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Antimicrobial activity of nanoemulsion containing *Moringa oleifera* seed protein

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ABSTRACT

Plant-based antimicrobial substances have been recognized as antimicrobial agents. These peptides demonstrate antimicrobial properties against a wide range of pathogens. This study reports the efficacy of *Moringa oleifera* nanoemulsion as an antimicrobial. *M. oleifera* seed was defatted and the protein was extracted from the grounded seed, characterized, and formulated into nanoemulsions by spontaneous nanoemulsification. This method is economically and environmentally safe as the components of nanoemulsion are biodegradable. The formulation was evaluated for particle size, viscosity, pH, antimicrobial activity, and kill time assay. The nanoemulsion was nanosized (43.440 nm - 74.430 nm) with increased encapsulation efficiency in a dose-dependent manner and a suitable pH (5.91 ± 0.01 to 6.14 ± 0.01), excellent dynamic viscosity (32 ± 7 to 39 ± 0). The antimicrobial and minimum inhibitory concentration study displayed a wide range of effectiveness on *Pseudomonas aeruginosa* and *Staphylococcus aureus*, the time-kill assay showed moderate biocidal activity. Therefore, *M. oleifera* seed protein nanoemulsion has the potential to act as antimicrobial.

Keywords: protein-based delivery, antimicrobial resistance, *Moringa oleifera,* nanoemulsion, therapeutic protein

INTRODUCTION

The World Health Organization (WHO) has recognized antimicrobial resistance (AMR) as a very serious (top-10) worldwide concern that strains public health systems (Walsh et al., 2023). The 2019 AMR report from the Center for Disease Control and Prevention (CDC) discloses that there are currently almost 3,000,000 instances of diseases that are not susceptible to antibiotics in the United States, leading to 35,000 fatalities (Zhang & Yang, 2022). AMR is known to occur when microorganisms -bacteria, viruses, fungi, and parasites, undergo changes over time that render them unresponsive to any pharmacological treatments (Arip et al., 2022; Tang et al., 2023). The transmission of AMR is essentially acquired through direct contact between humans, both within and outside of healthcare settings. AMR genetic materials are present in humans, animals, water sources, and the environment, serving as reservoirs for these genes. Transmission of these genes may occur in and between these storage spaces (Salam et al., 2023). The increasing menace of microbial resistance as a significant concern in public health emphasizes the need for innovative antibiotics that may either complement or replace existing antibiotics (Brandelli, 2012; Carmona-Ribeiro & De Melo Carrasco, 2014). Also, a high dose of currently available antibiotics can be employed to treat AMR microorganisms, but this approach is not beneficial due to toxicity (Zou et al., 2023).

Antimicrobial peptides (AMPs) are now under investigation as the next iteration of antibiotics, which can complement and/or replace traditional antibiotics (Srivastava et al., 2020). They are very potent antibiotics that have broad-spectrum activity against many pathogenic bacteria(including both Gram-negative and Gram-positive strains), fungi, viral envelopes, and other parasitic organisms (Tang et al., 2018). AMPs possess a broad therapeutic range, unique mechanisms of action, and the ability to selectively target microbial cells, resulting in deadly consequences at high doses. These characteristics make AMPs an appealing substitute for traditional antibiotics (Makowski et al., 2019; Zhang & Yang, 2022). Despite the significant antimicrobial activity of many AMPs, their minimal bioavailability limits their medical

application. This is due to their instability in the face of proteolysis and hydrolysis, as well as their poor permeability across barriers and short circulation-based shelf life (Brandelli, 2012). Some substances include unfavorable characteristics that render them inappropriate for therapeutic use, such as their toxicity towards cells of eukaryotes, leading to hemolysis, renal damage, and neurological damage, as well as an indeterminate pharmacokinetic profile (Biswaro et al., 2018; Craik et al., 2013; Falagas & Kasiakou, 2005; Grassi et al., 2017; NavonVenezia et al., 2002; Schmidtchen et al., 2002). AMPs can be protected from extracellular breakdown by nanotechnology, which can also be employed to improve medication pharmacokinetic profile and therapeutic selectivity (Biswaro et al., 2018).

M. oleifera is often referred to as drumstick, horseradish, miracle, benzolive tree, or mother's best friend. It is a widely distributed plant native to Asia, Africa, South America, the Caribbean, and Oceania. It appears as a fast-growing deciduous tree with soft and white wood and corky and gummy bark. The root, bark, fruits, and leaves of the plant are used to treat a variety of human maladies. The antibacterial and antifungal properties of the aqueous extract of *M. oleifera* seed have been shared (Mishra et al., 2011). These activities have been linked to the protein constituent of *M. oleifera* seed. Notably, no study has reported the formulation and characterization of *M. oleifera* seed protein (MOSP) nanoemulsion and its antimicrobial potential.

Stability is a crucial consideration in protein delivery. The stability of proteins is determined by their size and their secondary and tertiary structures, as they are prone to physical and chemical degradation. Furthermore, the size of therapeutic protein is crucial in the determination of its safety and efficacy as protein and peptides are large molecules. PEGylation plays a role in the delivery of therapeutic protein by altering its physicochemical properties thus enhancing the stability, pharmacokinetics, and therapeutic efficacy of the molecule (Pisal et al., 2010). Nanoemulsion, a nanotechnological approach, can be used as an innovative strategy to deliver therapeutic protein and enhance solubility and stability.

The objective of the study is to employ MOSP as an alternative AMP due to its biodegradability, safety, and availability in the local area. As the molecular weight of MOSP has been reported to be 13 kilodaltons (Kwaambwa & Maikokera, 2008), the utilization of eco-friendly and low-cost nanoemulsion for the delivery of MOSP as an antimicrobial agent is needed as it enhances its water dispersibility, resistance to environmental conditions and boosts their antimicrobial activity. Therefore, we hypothesized that the delivery of MOSP via nanoemulsion would improve its solubility, stability, and efficacy.

The novelty and originality of this study lie in the use of nanoemulsion to deliver MOSP. In light of the aforementioned, MOSP was extracted, formulated into nanoemulsion at different concentrations, characterized, and evaluated for its antimicrobial activity.

MATERIALS AND METHODS

Chemicals and Reagents

They include but are not limited to Mueller-Hinton agar (MHA) (LS Biotech, UK), Sabouraud dextrose agar (SDA) (LS Biotech, UK), distilled water procured from Anslem Chemicals, Nigeria; *M. oleifera* oil processed in our laboratory, soybean oil (Aromachem, UK), glycerylmonooleate (Sigma Aldrich, USA), Peceol® and Labrasol® were gifts from Gattefosse, Saint-Priest, France, Kolliphor® ELP was provided by BASF, Ludwigshafen, Germany, Tween 80 (Sigma Aldrich, USA), polyethylene glycol (PEG) 400 (Sigma Aldrich, USA), petroleum ether (Loba Chemie, India), sodium hydroxide (GH Tech, China), ammoniumsulphate (GH Tech, China), dimethylsulphoxide (Molychem, India).

Collection and Identification of *M. Oleifera* Seeds

The plant, which was cultivated on a farm in Katsina State, North-West Nigeria, was collected and preserved in sealed pouches. It was then cleansed to eliminate grit, desiccated leaves, and other debris. A plant taxonomist from the Department of Botany, Faculty of Biological Sciences, University of Nigeria, Nsukka, Enugu State, Nigeria, identified the seed.

Processing and Defatting of Seeds

The seeds were removed from their shells using a seed processor, and the desiccated nuts were ground into fine granules using a milling machine (Qasa Blender, Qlink Group, China) and sieved to obtain uniform-sized powders with a stainless-steel laboratory sieve of 1mm. The *M. oleifera* seed powders were defatted by soaking in some quantity of petroleum ether (boiling point 60-90 ℃) for 10 runs at 75 for 4h. The defatted *M. oleifera* seed powders were dried to remove residual solvent (Nebolisa et al., 2023).

Preparation of Microbial Isolates

The study was conducted using 24-hour cultures of *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, and Aspergillus niger,* which were obtained from the research parent cultures of the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Nigeria.

Moringa Oleifera Protein Extraction

A known quantity (120 grams) of desiccated and deoiled *M. oleifera* seed powders were measured using an analytical weighing scale (Ohaus Corporation, NJ, USA). The measured powders were then transferred into a pristine glass beaker. The protein was obtained by dissolving the powder in water and then separated by adding a small amount of diluted ammonium sulfate for 4 hours at room temperature. The mixture was then spun at a speed of 4,000 revolutions per minute using a centrifuge. The sediments were re-suspended in a solution of sodium hydroxide with a concentration of 1 mol/L. The mixture was then subjected to ultrafiltration using a membrane with a molecular weight cutoff of 13 kDa (Millipore Corp., Bedford, MA). Finally, the resulting solution was freeze-dried (Akasha et al., 2012).

Table 1. Nanoemulsion constituents

Test samples	Glyceryl monooleate(ml)	Tween 80 (ml)	Polyethylene glycol (ml)	*M. oleifera* protein (g)	Water q.s.p. (ml)
MNE1	20	2	1	0.1	100
MNE2	20	2	1	0.3	100
MNE3	20	2	1	0.5	100
MNE4	20	2	1	1	100
MNE5	20	2	1	---	100

Biuret Test for *M. Oleifera* Protein Extracts

A 2 ml sample of protein extract was mixed with 2 ml of biuret reagent in a test tube, and the combination was left undisturbed for five minutes. It was monitored for a transition in color from blue to a violet-purplish hue.

Ninhydrin Test for *M. Oleifera* Protein Extracts

In a test tube, 2 ml of test protein extract was mixed with a few drops of ninhydrin solution. The test tube was warmed for 5 minutes and observed for color change to deep blue color.

M. Oleifera Protein Solubility in Oils, Surfactants, and Co-Surfactants

Excess quantities of *M. oleifera* protein were added to soybean oil, *M. oleifera* oil, and glyceryl monooleate; surfactants such as Kolliphor® ELP, Tween® 80 and Labrasol® and co-surfactants such as PEG 400, glycerol, and propylene glycol in sealed vials (in triplicate). Following a 48-hour period of room temperature maintenance with constant magnetic stirring, the tubes were subjected to a 15-minute centrifugation run at 25 °C. After filtration, the recovered supernatant was preserved. After diluting the filtrate with petroleum ether, the protein content in the excipients was measured spectrophotometrically at 275 nanometers.

Preparation of *M. Oleifera* Seed Protein Nanoemulsions

Through spontaneous emulsification, the nanoemulsions were prepared. The organic phase consisting of oil (Pecol® - Glyceryl monooleate, 15%), surfactant (Tween 80), and MOSP homogenized to form a uniform phase, were introduced into the aqueous phase containing distilled water and a co-surfactant (PEG 400, 1%), under magnetic stirring. In order to achieve a state of balance the mixture was agitated using magnetic stirring for a duration of 30 minutes (Araújo et al., 2011). A unique series of formulations was created, consisting of one placebo and the remaining ones containing different quantities of the protein. The formulation design was to study the effect of various concentrations of MOSP. The constituents of the formulations are shown in **Table 1**.

Characterization of *M. Oleifera* Seed Protein Nanoemulsions

Particle size analysis

Photon correlation spectroscopy (PCS) was used at a temperature of 25 ℃ to determine the average particle size and polydispersity index using a Malvern Nanosizer/Zetasizer® nano-ZS ZEN 3600 instrument(manufactured by Malvern Instruments, USA). A volume of ten milliliters of high-purity water was used to dilute each twenty-liter sample. Three analytical cycles determined the mean values.

Dynamic viscosity

MOSP nanoemulsion viscosity was measured using a digital rotating viscometer (NDJ-5S, Searchtech Instruments, China), spindle number; one (1) at 60 r/min. The analyses were done in triplicate and the average value was recorded at the ambient temperature of 27 ℃.

pH

An electronic pH meter (Starter 5000, Ohaus Corporation, NJ, USA) initially configured with pH 4 and 8 standard solutions was used to measure the pH of the MOSP nanoemulsion. At a temperature of 27 ℃, the pH test was repeated three times, and the average pH was determined. On days 7, 14, 21, and 28, the pH tests were repeated (Shkreli et al., 2022).

Fourier Transform Infrared Spectroscopy

Fourier transform infrared (FTIR) (Buck Scientific, CT, USA) spectra identified protein concentrate secondary structures. Pellets were made from dried protein samples. FTIR spectra were captured at ambient conditions using a near-infrared (FTIR) instrument for 32 scans with 4 cm^{-1} precision in the 400-4,000 cm^{-1} wavelengths band.

Evaluation of Encapsulation Efficiency

The nanoemulsions were dialyzed by combining them with petroleum ether as the solvent at a ratio of 2:1 v/v and gently stirring the mixture. Afterwards, the amount of light absorbed by MOSP, which was not bound to other substances and dissolved in the liquid remaining after centrifugation, was determined at a wavelength of 275 nm with a spectrophotometer with an ultraviolet-visible spectrum (Jenway 6505, USA). Ultimately, the quantity of unbound MOSP was determined by using a calibration graph that was created using different strengths of MOSP.

$$Encapsulation\ efficiency\ (EE\ [\%\ ]) = (Actual\ protein\ content\ ) /(\ Theoretical\ protein\ content)\ \times 100 \quad (1)$$

Antimicrobial Activity Assay

The formulations were examined for their antimicrobial effectiveness against five distinct microbial species: *S. aureus, Pseudomonas aeruginosa, B. subtilis, E. coli*, and *A. niger*. The agar diffusion well test technique was used with few modifications. To prepare microbial suspensions for sub-culturing into MHA and SDA plates with a diameter of 90 mm, the suspensions were corrected to the 0.5 McFarland turbidity standard. Using a sterile cork borer, five wells with a diameter of 8 mm were made on both the MHA and SDA plates. Within the culture plates that had previously been prepared with the test organisms, a quantity of 80 µl of each formulation was

Table 2. *M. oleifera* seed protein solubility in oils, surfactants, and co-surfactants

Solubility in oils		Solubility in surfactants		Solubility in co-surfactants	
Oils	**Protein (mg)**	**Surfactants**	**Protein (mg)**	**Co-surfactants**	**Protein (mg)**
Soybean oil	160 ± 11	Kolliphor® ELP	186 ± 11	Glycerol	181 ± 10
Peceol®	301 ± 5	Tween® 80	276 ± 16	Propylene glycol	101 ± 13
M. oleifera oil	177 ± 6	Labrasol®	151 ± 9	PEG 400	270 ± 15

applied to each well (CLSI, 1998; Okezie et al., 2017). The positive controls consisted of ciprofloxacin at a concentration of 20 µg/mL and fluconazole at a concentration of 80 µg/mL. Negative control was sterile distilled water. The cultures were placed in incubators set at temperatures of 37 and 25 degrees Celsius for periods of 24 and 48 hours, respectively, to develop the bacterial and fungal cultures. Efficacy against bacteria of each formulation was determined by measuring the area of bacterial growth inhibition around each well, omitting well's diameter. Three replicates were performed for each organism.

Optimum Bacterial and Fungal Concentrations (MBC & MFCs)

Based on the preliminary inhibitory/susceptibility of the test organisms, MOSP nanoemulsion was diluted two-fold serially with 3 ml sterile nutrient broth according to the modified European Committee for Antimicrobial Susceptibility Testing 0.1 ml of each test microbial solution, which had been adjusted to the MacFarland standard, was added to each tube holding the diluted formulation. The tubes were then sealed and maintained at temperatures of 37 and 25 °C for 24 and 48 hours, respectively. After incubation, test tubes were checked for turbidity, suggesting growth. The lowest inhibiting dosage of each formulation against the subject of study was determined by turbidity in the test tubes after incubation. Then, the tubes with no growth(turbidity) were taken as the MBC or MFC (Balouiri et al., 2015).

Kill Time Assay of *M. Oleifera* Seed Protein Nanoemulsion

To evaluate the effectiveness of the nanoemulsion in killing microorganisms, the time-kill kinetics technique of testing for antibiotic susceptibility was employed. The formulations were tested against *S. aureus, P. aeruginosa, and A. niger* to determine their bactericidal and fungicidal effects, respectively. A sterile tube containing 3 ml of the formulation was inoculated with 0.1 ml of the standardized bacteria/fungal suspension to achieve a concentration of approximately 106 cfu/ml. Following a 7-day interval, 1ml samples were extracted from each reaction mixture and introduced onto the surface of matching sterile nutritional agar. The agar plates were then placed in an incubator(Techmel and Techmel Inc., China) for incubation at 25 °C for 24 and 48 h. A negative control (distilled water) was equally plated on the surface of a sterile nutrient /saboraud dextrose agar and incubated. The colonies formed were counted in duplicate and recorded as cfu/ml for each day interval. The number of viable organisms was measured in cfu/plates using a colony counter (Media Instrument Manufacturing Company, India), and log reductions were calculated for each formulation (ASTM, 2008).

Statistical Analysis

The outcome will be presented as the average ± standard deviation and examined using one-way ANOVA. The differences between means will be considered statistically significant at a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

M. Oleifera Seed Protein Solubility

Solubility of *M. oleifera* protein in oil, surfactant, and cosurfactant aids in the selection of a suitable vehicle for the formulation of nanoemulsion. **Table 2** illustrates that Peceol® (glyceryl monooleate) had the greatest solubilization for *M. oleifera* protein in the oil solubility test. Other oils produced lower solubility for *M. oleifera* protein. Thus, Peceol was selected for the formulation of nanoemulsion.

Biuret Test for *M. Oleifera* Protein Extracts

The test solution turned from blue to violet-purplish color in the presence of a biuret reagent. Biuret compound consists of copper sulfate, sodium hydroxide, and sodium potassium tartrate, which function to provide support for the cupric ion in the basic medium. The observed hue is due to the development of complex ions between cupric ions and peptide bonds in the proteins of *M. oleifera* under alkaline conditions (Nielsen, 2017).

Ninhydrin Test for *M. Oleifera* Protein Extracts

The test solution turned deep blue color in the presence of ninhydrin reagent after warming in water bath for five minutes. The ninhydrin reagent consists of ninhydrin dissolved in either ethanol or acetone. Heating a combination of water-based solutions of *M. oleifera* protein, composed of amine residues in the side strands of linear or aliphatic-aromatic amine, leads to the formation of a dark blue hue. The first step of the process entails the action of oxidatively deaminating the amino acid, which leads to the generation of ammonia and the reduction of ninhydrin to hydrindantin. Ammonia reacts with hydrindantin to form diketohydrindylidenediketohydrindamine, which causes a blue color to appear (Friedman, 2004; Yemm et al., 1955).

Coefficients of Dispersion and Measurement of Particle Size

The Malvern zetasizer was used to measure the particle size and coefficients of dispersion of MOSP nanoemulsion. This instrument functions based on the idea that smaller particles move at greater velocities. The MOSP nanoemulsion formed has particle sizes that vary between 43.440 and 74.430 nm as shown in **Table 3**. The MNE3 formulation has the least particle size of 43.440 nm (Gurpreet & Singh, 2018). The small droplet size confirms that the formulation technique is reliable, and it provides a large surface area to the formulations for any bioactivity. The polydispersity index of all formulations was less than 0.6 ranging from 0.348 to 0.542.

Table 3. Coefficients of dispersion and measurement of particle dimension

Formulation	Dimension of particles (nm)	Coefficients of dispersion
MNE1	57.550	0.356
MNE2	79.430	0.378
MNE3	43.440	0.542
MNE4	64.580	0.348
MNE5	74.430	0.431

Table 4. Viscosity of various formulations

Formulation	Viscosity (mPas)
MNE1	38 ± 2
MNE2	38 ± 3
MNE3	39 ± 0
MNE4	39 ± 0
MNE5	32 ± 7

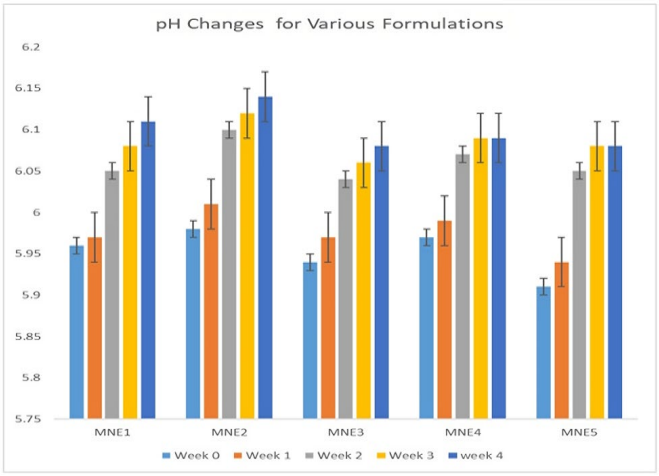


Figure 1. pH of nanoemulsions after formulation and weeks of storage (Source: Authors' own elaboration)

These results indicate that MOSP nanoemulsion formulations have homogenous particles and uniform nanoemulsion (Suhery et al., 2020).

Dynamic Viscosity Analysis

As an important parameter, the viscosity of MOSP nanoemulsion ranges from 32 ± 7 to 39 ± 0 mPa.s with MNE5 formulation having the least viscosity as shown in **Table 4**. The low viscosity would facilitate the flow property of the nanoemulsions and improve their use due to easy spreadability or extrudability.

pH of Nanoemulsion

Given that pH has the ability to regulate the antimicrobial properties of peptides that inhibit bacteria, pH plays a significant role in the complex relationships between biological barriers and the biological functions of these peptides. The pH values of the formulations shown in **Figure 1** varied from 5.9 ± 0.01 to 6.14 ± 0.01. The findings indicated that the samples' pH stayed consistently constant within the acidic region during the storage period, despite fluctuations in light and temperature.

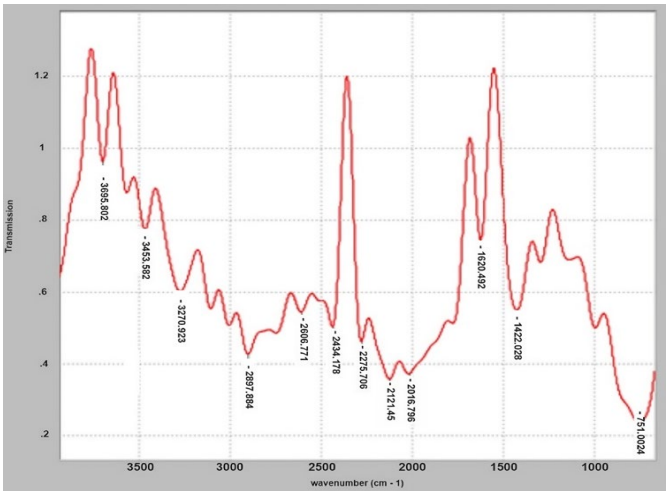


Figure 2. The FTIR spectrum of *M. oleifera* protein (Source: Authors' own elaboration)

Since the formulations were intended for use as antimicrobials, an alkaline pH is required for an effective antimicrobial activity. The reason for this is because basic pH facilitates the peptide's ability to possess a reduced positive electric charge and rapidly incorporate into the microbial partition. It can be inferred that acidic pH will reduce the antimicrobial action and efficacy of *M. oleifera* protein formulation (Abou Alaiwa et al., 2014). Furthermore, the formulations will be able to prevent the growth of bacteria more so than that of yeasts and molds, as many of these microorganisms may thrive at acidic pH values (Halla et al., 2018; Shkreli et al., 2022; Vavaresou et al., 2009).

Determination of Fourier Transform Infrared Spectroscopy

The structure of MOSPs was evaluated in this work using FTIR analysis. The prominent peaks seen at 1,680 and 1,560 cm^{-1} correspond to the polypeptide and protein iteration units, as shown in **Figure 2**. The peak seen at a wavenumber of 1,680 cm^{-1} is attributed to the amide structure 1 band, which corresponds to a secondary structural element of proteins. This band is associated with the stretching vibration of the C = O bond in the peptide backbone. The wavenumber maximal at 1,560 cm^{-1} is attributed to amide structure 2, which corresponds to the elongation of the C-N bonds and the bending of the N-H bonds in the peptide backbone (Magalhaes et al., 2021; Mune Mune et al., 2016). The major peaks observed for amide I and amide II in formulation MNE1 were at 1,721.180 and 1,544.210 cm^{-1}, in formulation MNE2 were at 1,696.730 and 1,566.270 cm^{-1}, in formulation MNE3 were at 1,669.570 and 1,533.060 cm^{-1}, and in formulation MNE4 were at 1,677.220 and 1,550.730 cm^{-1}, as depicted in from part a to part d in **Figure 3**. The change in wavenumbers of the prominent peaks is a result of structural modifications in the protein structure caused by the process called PEGylation.

Evaluation of Encapsulation Efficiency

The results of EE were observed to follow a concentration-dependent manner, MOSP yielding EE% of 76, 81, 82, and 85 for nanoemulsion formulated with 0.1 g, 0.3 g, 0.5 g, and 1 g, respectively as shown in **Table 5**.

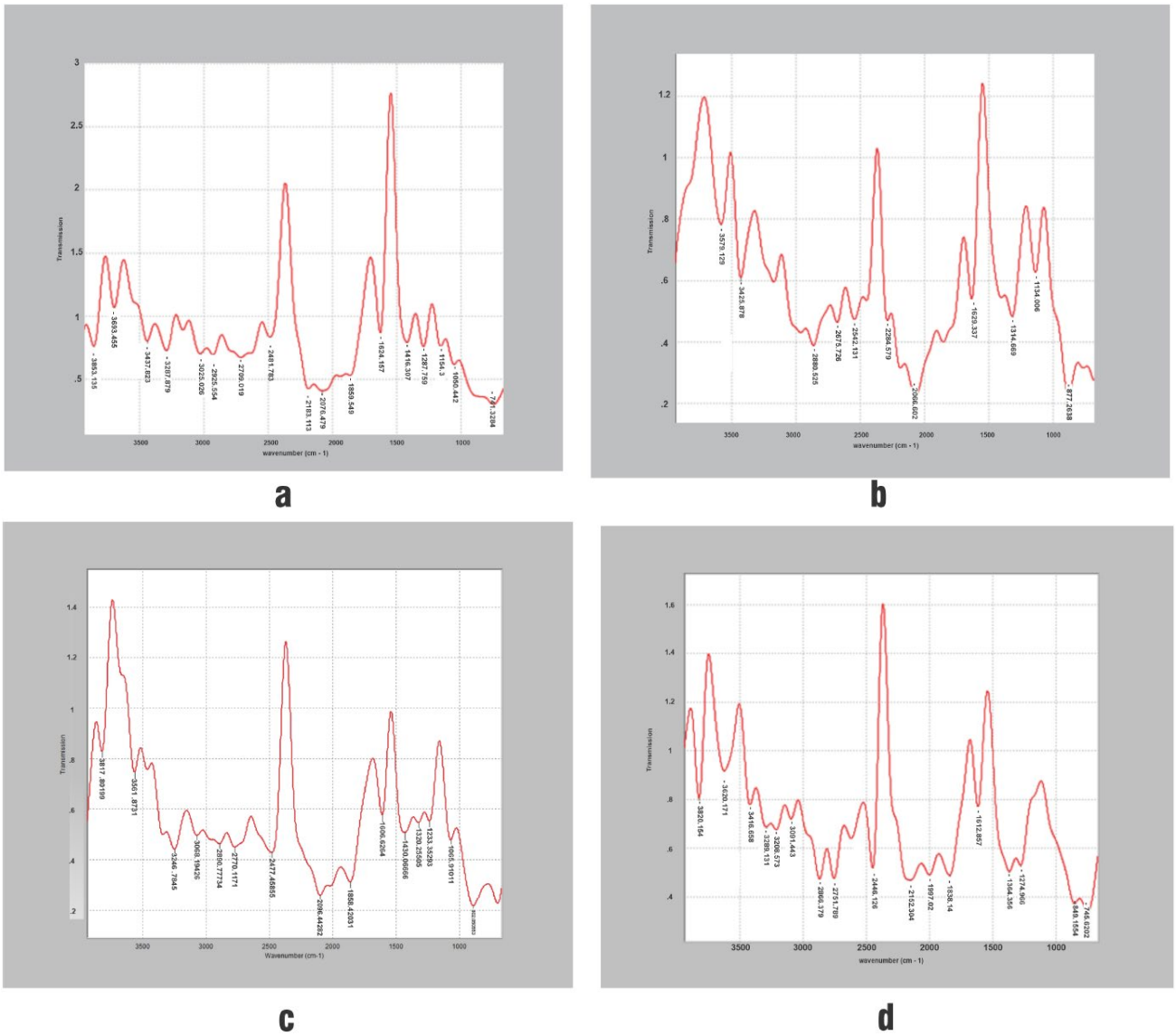


Figure 3. The FTIR spectrum of (a) MNE1 containing 0.1 g of *M. oleifera* protein, (b) MNE2 containing 0.3 g of *M. oleifera* protein, (c) MNE3 containing 0.5 g of *M. oleifera* protein, & (d) MNE4 containing 1.0 g of *M. oleifera* protein (Source: Authors' own elaboration)

Table 5. Encapsulation efficiency of the formulations

Formulation	Encapsulation efficiency (%) of MOSP
MNE1	76 ±1
MNE2	81 ± 2
MNE3	82 ± 3
MNE4	85 ± 1

Antimicrobial Activity Assay

The antibacterial activity of formulations MNE1, MNE2, MNE3, and MNE4 was evaluated in this study against the following microorganisms: the yeast *A. niger, S. aureus, E. coli, P. aeruginosa,* and *B. subtilis*. Due to its amphiphilic nature, which enables integration into the cellular membranes of microorganisms, MOSP exhibits antimicrobial activity when it interacts with negatively charged microbial surfaces (Suarez et al., 2005).

Table 6 shows that MNE1 had the greatest impact on *P. aeruginosa* with diameter of the inhibition zone (DIZ) of 4 ± 1 mm and followed by *S. aureus* with DIZ of 2 ± 0.5 mm. The formulation MNE2 exhibited the most effectiveness against *P.*

Table 6. Diameter of the inhibition zone (mm) of test formulations against studied pathogens

Test formulation	Test organisms/the diameter of the inhibition zone (in millimeters)				
	S. aureus	*P. aeruginosa*	*E. coli*	*B. subtilis*	*A. niger*
MNE1	2 ± 0.5	4 ± 1	0	0	0
MNE2	0	2 ± 0	0	0	0
MNE3	0	0	0	0	0
MNE4	0	0	0	0	0
Ciprofloxacin (20 µg/ml)	10 ± 0	6 ± 1	0	14 ± 0.5	-
Fluconazole (80 µg/ml)	-	-	-	-	0

Table 7. Minimum bacterial and fungal concentration of formulations

	Minimum bacterial & fungal concentration (µg/ml)			
	MNE1	MNE2	MNE3	MNE4
S. aureus	100	100	50	-
P. aeruginosa	50	50	-	100
E. coli	-	-	100	-
B. subtilis	-	-	-	-
A. niger	100	-	100	100

aeruginosa with DIZ of 2 ± 0 mm. The positive control, Ciprofloxacin, produced an inhibitory zone diameter of 10 ± 0 mm for *S. aureus*, 6 ± 1 mm for *P. aeruginosa,* and 14 ± 0.5 mm for *B. subtilis*. Therefore, the result of this study confirms the activity of MOSP nanoemulsion against *P. aeruginosa* and *S. aureus*.

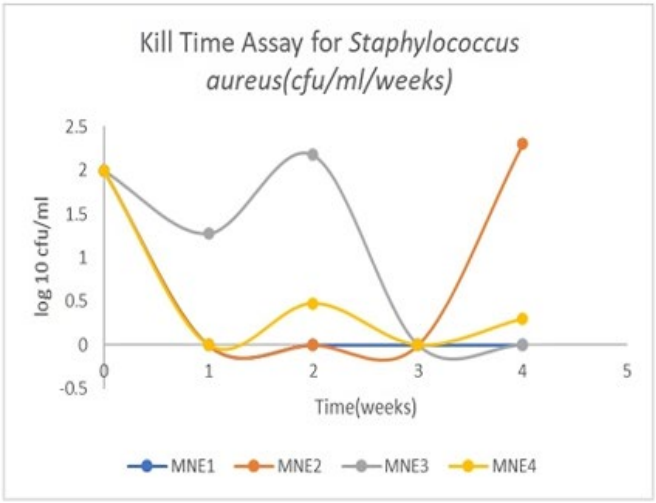
Minimum Bacterial and Fungal Concentrations (MBC & MFCs) Study

The MBC or MFC values of various formulations against test organisms are shown in **Table 7**. Formulation MNE1 exhibited minimal bacterial or fungicidal concentrations of 100 µg/ml, 50 µg/ml, and 100 µg/ml against *S. aureus, P. aeruginosa, and A. niger* correspondingly. Formulation MNE2 exhibited minimum bactericidal doses of 100 µg/ml and 50 µg/ml against *S. aureus and P. aeruginosa,* correspondingly. Formulation MNE3 exhibited minimum bactericidal or fungicidal concentrations of 50 µg/ml, 100 µg/ml, and 100 µg/ml against *S. aureus, E. coli, and A. niger,* correspondingly. Formulation MNE4 exhibited minimum bactericidal concentrations of 100 µg/ml against P. aeruginosa and minimum fungicidal concentrations of 100 µg/ml against *A. niger*. Also, the formulations had no activity against *B. subtilis*. Among the test pathogen, *P. aeruginosa* was the most sensitive microorganism to the formulation. Therefore, the result of this study confirms the activity of MOSP nanoemulsion against test pathogens.

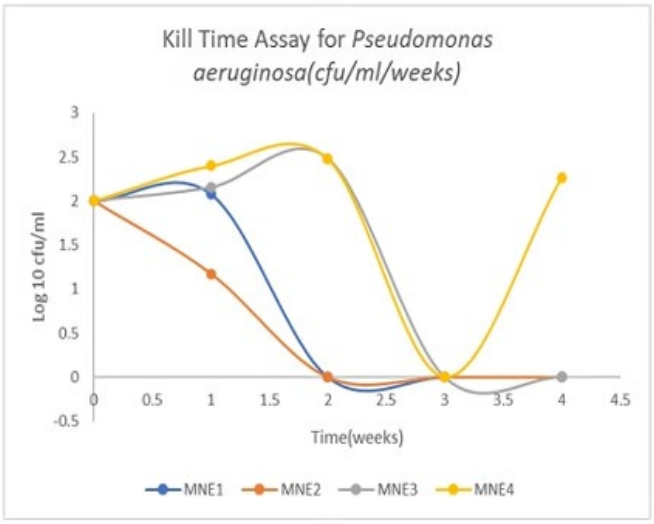
Kill Time Assay

The antimicrobial efficacy is determined by observing changes in the colony–forming units for microbial cells–*P. aeruginosa*, *S. aureus*, and *A. niger* for 4 weeks after exposure to the viable count of 106 cfu/ml of microbial cells. In part a in **Figure 4**, there is a recorded logarithmic reduction in the proliferation of *S. aureus* for all formulations in week 1 and week 3 and an increase in microbial load for MNE3 and MNE4 at week 2 and MNE2 and MNE4 at week 4. It could be due to the presence of PEG in the nanoemulsion as it affects its microbial activity. MOSP is probable to have formed a compound with PEG by bonding with the -OOH unit and the α amine moiety at the compound end. Chemical alteration may lead to a decline in its antibacterial effectiveness against *S. aureus*. This is because the modified chemical may not be able to penetrate the bacterial cell membrane as effectively, resulting in less harm being caused (Aryee et al., 2018; Gokran et al., 2012; Guiotto et al., 2003; Lawrence & Price, 2016; Ndabigengesere et al. 1995; Veronese & Pasut, 2005; Wang et al., 2020; Youle & Huang, 1981).

In part b in **Figure 4**, there is a recorded logarithmic reduction in the proliferation of *P. aeruginosa* for all formulations in week 1 and week 3; an increase in microbial load for MNE3 and MNE4 at week 2, and only MNE4 at week 4. All formulations showed no reduction of logarithmic cycles in the proliferation of *A. niger*. Also, it could be due to the presence of PEG in the nanoemulsion as it affects its microbial activity. It is likely that MOSP might have conjugated with PEG through the -OOH unit and the α amine moiety at the end. Such chemical modification could result in a reduction of antibacterial activity against *S. aureus* due to decreased permeation of bacteria cell membrane by MOSP to cause irreversible damage.



a



b

Figure 4. Time exposure viability curves for (a) the formulations to culture of *S. aureus* & (b) the formulations to culture of *P. aeruginosa* (Source: Authors' own elaboration)

AMR arises either through a genetic mutation or by the acquisition of antibiotic-resistant genes from another microorganism (Khan et al., 2021). Nanoemulsion containing protein from *M. oleifera* seed exhibited potent antimicrobial activity. As an AMP that contains a positively charged disulfide bond, the plant protein binds to negatively charged molecules like phospholipids, teichoic acids, and lipopolysaccharides of the microorganism to cause lyses of the cell membrane. This study reports that plant AMPs have the potential to act as antimicrobial agents (**Figure 5**).

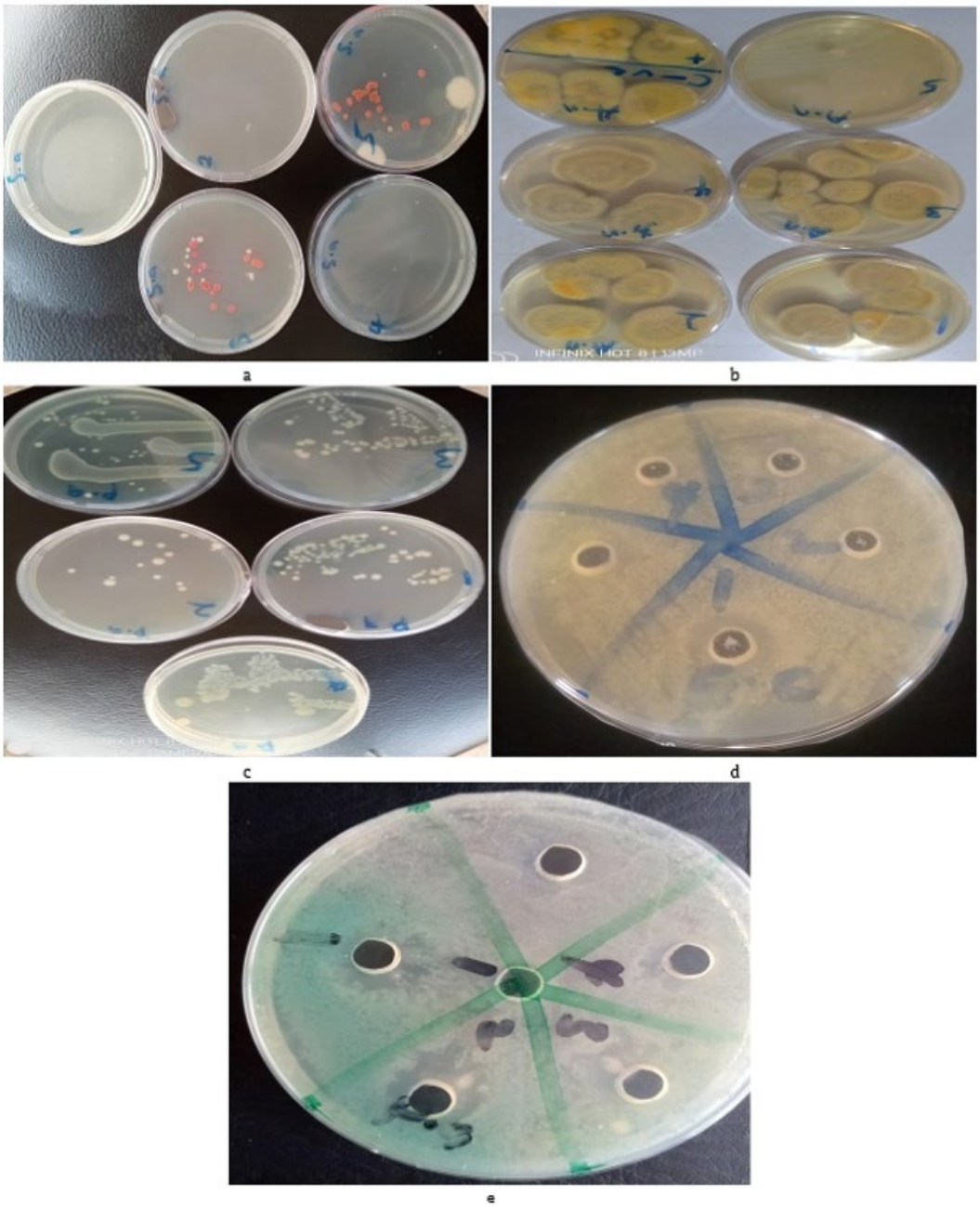


Figure 5. Colony morphology and diameter of the inhibition zone of formulations on test pathogens (Source: Authors' own elaboration)

CONCLUSION

It is beneficial and environmental–friendly to explore plant peptides as antimicrobial agents and formulating it into nanoemulsion provides an avenue to use biodegradable materials like glyceryl monooleate, polysorbate 80, and PEG 400 as the components of nanoemulsion. Polysorbate 80 helped to preserve the biological activity of *M. oleifera* protein by minimizing the exposure of three-dimensional conformation of protein during formulation, storage, and transportation. PEG offers support to the plant protein thereby reducing protein aggregation and proteolysis resulting in increased protein shelf-life.

The nanoemulsion was nanosized with an acidic pH, and excellent dynamic viscosity. The antimicrobial and minimum inhibitory concentration study demonstrated significant antimicrobial efficacy against *P. aeruginosa* and *S. aureus*, but the time-kill assay showed moderate biocidal activity. Further study is necessary to determine the molecular mechanism by which *M. oleifera* nanoemulsion had reduced activity against microorganisms. There should be further studies to explore possible improvement of antimicrobial activity through modification of the lipids.

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Declaration of interest: No conflict of interest is declared by the authors.

Data sharing statement: Data supporting the findings and conclusions are available upon request from corresponding author.

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