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Development of Nano-Emulsion Formulation of Chrysopogon Zizanioides for Targeted Antibacterial Activity Against Pyogenic Bacteria

Dhanyasri R P[1], Kiran Kumar S[2]
[1,2] B.Sc., Department of Microbiology, Hindusthan College of Arts & Science, Coimbatore (Afflicted to Bharathiar University)

Arun Kumar G*[3]
*[3]Corresponding Author, Assistant Professor, Department of Microbiology, Hindusthan College of Arts & Science, Coimbatore (Afflicted to Bharathiar University). E-Mail: Arunkumar.G@Hicas.Ac.In

ABSTRACT:

Chrysopogon zizanioides (Vetiver) is a medicinal plant known for its effective antibacterial activity. In the present study, we demonstrate the evidence of developing a Nanoemulsion formulation of *C. zizanioides* essential oil for its enhanced antibacterial activity against pyogenic infection caused by bacteria. Nanoemulsions offer improved solubility, stability, tunable rheology, optical transparency and bioavailability, making them ideal for drug delivery applications. The formulation was prepared using a high-energy emulsification method and characterized for particle size, zeta potential and stability. Antibacterial efficiency was evaluated against clinically relevant pyogenic bacteria, including *Staphylococcus aureus and Pseudomonas aeruginosa*, using standard microbiological assays. Results demonstrated that the Nanoemulsion formulation exhibited superior antibacterial activity compared to the crude essential oil, indicating the Nanoemulsion to be a potent antibacterial agent for treating pyogenic infections. This study highlights that the essential oil obtained from *C. zizanioides* serves to be a natural alternative for treating bacterial infection caused by antibiotic-resistant strain.

1. INTRODUCTION:

Antibiotic resistance occurs when bacteria evolve to withstand the effects of antibiotics, making infections harder to treat. Nowadays, this is one of the concerning problems that has aroused and led to the development of novel therapeutic strategies. Pyogenic bacteria namely, *Staphylococcus aureus, Streptococcus pyogenes, Streptococcus pneumonia, Pseudomonas aeruginosa* are responsible for causing pyogenic infection that includes both localized and systemic infection targeting soft tissues, respiratory systems, bone and joints etc. with an ability to induce the formation of pus. Nowadays, the traditional antibiotics aren't effective against these set of pyogenic infection .The rise of these resistant strains has emphasized the need for alternative antimicrobial therapies, particularly those derived from natural sources, which may offer novel mechanisms of action that circumvent traditional antibiotic resistance. An effective alternative to treat these infections are essential oils obtained from medicinal plants such as *C. zizanioides* that has antibacterial properties. The *C. zizanioides* is commonly known as vetiver known for its wide range of pharmacological properties, including antimicrobial activity against several bacteria, including pyogenic strains. The essential oil obtained from the roots of vetiver has low solubility and volatility that serves to be the limiting factor for practical use. Hence, with the recent advancements the Nanoemulsions were introduced that has improved the properties of essential oil in the aspect of solubility, stability and controlled release (Lima, G. M., *et al.*, 2020).

Chrysopogon zizanioides, is a perennial grass species native to India and widely known for its aromatic and antibacterial properties. The essential oil derived from this plant has been extensively studied for its various therapeutic benefits, which are critical for understanding the oil's potential in pharmaceutical and therapeutic applications activity. Its roots yield an essential oil that is high in vetiverol, vetivenes and other sesquiterpenes, which give it antibacterial, anti-inflammatory and antioxidant qualities. There are two uses for vetiver oil: medications and fragrances. The roots of plants are utilized in India to make baskets, hand fans, carpets, and other everyday items. The non-invasive grass *C. zizanioides* is indigenous to tropical Asia and belongs to the Poaceae family, subfamily Panicoideae, and tribe Andropogonae. The root (vetiver), it is extensively used in India and Southeast Asia. The plant's uppermost portion is around 4 meters tall. Furthermore, vetiver is not tolerant of shade because it will reduce its growth and, in some situations, can kill the vetiver plant. Vetiver grass should be planted in an open environment and free of weeds (S. S. Yadav., et al., 2019).

A Nanoemulsion is a kinetically stable dispersion of two immiscible liquids for i.e. water and oil, where one liquid is dispersed as nanoscale droplets within the other (in the range of 20 - 200 nm). The nano sized droplets, such as enhanced solubility of hydrophobic substances, greater penetration into bacterial cell membranes, and increased surface area for contact with the target. In order to provide a targeted therapy against pyogenic infection the modernization included the exploitation of essential oil obtained from vetiver into a Nanoemulsion with improvised therapeutic potential. The essential oil plays a crucial role in destroying the cell membrane of the pyogenic bacteria which was the major reason it was used as an antibacterial agent. Furthermore, essential oils such as *Chysopogon zizanioides* help prevent the production of bacterial biofilms. Since biofilm is a layer composed of extracellular polymeric substance which are synthesized by a group of bacteria are resistant to therapies preventing the cure of an infection. The essential oil of *C. zizanioides* may be utilized as a powerful supplement or substitute for traditional antibiotics in a Nanoemulsion formulation, providing a more secure and efficient means of treating pyogenic bacterial infections (M. A. B. Silva., *et al.,* 2017).

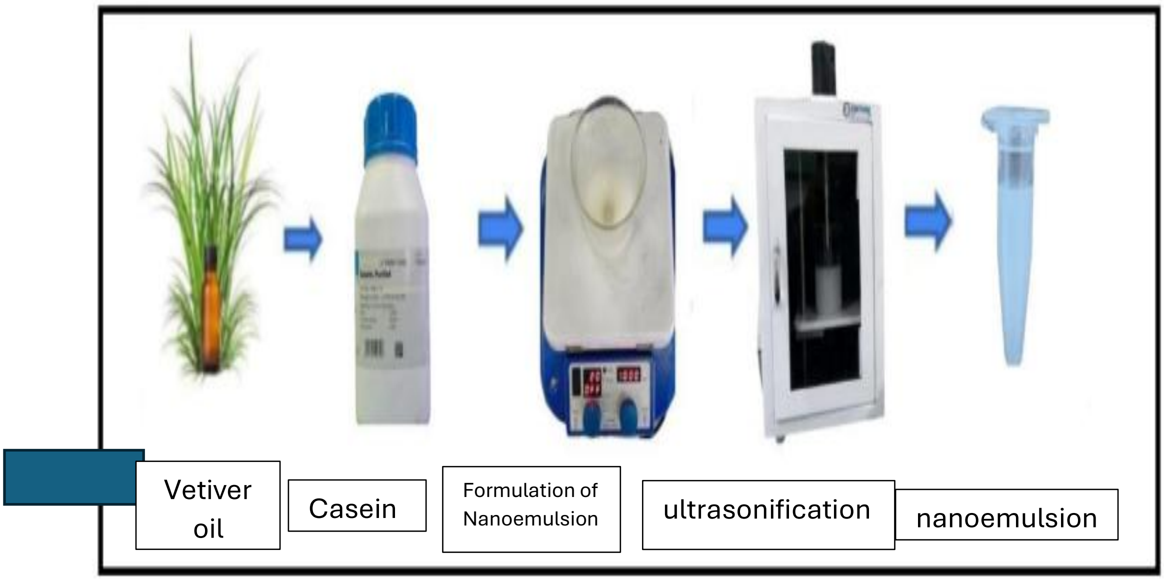


Fig 1: Preparation of oil in aqueous phase

Formulating Nanoemulsions (Fig 1) requires the selection of suitable oil, surfactants, and co-surfactants to stabilize the emulsion and ensure its functionality. In the case of *Chrysopogon zizanioides* essential oil, the oil phase is composed of the essential oil, while the aqueous phase contains surfactants such as polysorbates and co-surfactants like ethanol or propylene glycol, which helps to stabilize the emulsion and control droplet size (Narang, N., *et al.*, 2020). The most commonly used methods for preparing Nanoemulsions include high-energy techniques, such as, Sonication High-frequency sound waves are used to break down the oil droplets into the nanoscale range, leading to the formation of stable Nanoemulsions (or) High-Pressure homogenization technique uses high pressure to force the oil-water mixture through a small orifice, generating shear forces that reduce the droplet size (Santos, E. R., et al., 2018) (Bhatia, 2020).

2. Results:

2.1 Essential Oil:

The store-bought oil is processed through distillation process to confirm its purity and composition.

2.2 Characterization of Nanoemulsion:

2.2.1 Dynamic Light Scattering (DLS):

DLS is carried out to find the particle size of the nanoparticles dispersed in a solution. With the help of DLS the surface charge can be analysed which is determined by zetasizer instrument, which is used in quantifying the magnitude of charge. Therefore, the peaks at different concentrations levels at 0.12%, 0.25% and 0.5%. The concentration of emulsion sample with 10ml water + 100ul sample dilution gives the correct size of Nanoemulsion. Using zetasizer the zeta potential was calibrated *for Chrysopogon zizanioides* and aqueous phase of Nanoemulsion. From the analysis it can be seen that the composite is positively charged with potential of 330 mV, 255.3 mV and 301.9 mV, which means the net charge of the scattering object is positive. If the potential is between -20 to +20 then the particle suspension is stable. Hence, the *Chrysopogon zizanioides* Nanoemulsion is stable in aqueous medium.

2.2.2 Contact angle measurement:

Contact angle was observed to be 44.72° and right contact angle: 40.10° in the presence of glycerol and water. Wettability of the emulsion sample surface is likely partially wettable, meaning the liquid spreads but does not fully absorb into the surface. Contact angles below 90 indicate that the surface is hydrophilic. The contact angles left and right were 70.96° and 80.35° respectively form the emulsion sample. A higher contact angle suggests that this surface is less wettable. The first sample showed better spreading behaviour, indicating a more hydrophilic nature.

2.2.3 Fourier-Transform Infrared (FT-IR) spectroscopy:

FT-IR is a technique that analyses the presence of different functional groups from the given sample based on different bending frequency which is unique to each of the functional group. FT-IR spectrum of *Chrysopogon zizanioides* oil sample was recorded in the wave number range of 1026.13 -2.924.09 cm3. The peak stretching 2924.09 cm-1, 2870.08 cm-1 confirms the presence of C-H in the extract and peak at 1705.07 cm-1, 1658.78 cm-1 confirms the presence of C=H group. The peaks at 1458.18 cm-1 and 1373.32 cm-1 confirms the presence of -CH3 and –CH2-. The stretching due to C-O was 1172.72 cm-1, 1026.13 cm-1 were observed significantly. Aqueous phase emulsion was recorded in the wave number range of 3857.63,1643.35cm3 the peaks stretching 3857.63 cm-1, 3749.62 cm-1 confirms the presence of O-H in the emulsion and peak at 3363.86 cm-1 confirms the presence of O-H hydrogen-bonded group. The peaks at 2337.72 cm-1 confirms the stretching due to C=N. The stretching due to C=O 1643.35 cm1 were observed significantly.

2.3 Minimum Inhibitory Concentration:

Biosynthesized *Chrysopogon zizanioides* (OIL) with concentration (1%, 2.5%, 5% and 10%) showed significant zone of inhibition against *Pseudomonas aeruginosa* (12mm, 14mm, 15mm and 18mm) MRSA- *Staphylococcus aureus* (2mm, 6mm, 7mm and 8mm) and MSSA - Staphylococcus aureus (20mm, 22mm, 23mm and 25mm) respectively. The aqueous phase was compared and results were tabulated. The zone of inhibition shown for selected bacteria isolates against MRSA- *Staphylococcus aureus*, (12mm, 16mm, 18mm and 19mm), MSSA-*Staphylococcus aureus* (11mm, 15mm, 20mm and 21mm), *Pseudomonas aeruginosa* (9mm, 14mm, 16mm and 19mm) respectively. The difference in the above results is due to the use of essential oil and Nanoemulsion in the first and later case. Nanoemulsion sample were tested with concentration (1%, 2.5%, 5% and 10%) for the selected bacterial isolates.

2.4 Antibacterial assay:

The microbial properties of the *Chrysopogon zizanioides* (Table 2) and aqueous phase of Nanoemulsion (Table 3) were evaluated to be, whereas in the aqueous phase the antimicrobial activity was found to be,

Antibacterial assay with different oil concentration

OIL Concentration	Pseudomonas	MRSA	MSSA
1%	12mm	2mm	20mm
2.5%	14mm	6mm	22mm
5%	15mm	7mm	23mm
10%	18mm	8mm	25mm

Table 1: Antimicrobial activity with different oil concentration

Antibacterial assay with different sample concentration

SAMPLE Concentration	Pseudomonas	MRSA	MSSA
1%	9mm	12mm	11mm
2.5%	14mm	16mm	15mm
5%	16mm	18mm	20mm
10%	19mm	19mm	21mm

Table 2: Antimicrobial activity of different sample concentration

2.5 Antioxidant activity:

Antioxidant activity refers to the ability of compounds to neutralize free radicals and prevent oxidative damage in biological systems. This was carried out based on two different assays DPPH and ABTS, through which it has revealed that the concentration of Nanoemulsion is directly proportional to the antioxidant activity (Fig 2). Implication suggests that the Nanoemulsion has good antioxidant potential, with higher concentrations resulting in greater inhibition of free radicals.

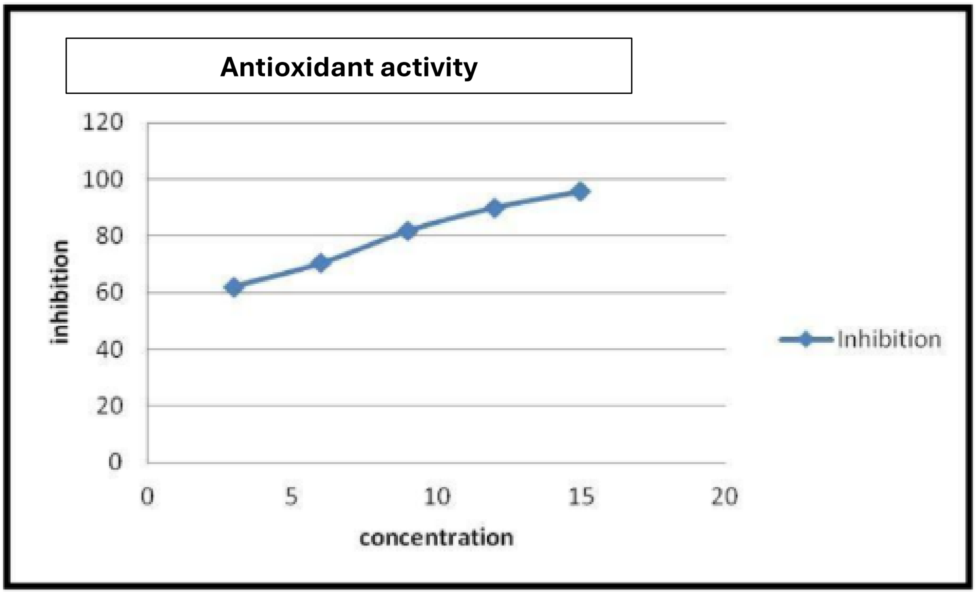


Fig 2: Antioxidant activity in standard curve concentration

3. Discussion:

This study focuses on the development of Nanoemulsion from *Chrysopogon zizanioides* – Essential oil wherein the goal is to improve the delivery and potency of the essential oil by utilizing Nanoemulsion technology. Stability is crucial because the active compounds in the oil need to be preserved while ensuring the formulation can be applied consistently. The efficacy of the Nanoemulsion will be optimized to maximize the antimicrobial properties of *Chrysopogon zizanioides* oil, allowing it to combat bacterial infections more effectively. Through the DLS analysis (Fig 3) the particle size were found from the graph which was up to an average of 288.36 nm.

Through the contact angle measurement, the efficiency of Nanoemulsion when applied on surface was found in presence of glycerol (left) and water (right) (Fig 4) in the below figure.

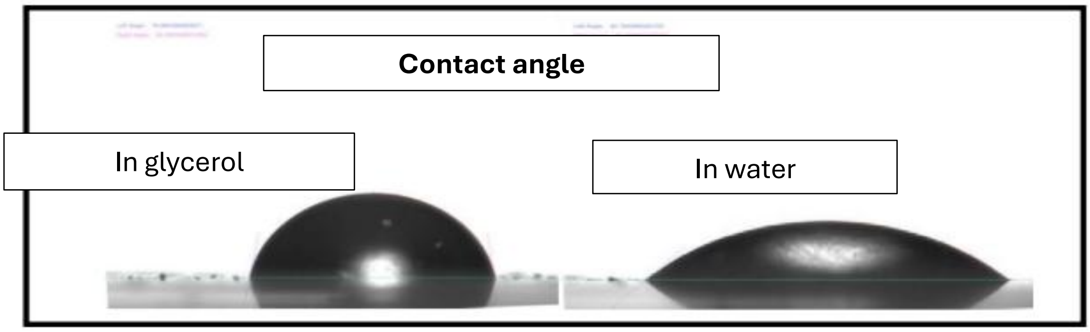


Fig 3: Contact angle of emulsion sample added on glycerol and water

With the usage of FT-IR analysis the presence of various functional groups in the emulsion sample were found from graph as given below (Fig 5).

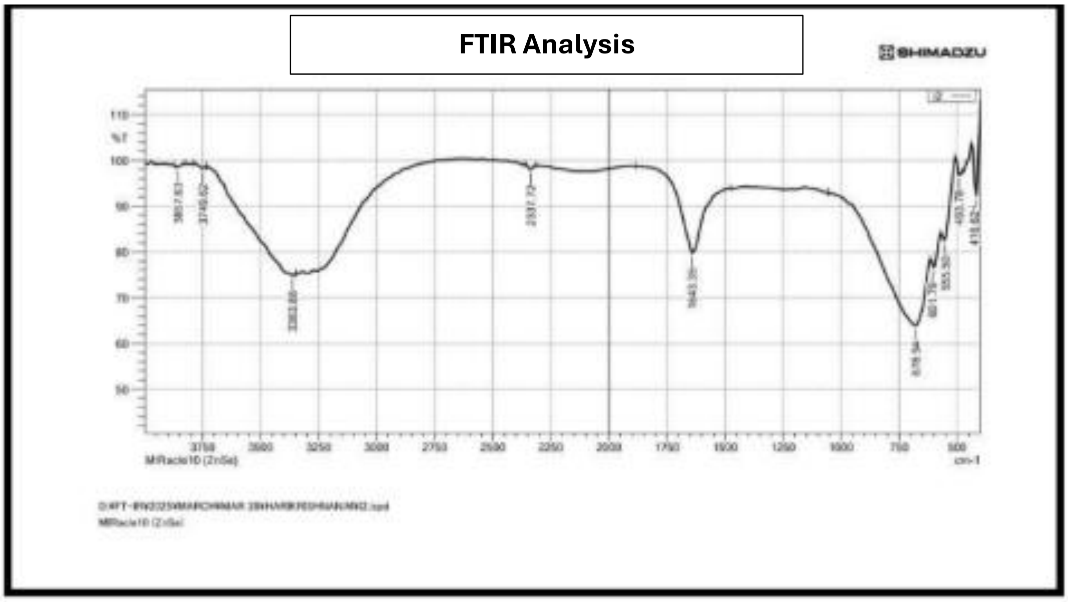
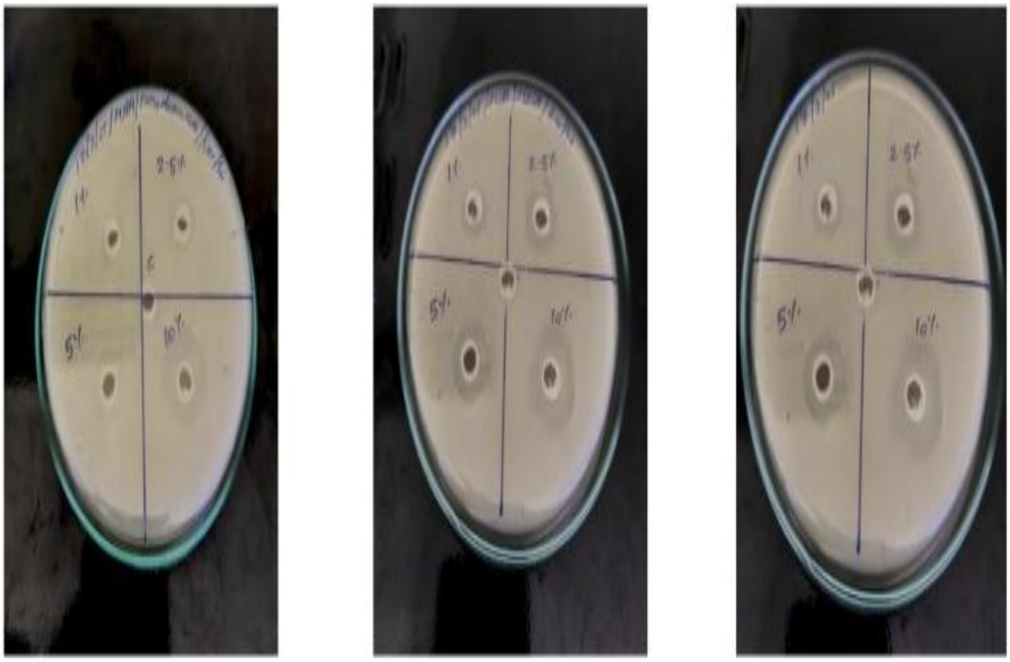


Fig 4: IR spectrum of *C. zizanioides* emulsion sample

With the use of MH Agar the Antibacterial assay was carried out and zone of inhibition were measured in terms of (mm) (Fig 6) for bacterial isolates including Pseudomonas, MRSA & MSSA with different sample concentration in aqueous phase.



Zone of inhibition of different Sp at different concentration of

Fig 5: Antimicrobial activity of emulsion sample Pseudomonas, MRSA & MSSA

In the Antioxidant assay, the concentrations range from around 0 to 750 µl in which, as concentration increases (Fig 7), the % inhibition also increases, indicating a dose dependent antioxidant activity. At the lowest concentration, the inhibition is around 50%, showing that the sample already has significant antioxidant effect even at low levels.

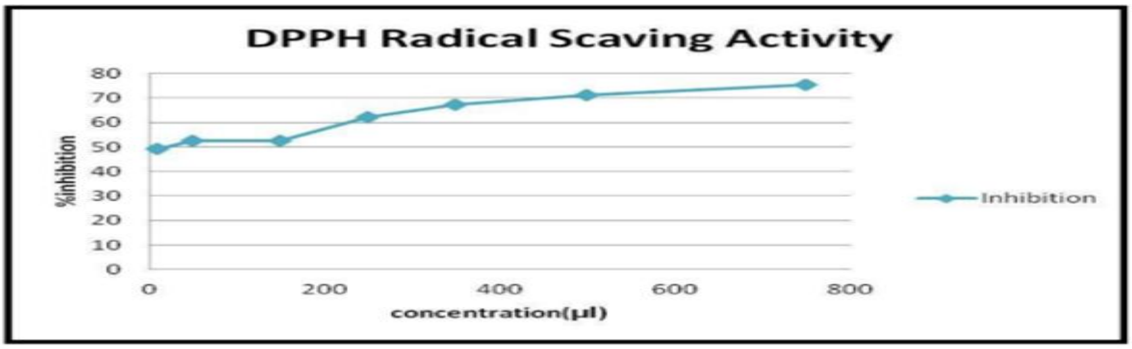


Fig 6: Antioxidant activity in Nanoemulsion sample concentration

4. MATERIALS AND METHODS:

4.1 Essential oil – Raw material:

Vetiver oil that is commonly sold in stores, especially those specializing in aromatherapy products, are utilized as a source of raw material. A much more crucial aspect of choosing the essential oil from stores include the Grade of the oil and some premium brands have the report indicating GC-MS (Gas Chromatography-Mass Spectrometry) which gives information about the purity and composition.

4.2 Preparation of Aqueous phase:

The aqueous phase in the (oil in water) emulsion consist of casein polymer that naturally acts as an emulsifying agent. The process begins with the addition of essential oil into the aqueous phase that is composed of casein polymer in the present of magnetic stirrer to create a uniform distribution of oil within the aqueous phase.

4.3 Preparation of Nanoemulsion:

The above mixture is ultrasonically treated with the ultrasonicator to produce Nanoemulsion. In the presence ultrasonicenergy the oil droplets from the mixture are broken down into Nanometer range. The formed oil droplets now has an improvised stability and bioavailability with decreased size. The ultrasonicator helps to create a stable Nanoemulsion and guarantees that the active ingredients in the vetiver oil are efficiently supplied in a form, that is easier for the skin to absorb.

4.4 Preparation of oil in aqueous phase by emulsification:

Oil-in-water Nanoemulsions were created in two steps technique, with minor adjustments. The two steps were as follows: The final stable emulsion containing nanoparticles was obtained by:

1) ultrasonication and 2) pre-emulsification employing a high-speed magnetic stirrer to create coarse emulsions (Jafari *et al*., 2007).

4.5 Formulation of Nanoemulsion:

Nanoemulsion formulations were then created from the coarse emulsion using a 20 kHz ultrasonic processor (Model: CP750, Power: 750 watts) from Cole-Parmer Instruments, Illinois, U.S.A. to create disruptive forces, a 13 mm-diameter ultrasonic probe tip was employed. Cavitation bubbles are created when ultrasonic vibrations are released after the probe has been immersed in a sample. These bubbles grow until they burst. The ultrasonic pulser was run at 30-second on and 30-second off intervals to avoid overheating. To lessen the heat produced during sonication, the sample was processed in an icy water bath. With amplitudes of 20%, 40%, and 60%, durations of 2, 4, 6, 8, and 10 minutes, the sonication parameters were changed. All studies were performed in a 200 ml glass beaker. The resultant Nanoemulsion formulations which were processed through ultrasonification (Table 1) were stored at a temperature of 4°C for subsequent tests.

	Aqueous phase	Oil phase	Emulsionfication(Time)	
Sample	CASEIN	OIL	strring	Ultra Sonification
CZ01	0.12%	0.1%	30 minutes	40 minutes
CZ02	0.25%	0.1%	40 minutes	50 minutes
CZ03	0.50%	0.1%	45 minutes	50 minutes

Formulation of Nanoemulsion

Table 3 : Formulation of Nanoemulsion

4.6 Characterization of Nanoemulsion:

4.6.1 Dynamic Light Scattering (DLS):

Using Nanoplus 3, MICROMERITICS, Japan, Dynamic Light Scattering (DLS) was used to ascertain the O/W Nanoemulsion's standard droplet size and Polydispersity Index (PI). Measurement parameters were an unchanged temperature of 25°C and a fixed angle of 165°. Deionized water was used to dilute the Nanoemulsion samples at a ratio of 1:9 in order to prevent multiple-scattering effects during droplet analysis. Each measurement was carried out five times, and calculations were made using the nanoplus software version 5.10/3.00. The best formulation for additional testing was determined to be the Nanoemulsion with the lowest droplet size.

4.6.2 Contact angle measurement:

To calibrate the contact angle measurement, the resultant droplet is fixed at a specific spot on the tungsten tip using a recently created method of forced wetting by ion implantation.

4.6.3 Fourier-Transform Infrared (FT-IR) spectroscopy:

Analysis using Fourier-Transform Infrared (FT-IR) spectroscopy A FT-IR spectrometesr (Invenio-s, Bruker) was used to gather FT-IR spectra spanning the wavenumber range of 4000–400 cm−1 in order to determine the functional groups and covalent connections, if any, present in the pure EO, coarse emulsion and optimum Nanoemulsion. For every spectrum, thirty-two scans were acquired at a resolution of 2cm−1. Four replications and a fully randomized design were employed. To validate the estimated droplet size, the ideal Nanoemulsion's droplet morphologies were examined. To examine the morphology of the Nanoemulsion, an A transmission electron microscope (HITACHI HT7700, Japan) running at 80 KV was employed. A drop of 2% uranyl acetate was added to a drop of Nanoemulsion that had been allowed to settle on a carbon-covered grid for ten minutes. The combination was then allowed to stand for thirty seconds. Four replications and a fully randomized design were employed.

4.7 Antimicrobial Assay:

4.7.1 Minimum Inhibitory Concentration (MIC):

Muller Hinton Broth was taken in weight of 4.4 Grams and added with 200ML of water for preparation of the broth. Through the use of a modified agar well diffusion experiment, the MIC was established. Using sterile petri plates, nutritional agar was cooled in an incubator set to 37°C for 24 hours in order to measure its antibacterial activity. In 7 ml of nutrient soft agar, 100 μL of bacterial cultures (~ 106 cfu ml−1) were then injected onto the plates' surface. A sterile 9 mm diameter well borer was used to punch wells out of the agar. The wells were then filled with varying amounts (10–200 μl) of free *Chrysopogon zizanioides* oil and Nanoemulsion. *C. zizanioides* oil served as a positive control and sterile saline as a negative control in order to compare the antibacterial activity of the Nanoemulsion (Bertrand-Harb, I., *et al.*, 2003). At 37°C, the well plate was incubated for 24 hours.

4.7.2 Antibacterial Assay:

The concentrations of vetiver were prepared with different percentages (%) of volume of oil + solvent volume (DMSO). When 1% and 2.5% volume of oil were added to level the percentage of solvents volume to 99% and 97.5%. The 95% of solvent is added along with 5% of oil and 10% of oil were added with 90% of solvent for preparation of concentration of vetiver oil. Nanoemulsion sample of (1%, 2.5%, 5% and 10%) were added with different percentages of (DMSO). The solvents volume of 99% and 97.5% was added with the level 1% and 2.5% percentage of Nanoemulsion sample. The 95% of solvent is added along with 5% of Nanoemulsion sample and 10% Nanoemulsion sample were added with 90% of solvent (DMSO).

4.7.3 Antioxidant Activity:

The antioxidant activity of Nanoemulsions is primarily attributed to their ability to neutralize free radicals, thus preventing oxidative damage to cells and tissues. This is commonly evaluated using commonly evaluated using radical scavenging assays such as the DPPH (2,2 Diphenyl-picrylhydrazyl) assay. Using 0.5 G/ML of *Chrysopogon zizanioides* and *C. zizanioides* - NEs (stored for 7 days), the DPPH scavenging assay was measured in accordance with the kit's instructions. The kit's extraction buffer was used to dilute the Eos. First, 20 μL of sample was mixed with 380 μL of Reagent 1 and forcefully shaken for 20 minutes. The microplate reader (Thermo Scientific, Waltham, MA, U.S.A.) was used to measure the change in absorbance at 515 nm. The following formula was used to determine DPPH's percentage inhibition free radical scavenging rate:

DPPH scavenging activity (Inhibition %) = [(A control – A sample) / A control] × 100

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1. **NO Funding is provided for this project.**
2. **Clinical trail : Not Applicable.**
3. **Ethics, Consent to Participate, and Consent to Publish declarations: not applicable.**
4. **No competing interest.**