of biofilm.
In the present study, an attempt has been made to formulate a nanoemulsion from the extracts of Tulsi, Neem and Nochi_with different concentration and is studied for their potential inhibitory action against mosquitoes and biofilm formation. In future, we are going to formulate nanocolloidal biopaint to eradicate mosquitoes and biofilms using essential plant oils.

MATERIAL AND METHODS

Extraction of essential oil

The fresh plant leaves of Tulsi, Neem and Nochi were collected from in and around Sathyamangalam and washed with distilled water and shade dried for one day. The grinded powder samples was seived and subjected for extration using Hexane solvent. The essential oil extraction was done by steam distillation for 8

hours and the temperature was maintained at 55℃. The separated oil from aqueous solution was stored in dark glass bottle and kept it for 4 °C for until further use (**Mindaryani and Rahayu, 2007**). Measure the amount of oil obtained (**Navarro-Rocha *et al*., 2020**).
The yield of essential oil can be calculated by,

X1 = final yield of oil (g)

X2 = total weight of fresh leaves (g)

Essential oils yield (%) = [X1 / X2]* 100

Nanoemulsion preparation

Nano emulsion was prepared using 6% (v/v) mixed plant oil (Tulsi, Neem and Nochi), nonionic surfactant Tween 80 (high HLB value) and distilled water by ultrasonication method. The nonionic surfactant is used to stabilize the droplets of emulsion by stearic stabilization. The core emulsion was formulated by mixing essential oil and surfactant in different oil-to-water ratios (5/95, 10/90, 15/85, 20/80, 25/75, 30/70) followed by addition of distilled water. After that, the emulsion was subjected to high energy ultrasonication using a 20 kHz sonicator. The emulsion is placed in a beaker containing ice to nullify the heat during ultrasonic emulsification process (**Kalita *et al*., 2013**).

Stability Study

The thermal stability analysis of formulated nanoemulsion was performed to investigate the effect of temperature. Samples were stored between 4 and 40 ℃ temperature each for a period of 48 h. The heating–cooling cycle was repeated four times. Absence of cracking, creaming and phase separation indicates that prepared emulsion is highly stable (**Kuhn *et al*.,2002**).

Zeta-Potential analysis

The stability of the emulsions depends on its electrostatic potential on the surface of emulsion and magnitude of the zeta potential is predictive of the colloidal stability. Nano emulsions with Zeta Potential values greater than +25 mV or less than -25 mV typically have high degrees of stability. The zeta potential values for combined oil emulsion can be obtained using zeta potential analyzer (**Sharma *et al*.,2020**).

Particle size analysis

The droplet size measurement and PDI of mixed nanoemulsion was investigated using particle size analyzer at room temperature (Malvern, UK). The Polydispersity Index is used to measure the homogeneity and stability of the droplet size in the nanoemulsion system. PDI values below 0.2 designate a narrow size distribution and as a result of long-term stability of the formulated nanoemulsion. Prior to analysis, the formulated emulsion was diluted with milli-Q (Millipore corporation) double-distilled water to avoid down multiple scattering effects (**Osanloo *et al*., 2017 ; Roy and Guha *et al*., 2018**).

Isolation of Biofilms

The contaminated wall samples were collected from nearby sathyamangalam region. The biofilm producing bacteria was isolated by serial dilution method and grown over Nutritive agar plates (**Arciola *et al*., 2005**).

Biofilm screening assay

The three bacterial colonies were cultured in nutrient broth media. 5ml of each bacterial culture is taken in a test tube and stored at 37°C for 5-10 days. Formation of biofilm is confirmed by adding crystal violet over the inner surface of the test tube. Crystal violet dye binds to negatively charged molecules such as polysaccharides present in the biofilm. After addition of dye, the biofilm appears violet in colour which can be observed with naked eye (**Qu *et al*., 2020**).

Antibiofilm activity of nanoemulsion

The antibacterial activities of the nanoemulsions against biofilm forming bacteria were determined using agar diffusion and microtiter plates. In agar diffusion method, the bacterial cultures were inoculated in petriplates containing agar medium. A filter paper was cut into round shaped pieces, soaked in the nanoemulsion and placed on the agar surface. The plates are incubated at 37°C overnight. The size of the zone of inhibition indicates the antibacterial activity of the nanoemulsion (**Muthuchamy *et al*., 2020**). Using micro titer plate, the nanoemulsion is added to nutrient broth media in different concentrations - 50%, 25%, 12.5% and 6.25%. 1µl of bacterial culture is added to each well. After one day of incubation of the microtiter plate at 37°C, the absorbance value is measured using ELIZA reader. Results of agar diffusion method and ELIZA reader were compared (**Blando *et al*., 2019**).

Vector larvae growth

The Aedes aegypti was *cultured* in our laboratory conditions using plastic trays (30*20*10cm) with a photo period of 13hrs light and 11hrs dark. The larvae's were cultivated in the plastic container which covered with muslin cloth to avoid emerged adult mosquito outside. The Larvae were fed with algae (or) dog biscuits and yeast extract in the ration of (3:1). The temperature is set at 28±1ºC with relative humidity of 80-85% (**Ramar *et al*., 2013**).

Larvicidal Activity

The larvicidal effect of the mixed oil nanoemulsion was evaluated according to the WHO procedures for testing mosquito larvicides (WHO, 2005). The larvae of *Aedes aegypti* was treated with different concentration of nanoemulsion and control larvae were kept in water without any treatment. According to the WHO protocol, 40 larvae of *Aedes aegypti* was added to the beaker with 200 ml of water using droppers in addition to that mixed oil nanoemulsion were added in different concentrations separately. The beakers containing the mosquito larvae were incubated at room temperature of 25°C and a photoperiod of 12 hr light followed by 12 hr dark (12L:12D) (**Oliveira *et al*., 2017**). Larval mortality of the mixed oil nano emulsion was monitored after different interval of exposure time without food as the maximum exposure period is 24 hr. The larvicidal studies were performed with three replicates. The larval mortality was corrected according to Abbott's formula (**Abbott, 1925**).

Mortality (%) = [X-Y / X]*100

Where X denotes the survival percentage in the untreated control group
Y denotes the survival percentage in different treated groups.

RESULTS AND DISCUSSION

The essential oils obtained from neem, tulsi and nochi are volatile complex mixtures with a various biological activities, including insect repellent and larvicidal properties (**Sugumar *et al*., 2016**). Bioactive compounds are ability to modulate the metabolic process and include positive properties such antioxidant, antiinflammatory and antimicrobial activities. This review examined the main compounds of medicinal plants such as Tulsi, Nochi and Neem with antimicrobial and anti-inflammatory activity and also inducing their physiological role and the different modes of action and synergies. Among the studied compounds, phenolic substances, which include phenylpropanoids, flavonoids, catechins, tannins, and lignans, could display various role in biochemical and pharmacological activities, as single compounds or complex phytochemical mixtures. These compounds are shown to be effective in inhibiting microbial biofilms and against mosquitoes (**Singh *et al*., 2019**). The development of A.aegypti occurs in water, consequently, active substances could be dispersed in this medium. On this perspective, an O/W nanoemulsion used to rectify the water solubility problems.
The formation of stable droplet size nano emulsion is mainly based on the type of oil (cloud and flavour oil) used in the emulsification process. The plant derived essential oil exhibits less viscosity and are highly refined and as called as flavour oils. Formulation of nanoemulsion with flavor oil resulted in stable emulsion with a droplet size in the range 100 - 200 nm. Therefore the flavour oils were used for nanoemulsion preparation.

Thermodynamic stability study

The Nano emulsion was prepared by ultrasonication method were subjected to different stress tests such as centrifugation, heating-cooling and freeze-thaw cycle. The emulsion is kept at different temperatures to check the stability of the nano emulsion at different temperatures 4, 26, 35, 40ºC. The best temperature for storage of Nano emulsion is found to be 26ºC respectively (**Gurpreet and Singh S, 2018**). The various concentration of neem, tulsi and nochi oil emulsions are taken for emulsion stability are 5/95, 10/90, 15/85, 20/80, 25/75 and 30/70. The Mixed oil nanoemulsion (10/90) formulated with Tween 20 as a surfactant, was found to be stable formulation. Based on these results, the stable nanoemulsion formulations with reduced surfactant concentration were chosen for size characterization studies. Singh et al. (2019) investigated stability studies on nanoemulsion and found that no change in physical appearance and viscosity, at 25° C /60 % RH and 30°/65 % RH.

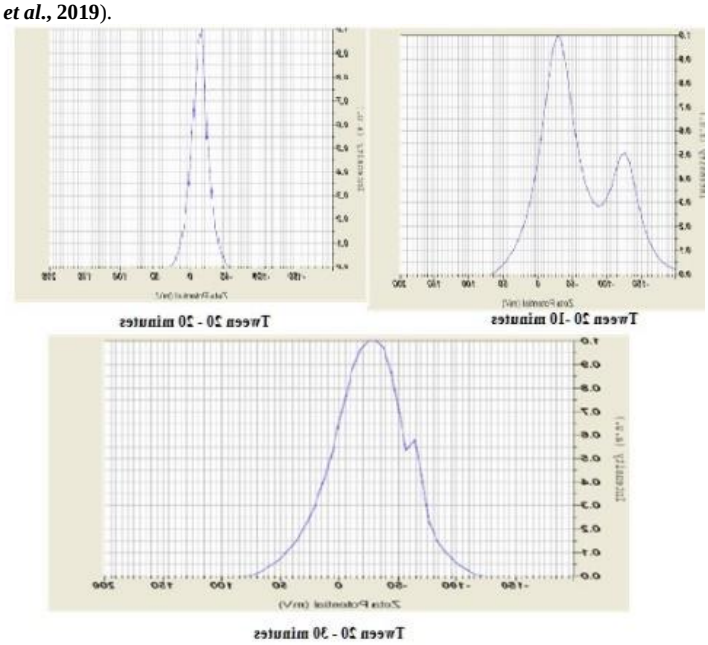
Droplet size distribution of selected nanoemulsion formulation

The nanoemulsion was formulated by ultrasonication method which yields low polydispersity index with minimized droplet size.Emulsification time has a direct correlation with droplet diameter of the nanoemulsion. When emulsification time increased from 10 min to 30 min using mixed oil nanoemulsion (10/90) and

Tween 20, the mean droplet size of the formulation NE1, NE2 and NE3 were reduced from 607.7 to 351 nm and 351 to 311.9 nm respectively. The polydispersity index of formulated nanoemulsion was found to be minimum in the case of NE1 (0.649) and NE2 (0.427) as compared to NE3 (0.360). The polydispersity index (PI) of the nanoemulsions was below 0.2 which provide long-term stability to the nanoemulsions (**Blando *et al*., 2019**). While the other formuations were not stable because of phase separation. It is used to measure the stability and homogeneity of formulated nanoemulsion droplet size. A Similar report observed by **Leong *et al.* (2009) and Tang *et al*. (2013)** which showed a decreasing trend of droplet size and PDI with the increase in sonication time while formulating sunflower oil and aspirin nanoemulsion.

Zeta-Potential analysis

Zeta potential is a scientific term for electro kinetic potential in colloidal systems. In colloidal system, an electro kinetic potential difference between the dispersed particle in a fluid of stationary layer and the dispersed medium. Zeta potential value of ± 30 mV can be considered as the arbitrary values to differentiate low-charged surfaces from highly charged surfaces (**Preetz *et al*., 2010**). It can be the degree of repulsion among similarly charged adjacent particles in dispersed medium. For a small particles shows a high zeta potential will resist to aggregation that confers stability. At low potentials have a tendency to coagulate. Therefore, colloids with high zeta potential (negative or positive) are highly stabilized while colloids with low zeta potentials tend to form coagulate (**Blando *et al*., 2019**).



[NE1-Nanoemulsion at 10 mins emulsification; NE1-Nanoemulsion at 20 mins emulsification; NE1-Nanoemulsion at 30 mins emulsification]

Figure 1 Zetapotential analysis of formulated nanoemulsions (NE1, NE2 & NE3) at different time interval

From figure 1, emulsification time increased from 10 min to 30 min using mixed oil and Tween 20, the zeta potential values of NE1, NE2 and NE3 were reduced from -14.2 to -29.2 mV and -29.2 to -41.5 mV respectively. In brief, zeta potentials from 0 to 30 mV (positive or negative) shows instability, although higher than ±30 mV proved the stability of nanoemulsion (ASTM 1985). For instance, **Krithika *et al*. (2019)** investigated the zeta potential of nanoemulsions: the information given by the zeta potential allows stating that the nanoemulsions with highly charged surfaces are stable and will resist to droplet aggregation .

Isolation of biofilms

Antibiotic resistance and biofilm formation play a crucial role in clinical infections. In conventional therapy, due to the increase in complexity of microbial infections and the resistance, scientist has been incited to recognize alternatives for the microbial infections. The Plant based bioactive compounds have ability to treat infections with less side effects while ancient times. From 2 different contaminated wall samples, a total of 17 bacterial isolates were obtained out of which 3 bacterial isolates identified black crystalline colonies after 24 h of incubation on nutrient agar plate (**Famuyide *et al*., 2019**).

Biofilm formation assay

Biofilm forming capability of the bacteria was confirmed by applying crystal violet over the surface of the test tubes as shown in figure 2. Crystal violet binds to negatively charged molecules such as polysaccharides present in biofilm. The biofilm formation was higher in bacterial culture 'S3' compared to S1 and S2. Because of its high biofilm forming activity, only 'S3' culture is used for antifilm assay. This was determined by quantifying and comparing the biofilm producing ability by Microtiter plate method. An optical density of S1, S2 & S3 ranging from 0.274 to 0.542 at 550 nm. The results showed that S3 isolate have higher biofilm formation than S1 & S2 strains (**Blando *et al*.,2019**).

Figure 2 Formation of biofilm by S3 isolate

Antibiofilm activity

The antibacterial activity of nanoemulsions against the biofilm forming bacteria was confirmed by disc diffusion method. Emulsions NE3 controlled bacterial growth at a higher rate compared to NE1 and NE2. This can be due to the optimum proportion of surfactant and oil mixture.

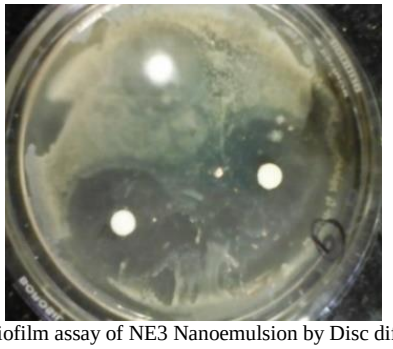


Figure 3 Antibiofilm assay of NE3 Nanoemulsion by Disc diffusion method

In addition, antibacterial activity of the emulsion NE3 was higher against bacterial culture S3 as shown in figure 3. This indicates that, antibacterial activity is enhanced in bacteria which forms thicker biofilm compared to other bacterial cultures. A similar result observed by microtiter plate method. (**Chaudhari *et al*., 2020; Seibert *et al*., 2019**)

Larvicidal activity of nanoemulsion

An experiment performed on mixed oil nanoemulsion (10/90) containing Tween 20 as a surfactant showed mortality in all the concentration such as at different emulsification time (10, 20 & 30 minutes). Results are shown in the figure 4. The larvicidal activity of NE1,NE2 & NE3 was investigated against third instar larvae A*edes aegypti*. Mixed oil nanoemulsion NE3 confirmed concentration and time dependent killing of mosquito larva. The study was investigated by varing concentration of nanoemulsion (5 ppm, 10 ppm, 15 ppm, 20 ppm, 25 ppm and 50 ppm) to check the larvicidal efficacy in different time interval (1 hr, 2 hr, 3 hr, 4 hr, 5hr and 6 hr). Five ppm of NE3 resulted in 40 % larval mortality in 4 hr, whereas 10 ppm of NE3 achieved 60 % larval mortality only in 3 hr (**Lucia *et al*., 2020; Osanloo *et al*., 2017**).

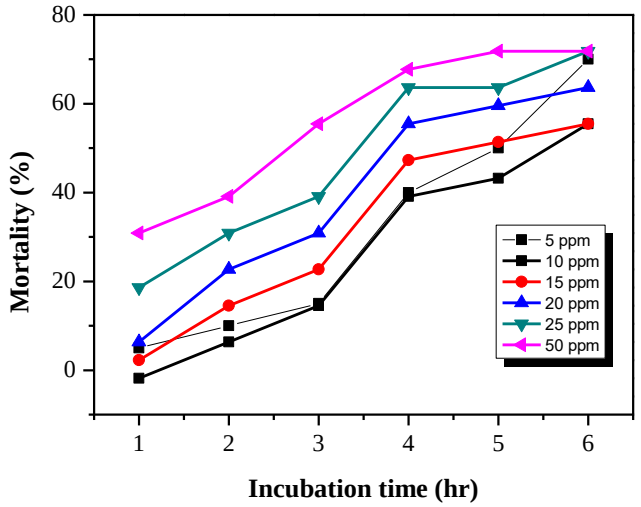


Figure 4 Larvicidal activity of formulated nanoemulsion

Similarly in NE1 and NE 2 achieved high mortality in 25 ppm and 50 ppm concentration than low concentration of nanoemulsion Increase in the nanoemulsion concentration to 25 ppm and 50 ppm resulted in complete loss of larval viability in 6 hr and 5 hr respectively. No significant difference was observed between larval viability after 6 hr and 12 hr in all the treatment groups.

CONCLUSION

The present study demonstrates formulated nanoemulsion using plant essential oils like neem, tulsi and nochi oil by ultrasonication method. Emulsification process was optimized with different process parameters like Tween 20 concentration, oil to surfactant ratio, and emulsification time. Mixed plant oil nanoemulsion showed significant larvicidal and antibiofilm activity even at lower concentrations. The interactions between nanodroplets and active ingredients present in the emulsion system played a major role in the application studies. Hence, the formulated nanoemulsions can be further used for different applications like control of mosquito larvae in the environment, and also for biopaint preparation. Thus, these nanoemulsions seem to be an effectual alternative to synthetic or semi-synthetic additives.

Acknowledgments: We would like to express our gratitude to the Department of Biotechnology, Bannari Amman Institute of Technology, Sathyamanagalam who helped us with the labwork.

REFERENCES

Abbott, W. S. (1925). A method of computing the effectiveness of an insecticide. J. econ. Entomol, 18(2), 265-267. https://doi.org/10.1093/jee/18.2.265a

Anjali, C. H., Sharma, Y., Mukherjee, A., & Chandrasekaran, N. (2012). Neem oil (Azadirachta indica) nanoemulsion—a potent larvicidal agent against Culex quinquefasciatus. Pest management science, 68(2), 158-163. https://doi.org/10.1002/ps.2233

Arciola, C. R., Campoccia, D., Gamberini, S., Baldassarri, L., & Montanaro, L. (2005). Prevalence of cna fnbA and fnbB adhesin genes among Staphylococcus aureus isolates from orthopedic infections associated to different types of implant. FEMS microbiology letters, 246(1), 81-86. https://doi.org/10.1016/j.femsle.2005.03.035

Blando, F., Russo, R., Negro, C., De Bellis, L., & Frassinetti, S. (2019). Antimicrobial and antibiofilm activity against Staphylococcus aureus of Opuntia ficus-indica (L.) Mill. cladode polyphenolic extracts. *Antioxidants*, *8*(5), 117. https://doi.org/10.3390/antiox8050117

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. International journal of food microbiology, 94(3), 223-253. https://doi.org/10.1016/j.ijfoodmicro.2004.03.022

Chaudhari, A. K., Singh, V. K., Deepika, S. D., Prasad, J., Dwivedy, A. K., & Dubey, N. K. (2020). Improvement of in vitro and in situ antifungal, AFB1 inhibitory and antioxidant activity of Origanum majorana L. essential oil through nanoemulsion and recommending as novel food preservative. *Food and Chemical Toxicology*, 111536. https://doi.org/10.1016/j.fct.2020.111536

Da Costa, S., Basri, M., Shamsudin, N., & Basri, H. (2014). Stability of positively charged nanoemulsion formulation containing steroidal drug for effective transdermal application. Journal of Chemistry, 2014. https://doi.org/10.1155/2014/748680

Famuyide, I. M., Aro, A. O., Fasina, F. O., Eloff, J. N., & McGaw, L. J. (2019). Antibacterial and antibiofilm activity of acetone leaf extracts of nine under-investigated south African Eugenia and Syzygium (Myrtaceae) species and their selectivity indices. *BMC complementary and alternative medicine*, *19*(1), 141. https://doi.org/10.1186/s12906-019-2547-z

Gurpreet, K., & Singh, S. K. (2018). Review of nanoemulsion formulation and characterization techniques. *Indian Journal of Pharmaceutical Sciences*, *80*(5), 781-789. https://doi.org/10.4172/pharmaceutical-sciences.1000422

Kalita, B., Bora, S., & Sharma, A. K. (2013). Plant essential oils as mosquito repellent-a review. *International Journal of Research and Development in Pharmacy & Life Sciences (IJRDPL)*, *3*, 143-150. https://doi.org/10.1055/s-0030-1270723

Karunakaran, S., & Niranjana, R. F. (2019). Laboratory evaluation of some selected indigenous plant leaf and garlic bulb extracts against Tribolium castaneum (Coleoptera: Tenebrionidae) IN CHICKPEA (Cicer arietinum). *ISA 2019*, 196. https://doi.org/10.16970/entoted.487623

Krithika, B., & Preetha, R. (2019). Formulation of protein based inulin incorporated synbiotic nanoemulsion for enhanced stability of probiotic. *Materials Research Express*, *6*(11), 114003. https://doi.org/10.1088/2053-1591/ab4d1a

Kuhn, R. J., Zhang, W., Rossmann, M. G., Pletnev, S. V., Corver, J., Lenches, E., ... & Baker, T. S. (2002). Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. Cell, 108(5), 717-725. https://doi.org/10.1142/9789814513357_0037

Leong, T. S. H., Wooster, T. J., Kentish, S. E., & Ashokkumar, M. (2009). Minimising oil droplet size using ultrasonic emulsification. Ultrasonics sonochemistry, 16(6), 721-727. https://doi.org/10.1016/j.ultsonch.2009.02.008

Lucia, A., Toloza, A. C., Fanucce, M., Fernandez-Pena, L., Ortega, F., Rubio, R. G., ... & Guzman, E. (2020). Nanoemulsions based on thymol-eugenol mixtures: Characterization, stability and larvicidal activity against Aedes aegypti. *Buletin of Insectology*, *73*, 153-160.

Mindaryani, A., & Rahayu, S. S. (2007, October). Essential oil from extraction and steam distillation of Ocimum Basillicum. In *Proceedings of the World Congress on Engineering and Computer Science* (pp. 24-26).

Mohammadi, M., Masoumipour, F., Hassanshahian, M., & Jafarinasab, T. (2019). Study the antibacterial and antibiofilm activity of Carum copticum against antibiotic-resistant bacteria in planktonic and biofilm forms. *Microbial pathogenesis*, *129*, 99-105. https://doi.org/10.1016/j.micpath.2019.02.002

Muthuchamy, M., Govindan, R., Shine, K., Thangasamy, V., Alharbi, N. S., Thillaichidambaram, M., ... & Alanzi, K. F. (2020). Anti-biofilm investigation of graphene/chitosan nanocomposites against biofilm producing P. aeruginosa and K. pneumoniae. *Carbohydrate Polymers*, *230*, 115646. https://doi.org/10.1016/j.carbpol.2019.115646

Oliveira, A. E., Bezerra, D. C., Duarte, J. L., Cruz, R. A., Souto, R. N., Ferreira, R. M., ... & Gama, P. E. (2017). Essential oil from Pterodon emarginatus as a promising natural raw material for larvicidal nanoemulsions against a tropical disease vector. *Sustainable Chemistry and Pharmacy*, *6*, 1-9. https://doi.org/10.1016/j.scp.2017.06.001

Osanloo, M., Amani, A., Sereshti, H., Abai, M. R., Esmaeili, F., & Sedaghat, M. M. (2017). Preparation and optimization nanoemulsion of Tarragon (Artemisia dracunculus) essential oil as effective herbal larvicide against Anopheles stephensi. *Industrial Crops and Products*, *109*, 214-219. https://doi.org/10.1016/j.indcrop.2017.08.037

Preetz, C., Hauser, A., Hause, G., Kramer, A., & Mäder, K. (2010). Application of atomic force microscopy and ultrasonic resonator technology on nanoscale: distinction of nanoemulsions from nanocapsules. European Journal of Pharmaceutical Sciences, 39(1-3), 141-151. https://doi.org/10.1016/j.ejps.2009.11.009

Qu, Y., Yang, S., Chen, Z., & Su, F. (2020). A modified crystal violet assay is comparable to XTT reduction assay for quantification of biofilm formation by Candida albicans. https://doi.org/10.21203/rs.3.rs-42661/v1

Ramar, M., Paulraj, G. M., & Ignacimuthu, S. (2013). Preliminary screening of plant essential oils against larvae of Culex quinquefasciatus Say (Diptera: Culicidae). African Journal of Biotechnology, 12(46), 6480-6483. https://doi.org/10.5897/ajb2013.12967

Roy, A., & Guha, P. (2018). Formulation and characterization of betel leaf (Piper betle L.) essential oil based nanoemulsion and its in vitro antibacterial efficacy against selected food pathogens. *Journal of food processing and preservation*, *42*(6), e13617. https://doi.org/10.1111/jfpp.13617

Sharma, A., Singh, A. P., & Harikumar, S. L. (2020). Development and optimization of nanoemulsion based gel for enhanced transdermal delivery of nitrendipine using box-behnken statistical design. *Drug Development and Industrial Pharmacy*, *46*(2), 329-342. https://doi.org/10.1080/03639045.2020.1721527

Singh, S., Jangde, R., & Daharwal, S. J. (2019). An Updated Review on Herbal Drug in Wound Healing. *Research Journal of Pharmacy and Technology*, *12*(6), 3089-3097. https://doi.org/10.5958/0974-360x.2019.00523.7

Sugumar, S., Singh, S., Mukherjee, A., & Chandrasekaran, N. (2016). Nanoemulsion of orange oil with non ionic surfactant produced emulsion using ultrasonication technique: evaluating against food spoilage yeast. *Applied Nanoscience*, *6*(1), 113-120.. https://doi.org/10.1007/s13204-015-0412-z

Tang, S. Y., Shridharan, P., & Sivakumar, M. (2013). Impact of process parameters in the generation of novel aspirin nanoemulsions–comparative studies between ultrasound cavitation and microfluidizer. *Ultrasonics sonochemistry*, *20*(1), 485-497. https://doi.org/10.1016/j.ultsonch.2012.04.005