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RESEARCH ARTICLE

Design and Development of Bio-based *Anisomeles indica* Kuntze Leaf Essential Oil Nanoemulsion: Antibacterial Activity, Antibiofilm Activity and Antiquorum Sensing Activity Against Skin Pathogens

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Abstract

Background: Plant aromatic oils have the phytochemicals to inhibit bacterial growth. However, an appropriate encapsulation method is required to enhance their activity and stability. *Anisomeles indica* is a weed and does not affect human health. Hence, *A. indica* aromatic oil nanoformulation could be used as a medication to treat skin infections. **Aim:** In this study, aromatic oil nanoformulation of *A. indica* was prepared and characterised. Further, antibacterial, antibiofilm and quorum quenching properties of nanoformulation were evaluated. **Methods:** Hydro-distillation process was used to extract aromatic oil. Further nanoformulation was prepared and characterised by the following methods: Ultrasonication method, Dynamic light scattering method, Transmission Electron Microscopy (TEM) analysis, Viscosity determination, pH measurement, Turbidity, % Transmittance and % Creaming index measurements. Agar disc diffusion, well diffusion methods and crystal violet staining assay were used to evaluate the anti-bacterial, antibiofilm, and quorum quenching activity respectively. **Results:** Nanoformulation 20AIU4 (Oil-in-water) was prepared using *A. indica* aromatic oil, Tween-20 and water using the ultrasonication method. Nanoformulation contains particles with an average size of 52nm, Polydispersity Index (PDI) value of 1.000, 99.9 % transmittance, and thermodynamically stable for three months. 20AIU4 nanoformulation inhibits the bacterial growth of the *Staphylococcus aureus* Microbial Type Culture Collection (MTCC) 902 and *Pseudomonas aeruginosa* MTCC 424 with the inhibition zone of 14.76 ± 0.4nm and 13.50 ± 0.5nm respectively. *A. indica* aromatic oil nanoformulation was inhibited the (85.71% and 85%) of bacterial attachment and biofilm development (80.55% and 85.71%) against *S. aureus* MTCC 902 and *P. aeruginosa* MTCC 424 respectively. Nanoformulation shows anti-quorum sensing activity with inhibit *Chromobacterium violaceum* MTCC 2656 growth with an inhibition zone of 23.9 ± 0.36nm. **Conclusion:** The present study reveals that the *A. indica* aromatic oil nanoformulation 20AIU4 is a potent antibacterial agent that can be used against bacterial skin infections.

Keywords: *Anisomeles indica* Kuntze, Antibacterial Activity, Antibiofilm Activity, Antiquorum Sensing Activity, Nanoemulsion

1. Introduction

Nowadays, the emergence of multidrug-resistant pathogenic organisms poses a significant threat to the health system and the phenomena have been defined by the World Health Organisation (WHO) under the following slogan: "Combat drug resistance: no action today, no cure tomorrow"[1]. Furthermore, several strains of both Gram-negative bacteria and Gram-positive have developed resistance to all classes of antibiotics, in addition to regularly used ones. While difficult to create novel antibacterial

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agents to treat infections induced by multidrug resistance microorganisms, it is also crucial and urgent to prepare new antibacterial agents. For this purpose, scientists are attempting to develop of nanoemulsion of plant essential oil with several possible targets, intricate mechanisms and desired biological and physicochemical features. There are charged lipids in the bacterial cell membrane that have a strong binding affinity and the capacity to attract electrostatically[2]. The nanoemulsions have charged lipids in the outer shell layers of nanodroplets and a core portion that contains antibacterial and therapeutic bioactive compounds. Ultrasonic emulsification is a high-energy technology that can quickly and effectively create nanoemulsions with small droplet diameters and narrow size distributions and stability for a prolonged period[3]. Herbal plant essential oil nanoemulsions have broad bactericidal activities and disturb the bacterial outer membranes. For the treatment of both Gram-negative and Gram-positive bacterial infections, nanoemulsions offer a novel class of antimicrobials[4]. Quorum sensing, a density-dependent communication mechanism used by many pathogenic bacteria, controls a variety of bacterial processes, including motility, virulence factors, resistance to antibiotics and the production of biofilms. A novel class of antibiotics known as nanoemulsions is intended to combat both Gram-positive and Gram-negative bacterial infections. For this reason, the quorum sensing system is a desirable target in the development of new drugs to stop quorum sensing-dependent bacterial harm in the food and medical industries[5].

A. indica Kuntze (*Lamiaceae* family), is a medicinal shrub that grows as a weed in India, Vietnam, Australia, China, Sri Lanka the Philippines, Taiwan and Thailand. The whole plant is used as a traditional medicine to treat several human ailments in Asian countries[6]. Antibacterial activity and antibiofilm activity of its aromatic oil and nanoformulation against phytopathogens and human pathogens have been reported[7-11]. In the present study, nanoformulation (20AIU4) was prepared using the ultrasonication method and characterisation of nanoformulation was carried out. Further, nanoformulation was assessed for bactericidal properties, inhibition of biofilm formation and signalling.

2. Materials and Methods

2.1 Collection of Plant Material and Extraction of Essential Oil

Fresh leaves of *A. indica* were collected in June near the Davangere University in Shivagangothri, Davangere, Karnataka. Davangere is situated in the Maidan region of the Deccan plateau, in western South India. Fresh leaves of *A. indica* were cleaned with distilled water and hydrodistillated for 3 hours to extract essential oil by Clevenger's apparatus[12]. Antibacterial activity containing phytoconstituents of *A. indica* essential oil was identified by Gas Chromatography Mass Spectrometry (GCMS) analysis.

2.2 Preparation of Nanoemulsion

The essential oil of *A. indica* leaf, Tween 20 (non-ionic surfactant) and water were used to create the oil-in-water nanoemulsion. Initially, a magnetic stirrer running at 400 rpm for 30 min was used to prepare the coarse emulsion by adding water to the organic phase comprising oil and surfactant. Subsequently, the coarse emulsion performed ultrasonic emulsification via a 25kHz ultrasonic cell crusher (Lark, India) capable of producing up to 150W of power. The droplet diameter is reduced by the disruptive forces produced by the sonicator probe, turning the coarse emulsion into a nanoemulsion. During the drawn-out emulsification process, heat is produced. By setting the sample into a beaker filled with ice, this heat is dissipated. As a result, there was little temperature variation. Next, the prepared nanoemulsion was examined for stability and its characteristics were noted. The entire process of characterisation was done at room temperature[13].

2.3 Characterisation of Nanoemulsion

2.3.1 Effect of Surfactant Concentration

A nanoemulsion was created using varying surfactant concentrations while maintaining a constant essential oil content of 5% v/v. Different ratios, including 1:1, 1:2, 1:3, and 1:4, were used to combine the oil-containing organic phase with surfactants (Tween 20). After 30 minutes of magnetic stirring the system at 400rpm, water was introduced dropwise to the organic phase.

2.3.2 Creaming Index (CI), Measurements and Evaluation of Stability of Nanoemulsion

To evaluate the phase separation intensity of nanoemulsion after 3 months of storage, % CI was measured using the following equations (1) and (2)[14].

$$\%CI = \frac{H_S}{H_T} \times 100 \quad (1)$$

$$H_S = H_T - H_C \quad (2)$$

Where H_S indicates stable phase height, H_C stands for the top of the emulsion with the height of the creamy layer, H_T for the overall emulsion height and % CI for the CI.

According to Shafiq and Shakeel[15], each nanoemulsion performed a stability assessment, which involved centrifuging the sample for 30 min at 3500 rpm. Next, the nanoemulsion's stability was examined at 4°C and 25°C. Additionally, the nanoemulsion was evaluated for 48 hours at two different temperatures 4°C and 40°C (Heating-Cooling cycle). The cycle was repeated thrice. Nanoemulsion was maintained in a freeze-thaw stress condition for 48 hours at two different temperatures -21°C and 25°C. Three duplicates of the experiment were carried out.

2.3.3 Percentage Transmittance and Turbidity Measurement

UV-visible spectrophotometer (Systronics 119, India) was used to measure the % transmittance and turbidity of the nanoemulsion at 600 nm[16]. Every measurement was carried out three times, and the results were computed and displayed as mean ± SD[17].

2.3.4 pH Measurement and Viscosity Determination

pH meter (ELICO Private Limited, India) was used to measure the pH of each nanoemulsion (20AIU1-20AIU4) at room temperature. To measure the viscosity of the nanoemulsion, an Ostwald viscometer was employed. 5ml of each nanoemulsion was put into the filling tube, and the capillary tube was then carefully suctioned full of the contents. The capillary tube's upper limit to the mark was reached at a time measured in seconds by the liquid's flow[18].

2.3.5 Determination of Polydispersity Index and Particle Size Distribution

Particle size distribution, droplet diameter and polydispersity index of selected nanoemulsion (20AIU4) were measured using Zetasizer Ver. 7.11 (Malvern Instruments Ltd., UK). The nanoemulsion was diluted with water in a 1:30 ratio before each experiment to mitigate the effects of repeated scattering. The droplet diameter was reported in nanometers (nm) and the Polydispersity Index (PDI) was measured.

2.3.6 Transmission Electron Microscope (TEM)

Field Emission Gun-Transmission Electron Microscope (TEM) was used to study and evaluate the morphology and nanodroplet form of 20AIU4 formulation[11]. For the TEM investigations, 20AIU4 was coated to a copper grid with a carbon covering (200 mesh/inches) and it was stained with phosphotungstic acid (1%). After being allowed to dry for about 30 min at room temperature, it was carefully cleaned using filter paper to remove any remaining phosphotungstic acid. The Field emission Gun-Transmission electron microscope at 200kV (JEOL JEM 2100F, United States) and tungsten source were used to capture the micrographs.

2.4 Evaluation of the Antibacterial Activity of Nanoemulsion.

2.4.1 Collection of Bacterial Strains and Culture Condition

P. aeruginosa MTCC 424, *S. aureus* MTCC 902 and *C. violaceum* MTCC 2656 were collected from IMTECH, Chandigarh. *C. violaceum* MTCC 2656 was cultured on nutrient agar media at 30°C. *P. aeruginosa* MTCC 424 and *S. aureus* MTCC 902 were cultured on nutrient agar media at 37°C.

2.4.2 Agar Disc Diffusion Assay

An agar disc diffusion experiment was used to evaluate the antibacterial activity of a particular nanoemulsion (20AIU4)[11]. A 6mm diameter of paper discs (Whatman No. 1 filter paper) with *A. indica* essential oil nanoemulsion (20AIU4), Ampicillin (positive control), 10% DMSO (negative control) and *A. indica* essential oil were placed on the *P. aeruginosa* and *S. aureus* (0.5 McFarl and standard) seeded agar plate. Plates were kept for 24 hours at 37 °C. The antibacterial capability

of the nanoemulsion was calculated using the mean diameter of the zone of inhibition surrounding the paper discs and expressed in millimetres (mm). Every assay was finished in triplicate.

2.4.3 Evaluation of Antibiofilm Activity of Nanoemulsion

The crystal violet staining technique was used to assess the inhibition of biofilm growth and bacterial cell attachment, modified as per the method suggested by Savitharani *et al.*[11] with slight modification. In 96-well microtiter plates, 40µL of each bacterial culture that was developing exponentially were dispensed. After adding the 20AIU4 nanoformulation to the wells, they were kept for 24 hours at 37 °C. The negative control was the medium that did not include nanoemulsion. Following incubation, the medium and detached cells were poured out and cleaned using PBS (Phosphate Buffer Saline). Following air drying, 0.1% (w/v) Crystal violet was used to stain the plate. To evaluate the effect on bacterial development, treated with nanoemulsion after 4 hours of development of biofilm in wells. Destain the wells after staining of crystal violet. The destained solution was transferred to a new microtiter plate for measuring optical density at OD590nm using a Bio-Rad microplate reader. Further, the percentage of biofilm development and bacterial cell attachment was calculated using the following equation (3).

$$Percentage\,inhibition = \frac{ODNegative\,control - ODExperimental}{ODNegative\,control} \times 100 \quad (3)$$

2.4.4 Quorum Sensing Inhibition Assays

The anti-quorum sensing activity of 20AIU4 nanoformulation was determined by the agar well diffusion method according to Mulya *et al.*,[19] with small modifications. A fresh suspension culture of *Chromobacterium violaceum* MTCC 2656 (0.5 McFarland standard) was spread on the nutrient agar media-containing plate. Wells were made using a sterile cork borer. The 100µL of 20AIU4 nanoformulation, Essential oil with 10% DMSO, Ampicillin (Positive control) and 10% DMSO (Negative control) were added into the wells and kept for 24 hours at 30°C. Quorum sensing activity was observed through the measurement of violacein inhibition around the wells and expressed in millimetres (mm).

2.5 Statistical Analysis

Every experimental trial was conducted in triplicate, and all data were presented using Microsoft Excel as mean ± standard deviation (SD). Using ANOVA (one-way analysis of variance), the data was statistically examined. Considered statistically significant was a P value of less than 0.05.

3. Results and Discussion

3.1 Preparation and Characterisation of Nanoemulsion

The four different nanoformulations of *A. indica* essential oil were prepared and essential oil concentration (5%) was constant for all the nanoformulations and Tween-20 (non-ionic surfactant) concentration was increased from 5-20% (20AIU1 to 20AIU4). The outcome of the standardized parameters of nanoemulsion preparation, its effect on visual appearance, and the turbidity of nanoformulations are mentioned in Table 1. The increase in the concentration of surfactant of *A. indica* essential oil nanoformulation was 5%, 10%, 15% and 20% resulting in a color change of the nanoemulsion from opaque to transparent (Figure 1). Also, the % Transmittance of nanoemulsion changes from 1.36 to 99.9%. The prepared nanoformulation of *A. indica* essential oil with a non-ionic surfactant (Tween-20) and 1:4 ratio of oil and surfactant concentration containing formulation (20AIU4) showed extremely transparent, low viscous, and 99.9% transmittance[20]. A non-ionic surfactant has steric stabilisation ability, is less toxic, and is cost-effective[21].

Table 1. Visual appearance, stability and % transmittance of nanoformulation of *A. indica* essential oil (20AIU1-20AIU4)

Formulation Code (Essential oil: Tween-20)	Visual Appearance	Stability	% Trans mittance
20AIU1 (1:1)	Opaque	Unstable	1.36 ± 0.153
20AIU2 (1:2)	Opaque	Unstable	1.58 ± 0.193
20AIU3 (1:3)	Transparent	Unstable	43.3 ± 0.964
20AIU4 (1:4)	Transparent	Stable	99.9 ± 0.057

3.1.1 Creaming Index (CI) and Stability Studies of Nanoemulsion

The 20AIU4 formulation was thermodynamically stable at room temperature for up to three months. This indicates that the 20AIU4 nanoformulation sustained the stability tests and the remaining three formulations are unstable after one month of storage (Table 1). The % CI of each formulation (20AIU1-20AIU4) was 82.4, 90.1, 92.5 and 99.9% respectively. 99.9% CI of 20AIU4 formulation indicates no phase separation was observed from 30 minutes of sonication[11].

3.1.2 pH Measurement and Viscosity Determination

The effect of surfactant (Tween-20) concentration (20AIU1-20AIU4) on the pH and viscosity of the nanoformulations was determined. The viscosity of the formulations was directly proportional to surfactant concentration and there are no considerable differences in the pH of the formulation with the increase of surfactant concentration (Figure 2). This indicates the Tween-20 had less effect on the pH of nanoemulsion[22].

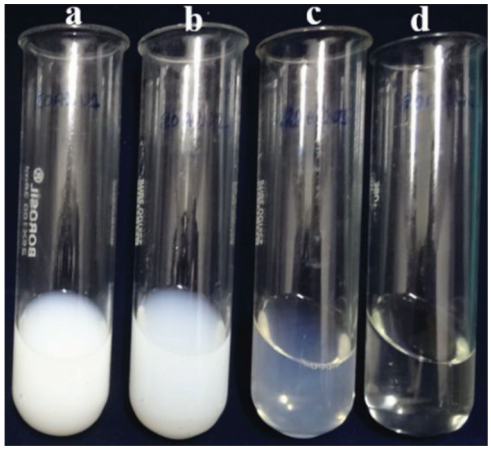


Figure 1. The visual appearance of *A. indica* essential oil nanoemulsion by ultrasonic emulsification: a-d (20AIU1-20AIU4).

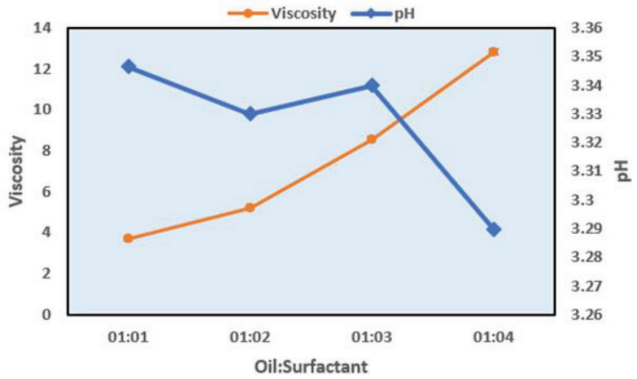


Figure 2. Effect of surfactant concentration on viscosity and pH of *A. indica* essential oil nanoformulations.

3.1.3 Particle Size Distribution and Polydispersity Index

A thermodynamically stable 20AIU4 formulation was selected to determine particle size distribution and polydispersity index. The Z-average particle size was found to be 52nm and the minimum particle size was 10.1 (Figure 3). The polydispersity index was found to be 1.000. From 0 to 1 PDI value of nanoemulsion indicates the uniformity and stability of particle size distribution of droplets[23].

3.1.4 Transmission Electron Microscopy (TEM)

To understand the morphological characteristics of 20AIU4 nanoformulation better, transmission electron microscopy was used. The results of the DLS of 20AIU4 nanoformulation were similar to the outcome of the TEM (Figure 4) which depicted a spherical form and

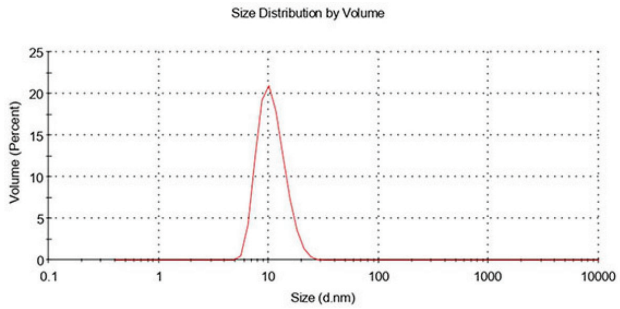


Figure 3. Droplet size distribution of *A.indica* essential oil nanoformulation (20AIU4).

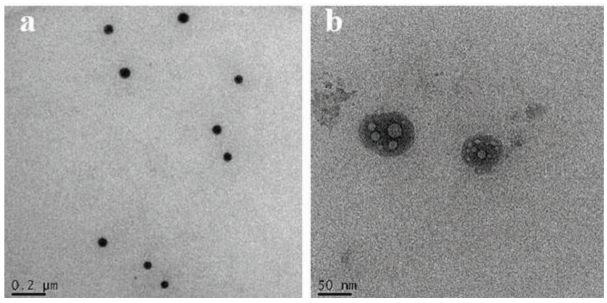


Figure 4. Morphology characterisation of 20AIU4 formulation analysed by Transmission Electron Microscopy (TEM).

a diameter of droplets below 100nm. Similar findings were reported by Savitharani *et al*[11].

3.2 Antibacterial Activity

20AIU4 formulation inhibited the growth of both Gram-negative and Gram-positive bacterial cultures by agar disc diffusion assay (Figure 5). The outcomes are mentioned in Table 2. 20AIU4 formulation showed more significant antibacterial activity than essential oil alone. 20AIU4 shows a higher inhibition zone than positive control (Ampicillin) against *P. aeruginosa* MTCC 424. In previous research studies, the antimicrobial activity of nanoemulsion against microorganisms (yeast, fungi and bacteria) has been reported[3,24,25]. However, the antibacterial activity of nanoemulsion of *A. indica* essential oil against *P. aeruginosa* and *S. aureus* has not yet been demonstrated in previous research studies and similar distinct activity of essential oil of aerial part and roots of *A. indica* was observed[26].

3.3 Antibiofilm Activity

Quorum sensing is the crucial mechanism for the production of biofilm in Gram-negative bacteria. The anti-biofilm activity (biofilm development inhibition and initial bacterial attachment inhibition) of the 20AIU4 formulation was determined using a crystal violet staining assay. The outcomes of the study are summarised in Table 3. Nanoemulsion has greater antibiofilm activity than alone *A. indica* essential oil. 20AIU4 nanoformulation showed significant antibiofilm activity. It inhibits both bacterial attachment and biofilm development against *P. aeruginosa* and *S. aureus*. Several previous studies confirmed the biofilm inhibition effect of plant essential oil nanoemulsion against drug-resistant bacterial strains[27, 28].

Table 2. Antibacterial activity of 20AIU4 nanoformulation

Sample Name	Zone of Inhibition (mm ± SD)	
	***Pseudomonas aeruginosa* MTCC 424**	***Staphylococcus aureus* MTCC 902**
20AIU4 (NE)	13.50 ± 0.5	14.76 ± 0.4
Essential oil (EO)	7.2 ± 0.26	12.8 ± 0.26
Ampicillin (A)	12.93 ± 0.378	19.9 ± 0.36

3.4 Anti-Quorum Sensing Activity

Anti-quorum sensing activity of *A. indica* essential oil nanoemulsion (20AIU4) against *C. violaceum* MTCC 2656 by agar well diffusion assay. The findings of the study were opaque and a colourless circle was noticed (Figure 6) around the nanoemulsion loaded well (23.9 ± 0.36 nm). The essential oil and positive control (ampicillin) also showed inhibition zones of 19.16 ± 0.85nm and 20.93 ± 0.4nm respectively. *P. aeruginosa* is a nosocomial human pathogen and opportunistic bacteria which infects defective immunity-containing patients. *P. aeruginosa* has a quorum-sensing system and it regulates the production of a variety of extracellular virulence factors and the formation of biofilm in bacterial pathogens[29]. Nanoformulation of *A.*

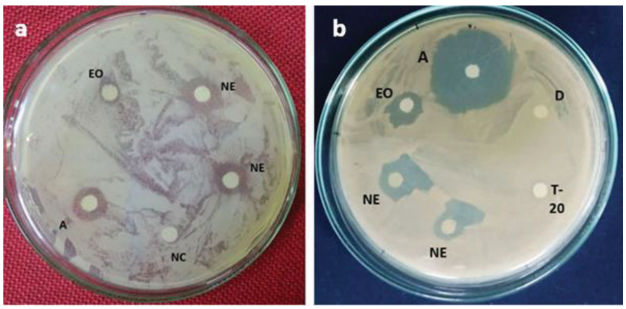


Figure 5. The antibacterial activity of 20AIU4 nanoemulsion by disc diffusion assay. a) *Pseudomonas aeruginosa* MTCC 424 and, b) *Staphylococcus aureus* MTCC 902. Essential oil (EO), 20AIU4 nanoemulsion (NE), Ampicillin (A), Negative control (NC), Tween-20 (T-20), and 10% DMSO (D).

Table 3. Antibiofilm activity of 20AIU4 nanoformulation of *A. indica.*

Sample Name	% Inhibition of bacteria attachment		% Inhibition of biofilm development	
	***Pseudomonas aeruginosa* MTCC 424**	***Staphylococcus aureus* MTCC 902**	***Pseudomonas aeruginosa* MTCC 424**	***Staphylococcus aureus* MTCC 902**
20AIU4 (NE)	85%	85.71%	85.71%	80.55%
Essential oil (EO)	67.5%	74.28%	71.5%	72.22%
Ampicillin (A)	82.5%	88.57%	88.09%	86.11%

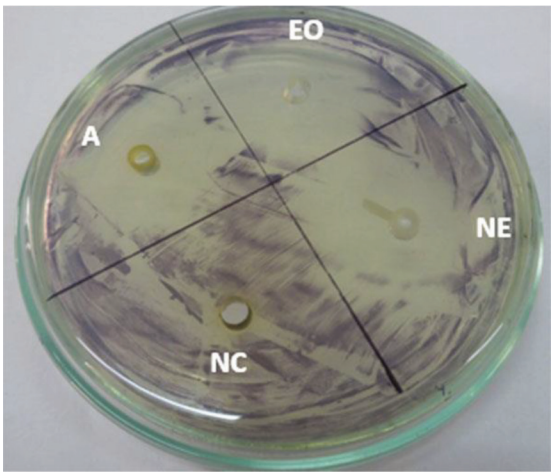


Figure 6. Anti-quorum sensing activity against *Chromobacterium violaceum* MTCC 2656. Essential oil (EO), 20AIU4 Nanoemulsion (NE), Ampicillin (A), Negative control (NC).

indica essential oil (20AIU4) significantly inhibits the growth of the violacein production against reference strain *C. violaceum* MTCC 2656 (Inhibition zone was 23.166 ± 1.04) and several previous studies confirmed the anti-quorum sensing activity of plant essential oil nanoemulsion against *C. violaceum*[5,30,31].

4. Conclusion

According to the results of the preparation and characterisation of *A. indica* essential oil nanoemulsion. Clear, uniform and stable oil-in-water nanoemulsions were prepared using *A. indica* essential oil, Tween-20 and water using ultrasonication with an average particle size of 52 nm, PDI value of 1.000, 99.9 % transmittance and thermodynamically stable for three months. Nanoemulsion (20AIU4) exerted significant antibacterial effects against gram-positive bacteria and gram-negative bacteria based on agar disc diffusion assay. 20AIU4 formulation significantly inhibited the biofilm formation and development against both *Staphylococcus aureus* and *P. aeruginosa*. Further, antibiofilm activity was confirmed by the anti-quorum sensing activity of 20AIU4 formulation against *C. violaceum*. From the outcome of the present study, it could be concluded that the *A. indica* essential oil nanoemulsion (20AIU4) was used as an antibacterial agent against skin pathogens.

5. Acknowledgments

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