European Journal of Clinical Pharmacy

Print ISSN: 2385-409X **Online ISSN**: 3105-0409

Website: https://farmclin.com/

Original Research Article

The antimicrobial and antioxidants properties of nanoemulsions synthesized with Moringa oleifera essential oil in laboratory conditions

Article History:

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Received: 12-11-2025
Revised: 26-11-2025
Accepted: 10-12-2025
Published: 27-12-2025

Abstract: ***Background:*** Herbal extracts are aromatic and volatile oil compounds that have gained attention due to their strong antibacterial and antioxidant properties. Objectives: In this study, the antimicrobial activity of Moringa oleifera essential oil nanoemulsion was evaluated agsinst Staphylococcus aureus and Escherichia coli was compared with the Moringa essential oil. Materials and methods: investigation of the chemical Chatatrization of Moringa oleifera Essential Oil Nanoemulsion by Fourier-Transform Infrared Spectroscopy (FTIR) and the morphological properties by Scanning electron microscopy, MIC of Moringa nanoemulsion were calculated for two bacteria S. aureus and E.coli according the Kirby-Bauer standardized method .Finally estimation the antioxidant activity nanoemulsion and oil M.oleifera by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method Results: FTIR analysis confirmed the presence of alkanes, aldehydes, aromatic compounds, aromatic amines, and halogen compounds in the range of 2000-1000 cm-1. Fe-SEM electron microscope, spherical nanoparticles in the size range of 60 to 70 nanometers were observed. The results indicated that nanoemulsions of Moringa essential oil increased the antibacterial activity, especially against the Gram-positive bacterium S. aureus.the antioxidant results found the half-maximal inhibitory concentration IC50 for M. oleifera essential oil and nanoemulsion are 23.97 and 17.78 mg/ml, respectively, and the difference between them is significant ($p < 0.05$). Conclusion: In comparison to M.oleifera essential oil the essential oil nanoemulsion, showed antimicrobial activity against S.aureus according MIC, the MIC of essential oil nanoemulsion was 1.25 mg/ml and MIC of essential oil was 156.0 mg/ml, while MIC of essential oil nanoemulsion 1.25 mg/ml and MIC of essential oil 2.5 mg/ml of E.coli , as well as the antioxidant activity showed the IC50 for M. oleifera essential oil and nanoemulsion are 23.97 and 17.78 mg/ml , respectively, the thus the study's results indicate that the essential oil nanoemulsion exhibited substantial antioxidant and antibacterial activity.

Keywords: Antimicrobial activity, Escherichia coli, Essential oil, Moringa oleifera, Nanoemulsion, Staphylococcus aureus.

INTRODUCTION

Moringa can serve as a natural plant growth enhancer, as its leaves are rich in zeatin, a plant hormone belonging to the cytokinin group. Leaf extract can stimulate plant growth and increase crop yields [1, 2].

It has been demonstrated that several moringa parts—fruits, leaves, flowers, and stems—help prevent cancer, a fatal illness. Moringa's isolated thiocarbamate and isothiocyanate chemicals suppress tumor cell growth [3]. It was discovered that the dichloromethane fraction was cytotoxic to MCF7 breast cancer cells [4]. In chemical carcinogenesis, niazimincin has been proposed as an effective chemopreventive agent [5]. Reactive oxygen spices have been studied using bioactive substances from Moringa pods, including flavonoids [9], thiocarbamates [8], glycosylates [6], isothiocyanates [7], and several other chemicals. It has been demonstrated that the aqueous extract is a strong scavenger of free radicals [10]. Previous studies suggest that the antioxidant potential might be due to

kaempferol, which is mainly found in plant leaves [11].

Research has highlighted the potent antimicrobial properties of Moringa oleifera seed extract, which can be utilized in the treatment of fungal diseases [12]. Antimicrobial peptides present in the extract lead to the separation of two microbial membranes (outer and inner), causing water to penetrate the cell, increase cell volume, and result in cell death [13].

Currently, the use of natural enzyme inhibitors, such as those found in essential oils, is gaining attention due to minimal side effects on the human body [14]. Additionally, nanoemulsions have numerous other advantages, such as minimizing the sensory impact on the taste of food products and enhancing bioactivity due to better size distribution and dispersion. Oil-in-water (O/W) nanoemulsions consist of oil droplets with an average size ranging from 20 to 200 nanometers, dispersed in an aqueous medium and stabilized by an emulsifier layer. Surfactants suitable for use in food (poly sorbates, sugar esters, natural gums, modified vegetable proteins, and even some fractionated alkali-hydrolyzed guar gums) serve as emulsifying agents, and in some cases, they contribute to the unique surface behavior of certain nanoemulsions. Nanoemulsions exhibit electrostatic forces, spatial repulsion, rheology, ons can be formulated with food components to have distinctive and unique properties [15,16]. Despite the enough information about the antimicrobial activity of oil M. oleifera there is a limited information about the the antimicrobial activity of oil nanoemulsion M. oleifera, the hypothesis of the current study is the oil nanoemulsion of Moringa will acquired new chemical and physical properties that will enhance its capacity as an antimicrobial .

The aim of this study was to evaluate the antimicrobial and antioxidant activities of Moringa oleifera oil nanoemulsion compared with the crude essential oil as well as study the chemical and physical characterization of the nanoemulsion . This comprehensive evaluation was conducted to provide a deeper understanding of the functional advantages of converting essential oil into a nanoemulsion system.

MATERIAL AND METHODS

Microorganism used in the study

Staphylococcus aureus bacteria (ATCC 29213, PTCC 1917) and Escherichia coli (ATCC 25922, PTCC 1399) and the ability to load and respond to environmental stresses. Depending on the size, nanoemulsiwere obtained from the microbial collection of the Iran Institute of Scientific and Industrial Research.

Extraction of Moringa oleifera Essence

The 150 grams of purchased leaf powder, along with water, were placed inside an ultrasonic bath (Elmap30h, Germany) for 40 minutes. Subsequently, the essence was extracted for 3 hours using the water distillation method in a Clevenger apparatus. The separated essence was then dried with sodium sulfate, and the essences were stored in sealed and dark containers in the refrigerator at 4 degrees Celsius until analysis [17].

Preparation of Moringa oleifera Essential Oil Nanoemulsion

To prepare the nanoemulsion, Tween 80 (30% by weight of the essential oil), distilled water, and M.oleifera essential oil (30% w/w) were used. The surfactant, along with the essential oil, was continuously and uniformly added drop by drop to distilled water and homogenized at a speed of 3000 rpm for 15 min . Then, the obtained emulsion solution was transferred to a sonicator probe device (OPTIMA, XL 100 K, 20 KHz, Germany). The 15 mm probe of the device was immersed in a 25 mm depth of the solution, and the nanoemulsion preparation process was carried out for 5 minutes at 200 watts and a temperature of 25 degrees Celsius [18].

Chatatrization of Moringa oleifera Essential Oil Nanoemulsion

1-Fourier-Transform Infrared Spectroscopy (FTIR)

The qualitative examination of the material was conducted using FTIR spectroscopy, a method commonly employed for identifying the structural features of biopolymers [19]. FTIR spectra of the samples were obtained in the range of 400–4000 cm-1 utilising a Thermo Scientific Nicolet iS10 Spectrometer with a Smart iTX attachment (Thermo Scientific, Inc., United States) at a spectral resolution of 4 cm-1 and 32 scans. FTIR spectra were acquired in reflection mode via the integrated diamond attenuated total reflectance (ATR) sampling method. The OMNIC Software facilitated the collecting, processing, analysis, and management of FTIR data inside a graphical interface.

2- Determining the morphological properties

Scanning electron microscopy was used to study the surface morphology of nanoparticles. After a 6-hour reaction, the colloidal solution was centrifuged for 4 minutes at 14,000 rpm to create the sample. After being re-dispersed in deionised water, the pellet was centrifuged once more. After three iterations of the procedure, acetone was used to wash the material. A drop of the solution was put on the carbon-coated copper grid after the purified nanoparticles had been sonicated for ten minutes to create the suspension. The sample was exposed to a lamp until it was totally dry. The prepared sample was examined using a scanning electron microscope[20].

Determination Minimum Inhibitory Concentration MIC of Moringa Nanoemulsion

Using the standard method recommended by Hudzicki [21], the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Moringa nanoemulsion were determined for two bacteria, S. aureus and E. coli .The concentration was prepared from 0.004 to 2.5 mg/ml of both M. oleifera nanoemulsion and essential oil)Initially, 13 sterile tubes containing 1 ml of Mueller-Hinton agar medium were prepared.

Then, 1 mL of Moringa nanoemulsion at a concentration of 5 mg/mL was added to each sterile tube containing 1 mL of Mueller-Hinton agar medium. The first tube was poured, and after shaking, 1 mL from the first tube was added to the second tube, and so on, until tube 13. Finally, 1 mL from tube 13 was poured out. Each bacterial suspension was separately added to the tubes at a concentration of 1.0 mL (CFU/mL 1.5 × 10^8), and the process continued from tube 1 to 12 . One tube was considered as a positive control (medium and bacterial suspension), and one tube was considered as a negative control (medium and essential oil without bacteria). The tubes were incubated at 37 degrees Celsius for 24 hr. After the incubation period, the first tube in which no turbidity was observed was considered as the MIC for each of the samples

Evaluation of Antioxidant Activity of Moringa oleifera Essential Oil and Nanoemulsion by DPPH Method

The antioxidant activity of Moringa oleifera nanoemulsion was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method figure 2. For this purpose, various concentrations of essential oil and nanoemulsion (5, 10, 20, 25, and 30 mg/ml in methanol) were prepared. Then, 200 microliters of each sample were added to 1.0 millimolar DPPH solution in 8.2 milliliters, and the mixture was incubated in the dark at 25 degrees Celsius for 30 minutes. The absorbance of the solution at 517 nanometers was measured using a spectrophotometer. Distilled water was used as a control in this test. The percentage of DPPH free radical inhibition was calculated using the formula:

SC (%) = 100 × (A blank –A sample)/A blank)

Then, the antioxidant activity of the essential oil and nanoemulsion was determined as the inhibitory concentration at 50% (IC50) using linear regression analysis of the SC values for various sample concentrations. All experiments were conducted in a completely randomized design with at least three repetitions [22].

Statistical Analysis

The results obtained from three repetitions was performed using version 9.1.0 of Graphpad Prism software. Two-way ANOVA was used for analysis, followed by the Tukey test to examine the significance level of the results. A significance level of 0.05 was considered ($P < 0.05$)

RESULTS AND DISCUSSION

Moringa oleifera Nanoemulsion Characterization

Fourier-transform infrared spectroscopy (FTIR) Analysis

As observed in Figure 2, the spectra obtained from FTIR analysis confirmed the presence of alkanes, aldehydes, aromatic compounds, aromatic amines, and halogen compounds in the range of 2000-1000 cm-1. Peaks in the region of 2927 and 2858 cm-1 indicate the CH bond related to the alkene functional group. The peak at 1740 cm-1 corresponds to the C=O bond related to the carbonyl group. The peak at 1116 cm-1 is related to the O-C bond. The peak in the range of 3300 cm-1 indicates the O-H bond related to the phenol functional group, which is observed in the spectrum of both samples. The presence of similar peaks in the nanoemulsion spectrum confirms the absence of chemical changes in the essential oil during sonication and the formation of the nanoemulsion. The softening of the peaks in the nanoemulsion spectrum compared to the essential oil indicates the formation of the nanoemulsion structure.

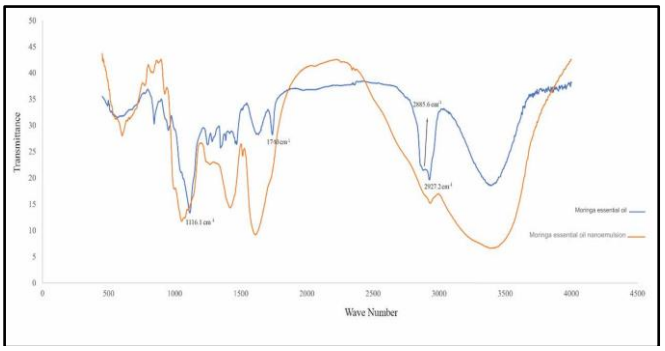

Figure 1. FTIR spectra of essential oil samples (blue plot) and Moringa oleifera nanoemulsion (orange plot).

Electron Microscope Image (Fe-SEM)

In the obtained images from the Fe-SEM electron microscope, spherical nanoparticles in the size range of 60 to 70 nanometers were observed, confirming the formation of Moringa essential oil nanoemulsion Figure 3.

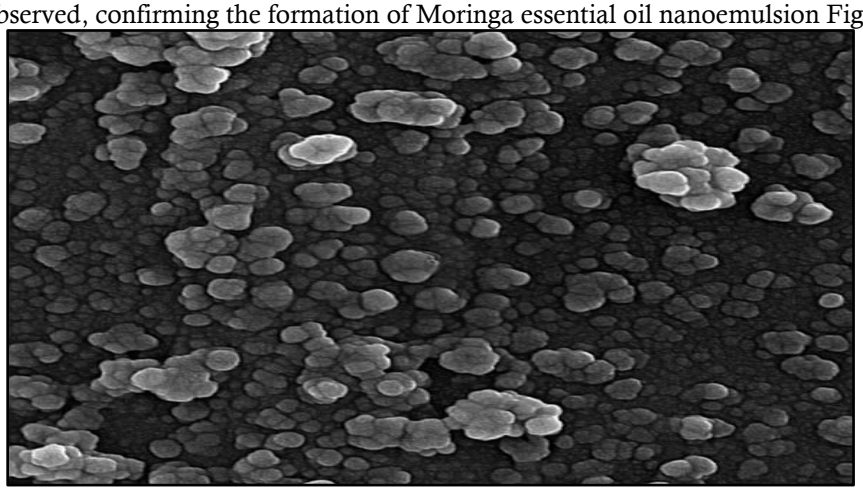

Figure 2. Fe-SEM Electron Microscope Image of Moringa Nanoemulsion with a magnification of 500 nanometers

Antimicrobial activity of *M. oleifera* Extract and *M.oleifera* Essential Oil Nanoemulsion

Due to the absence of turbidity in tubes containing concentrations of 1.25 and 156.0 milligrams per milliliter of essential oil and nanoemulsion for *S.aureus*, these concentrations were considered as the minimum inhibitory concentrations against the growth of this bacterium . As well as Considering the absence of turbidity in tubes containing concentrations of 2.5 and 1.25 mg /ml of essential oil and essential oil nanoemulsion for *E. coli*, these concentrations were considered as the minimum inhibitory concentrations (MIC) for inhibiting the growth of this bacterium, As shown in Table 1 .

1.25 and 156.0, 25 and 2.5 respectively.

Table 1. MIC of *M. oleifera* essential oil and essential oil nanoemulsion against studied isolated

Isolates	MIC mg/ml	
	Essential Oil Nanoemulsion	Essential oil
S. aureus (ATCC 29213, PTCC 1917)	1.25	156.0
E.coli (ATCC 25922, PTCC 1399	1.25	2.5

Antioxidant Properties of Moringa oleifera

Essential Oil and Nanoemulsion by DPPH method evaluation of antioxidant activity showed that the percentage of free radical inhibition by the nanoemulsion is higher than that of the essential oil ($p < 0.05$). Additionally, with an increase in concentration, the inhibition of free radicals occurs more effectively Figure 4. The half-maximal inhibitory concentration IC50 (the concentration of each sample capable of capturing or inhibiting 50% of free radicals) for Moringa oleifera essential oil and nanoemulsion are 23.97 and 17.78 micrograms per milliliter, respectively, and the difference between them is significant ($p < 0.05$) Figure 5.

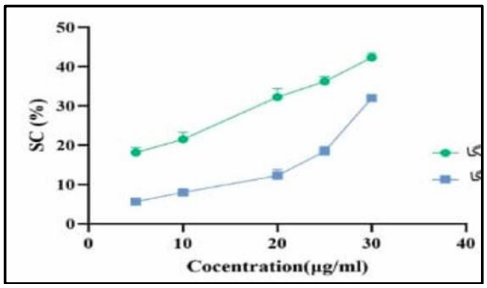

Figure 3. Percentage Inhibition Chart of Free Radical by Moringa oleifera Essential Oil d Nanoemulsion (blue) Using the DPPH Method Under Laboratory Conditions

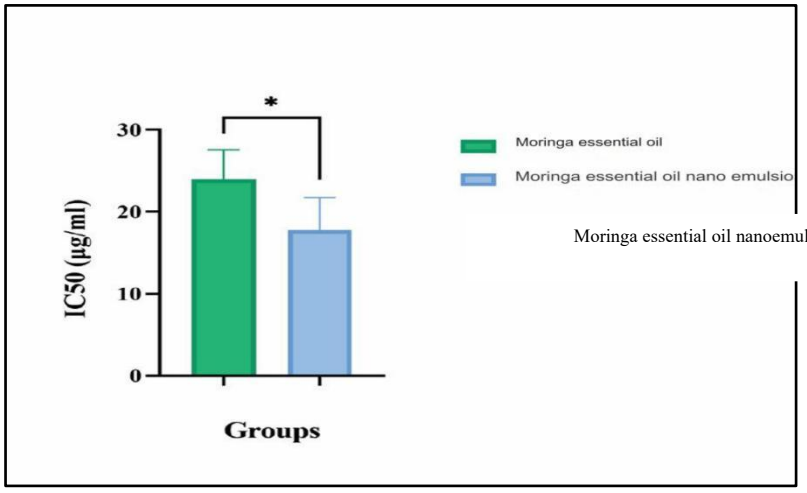

Figure 4. IC50 Diagram of Pomegranate Extract, Essential Oil, and Nanoemulsion.

In recent years, with the increasing awareness of consumers, the use of herbal medicines and natural products, in general, has significantly increased [23]. Moringa is a tropical tree from the Moringaceae family, which includes fourteen species. This fast-growing deciduous tree has adapted well to arid conditions, with drum-shaped pods containing seeds. The tree can reach a height of up to 12 meters and produces seeds in its first year of growth. Moringa has been used for centuries for nutritional and medicinal purposes. This plant contains vitamin C, vitamin A, calcium, potassium, and all essential amino acids [24, 25]. Essential oils are natural and plant-derived secondary metabolites that, due to their good functional properties such as antimicrobial and antioxidant properties, have been proposed as alternatives to chemical preservatives in food studies [26].

The essential oil of the Moringa plant is one of the few essential oils with industrial, health, food, and medicinal applications [27]. The antioxidant compounds present in this plant help the human body reduce oxidative damage. Due to concerns related to food safety and health, food industry specialists are seeking to replace synthetic antioxidants with various natural antioxidants [28, 29]. Despite the high potential of essential oils, their direct use in food products and aqueous solutions is accompanied by limitations such as volatility, low solubility, and changes in sensory properties. On the other hand, essential oils are susceptible to oxidation when exposed to temperature, light, and oxygen, reducing their effectiveness.

According to a study by Almuttairi and Abdulla, 2023 [30]. transforming essential oils into emulsion forms can enhance their physical stability, solubility, and biological activity. Emulsions can generally be classified into two particle size groups: microemulsions and nanoemulsions. Nanoemulsions, produced through various high-energy methods (such as ultrasonication) or low-energy methods (such as phase inversion), have particle sizes in the range of 1-100 nanometers. They consist of an oil phase dispersed in an aqueous phase, surrounded by a thin layer of surfactant or amphiphilic molecules, contributing to the stability of the nanoemulsion [30].

In the results of microbial analysis, the nanoemulsion of Moringa essential oil showed better effectiveness against the Gram-positive bacterium *Staphylococcus aureus* compared to the essential oil alone. In other studies, [31,32] were evaluated the antibacterial effect of Moringa oleifera extract and pomegranate extract on Porphyromonas gingivalis.. [33] investigated the antioxidant and antibacterial activities of the oil extract of Moringa oleifera showed inhibitory properties against all tested bacteria except *E. coli* and *K.pneumoniae* at a concentration of 125 microliters per milliliter. The maximum antibacterial activity at a concentration of 500 microliters per milliliter was observed against the tested bacteria, measuring 18.5, 14.16, 11.83, and 11.33 mm, respectively. The MIC of the oil extract for Staphylococcus aureus was reported to be 64 micrograms per milliliter. It was 128 microliters per milliliter for Bacillus cereus and 256 microliters per milliliter for both Escherichia coli and Klebsiella pneumoniae.

Accrording to Akintelu et al,(2021) [34] studied the antimicrobial activity of Moringa oleifera seed extract against some Gram-positive (*S. aureus* and *Streptococcus pneumoniae*) and Gram-negative bacteria (*E. coli*, *Pseudomonas aeruginosa*, and *K. pneumoniae*). Phytochemical analysis of the seed extract revealed the presence of alkaloids, tannins, saponins, phenols, and flavonoids as secondary metabolites. The antibacterial study demonstrated inhibitory zones for all tested bacterial strains. The study highlighted the effectiveness of Moringa oleifera seeds in combating certain bacterial infections.Based on the studies,

essential oil extracts, especially in the form of nanoemulsions, may affect Gram-negative bacteria by preventing the production of adenosine triphosphate (ATP) from dextrose and disrupting the cell membrane. Additionally, the hydrophobic property of essential oil nanoemulsions may contribute to their distribution in the structures of Gram-positive bacteria [35].

Various factors such as the extraction method, inoculum size, growth phase, growth medium, pH of the environment, duration, and temperature of incubation can influence the results of experiments, leading to reported differences [36]. Moreover, in a study by [30], investigating the antibacterial activity of micro and nanoemulsions of rosemary essential oil, the highest MIC and MBC for micro and nanoemulsions against Gram-negative bacteria Salmonella *enteritidis* and *E. coli* were observed at a concentration of 3.6 mg/ml. Staphylococcus aureus was the most sensitive bacterium, and the MIC and MBC of micro and nanoemulsions of rosemary essential oil against it were 0.9 and 1.8 mg/ml, respectively. In general, nanoemulsions can render bacteria inactive or cause their death through different mechanisms.

The mode of action of essential oils involves disrupting and destabilizing the structure of phospholipid membrane layers, membrane disruption, interaction with membrane enzymes and proteins as proton carriers, and reduction of pH across the membrane [37]. For instance, a nanoemulsion can release ions that react with thiol groups in bacterial cell surface proteins. As a result, proteins become inactivated, and cell membrane permeability is reduced, ultimately leading to bacterial death. Accumulation of nanoparticles in the bacterial cytoplasm and on its external membrane can be another mechanism involved in preventing bacterial growth and survival. Nanoemulsions produced by high-intensity and high-pressure homogenization methods exhibit higher antimicrobial properties compared to pure essential oils in additional the role of nanoparctical recorded in vivo [38, 39].

DISCUSSION

The current study demonstrated that the Moringa oleifera essential oil nanoemulsion exhibited markedly stronger antimicrobial activity than the crude essential oil. For S. aureus, the MIC of the nanoemulsion was 1.25 mg/ml compared to 156.0 mg/ml for the essential oil. Similarly, for E. coli, the MIC values were 1.25 mg/ml for the nanoemulsion and 2.5 mg/ml for the essential oil. The antioxidant assessment further showed that the nanoemulsion had a significantly higher free-radical inhibition percentage than the essential oil ($p < 0.05$), with IC_{50} values of 17.78 mg/ml and 23.97 mg/ml, respectively. These findings indicate that the essential oil nanoemulsion possesses enhanced antibacterial and antioxidant properties and may serve as a promising approach for use alone or alongside conventional methods in food preservation and in the management of bacterial infections.

Data availability

All data generated or analyzed during this study are available from the authors.

Acknowledgments

The authors are grateful to all person who helped improve the quality of this work.

Funding

The authors reported there is no funding associated with the work featured in this article.

Declarations of competing interests

The authors have declared no conflict of interest.

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