A dissertation

On

Interactive Role of Nanoemulsion against Pathogenic Bacteria

Submitted to

Department of Biosciences

Integral University, Lucknow

For the partial fulfillment of degree in Master of Science in Microbiology

Under the supervision of

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प्रमाण पत्र

सुश्री प्रीती मधेशिया, पुत्री श्री बृजेश कुमार, एम.एससी. माइक्रोबायोलॉजी इंटीग्रल यूनिवर्सिटी, लखनऊ ने दिनांक 02.03.2022 से दिनांक 22.06.2022 तक **"Interactive Role of Nanoemulsion against Pathogenic Bacteria"** शीर्षक में डा. आराधना मिश्रा, प्रिंसिपल साइंटिस्ट के पर्यवेक्षण (Supervision) में सफलतापूर्वक प्रशिक्षण प्राप्त किया।

CERTIFICATE

Ms. Preeti Maddheshiya, D/o Shri Brijesh Kumar, student of M.Sc. Microbiology, Integral University, Lucknow has successfully completed the training from 02.03.2022 to 22.06.2022 on the project entitled, **"Interactive Role of Nanoemulsion against Pathogenic Bacteria"** under the supervision of Dr. Aradhana Mishra, Principal Scientist.

for. 22/6/22

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TO WHOM IT MAY CONCERN

This is to certify that Miss Preeti Maddheshiya, a student of M.Sc. Microbiology (2nd year/4th semester) from Integral University, Lucknow has completed her four-month dissertation work entitled "**Interactive Role of Nanoemulsion against Pathogenic Bacteria**" from CSIR-National Botanical Research Institute, Lucknow under the supervision of Dr. Aradhana Mishra (Principal Scientist). The dissertation was a compulsory part of her M.Sc. Microbiology degree.

I wish her good luck and a bright future.

Dr. Snober S MIR

Head of Department of Bioscience

Integral University

DECLARATION

I Preeti Maddheshiya a student of M.Sc. Microbiology (2^{nd} year/4^{th} semester), Integral University, Lucknow has completed her four months' dissertation work entitled "**Interactive Role of Nanoemulsion against Pathogenic Bacteria**" from CSIR-National Botanical Research Institute, Lucknow, under the supervision of Dr. Aradhana Mishra, Principal Scientist.

I hereby declared that the work has been done by me in all aspects.

I have sincerely prepared this project report and the results reputed in this study are obtained.

Preeti Maddheshiya

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Preeti Maddheshiya

M.Sc. Microbiology

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LIST OF INSTRUMENTS

S.No.	Instrument	Company
1	Horizontal autoclave	Equitron
2	Vortex	GeNei
3	Refrigerator Incubator shaker	Innova 4230
4	Refrigerator	LG
5	Weighing balance	Denver
6	Refrigerator centrifuge	Sigma, Dynamico
7	Tabletop centrifuge	GeNei
8	Hot air oven	GeNei
9	pH meter	Inolab
10	Microwave	Samsung
11	Laminar air flow	Yorko
12	Ice machine	Icematic D101
13	Freeze	Sanyo
14	Ultrasonic homogenizer	Biochem
15	Deep freezer (-20 ℃)	Samsung
16	Zeta sizer	Malvaren

LIST OF ABBREVIATIONS/SYMBOL

%	Percentage
NMs	Nanomaterials
NPs	Nanoparticles
CNTs	Carbon nanotubes
DR	Drug-resistant
MRSA	Mecithilin-resistant Staphylococcus aureus
ESBL	Extended- spectrum beta lactamase
O/W	Oil in water
O/W/O	Oil water oil
PIT	Phase inversion temperature
EIP	Emulsion inversion phase
HPH	High pressure homogenization
DLS	Dynamic light scattering
DSC	Differential scanning calorimetry
AFM	Atomic force microscopy
EPR	Electron paramagnetic resonsnce
NMR	Nucleur magnetic resonance
EO	Essential Oil
O_9NE	Lab's oil coding
MIC	Minimum inhibitory concentration
NE	Nanoemulsion

INTRODUCTION

Nanotechnology, which is centered on the precise synthesis, manipulation, and application of materials at the nanoscale (10^{-9}m), is rapidly growing. The National Nanotechnology Initiative (NNI) of the United States defines nanotechnology as "atomic or molecular-scale research and development activities to construct structures and systems adaptable to a wide range of applications" (Balzani., 2005). Traditional medical practitioners in India and China succeeded in manufacturing gold colloids (known as **'Suwarna Bhasma'** in ancient Ayurveda in India) for therapeutic purposes in the 4th and 5th centuries BC, thereby beginning the history of nanotechnology (Paul *et.al.*, 2011). Paracelsus used colloidal gold to treat mental illnesses and syphilis in Europe throughout the Middle Ages (Dykman *et.al.*, 2012). In 1618, the Philosopher and Doctor Francisco Antonii released a book on the manufacture and therapeutic applications of colloidal gold. In 1857, Michael Faraday published the first scientific study of colloidal gold. Richard Feyman's famous address **'There's Plenty of Room at the Bottom'** in 1959 at Caltech, USA, sparked a genuine interest in the field of nanomaterial science (Feynman., 1960). Eric Drexler popularized the concept of nanomaterial science in the 1980s with his book Engines of Creation: The Coming Era of Nanotechnology. Nanotechnology is now widely used in the medical, pharmaceutical, industrial, food and agricultural, and environmental fields, with a diverse spectrum of applications (Fig. 1). It is now possible to build materials atom by atom and impose desired characteristics for numerous applications in almost every area, such as composite materials development, electronics, nano-electro-mechanical systems (NEMS), biomedical technologies, and renewable energy solutions and environmental remediation (Navya *et. al.*, 2016). According to the Business Communication Company (BCC), the global market of nanomaterials used in the biomedical, pharmaceutical, and cosmetic industries has increased from $170.17 million in 2006 to $684.4 million in 2012 with an expected compound annual growth rate of 27.3% by 2020 (https:// www.bccresearch.com/market-research/nanotechnology).

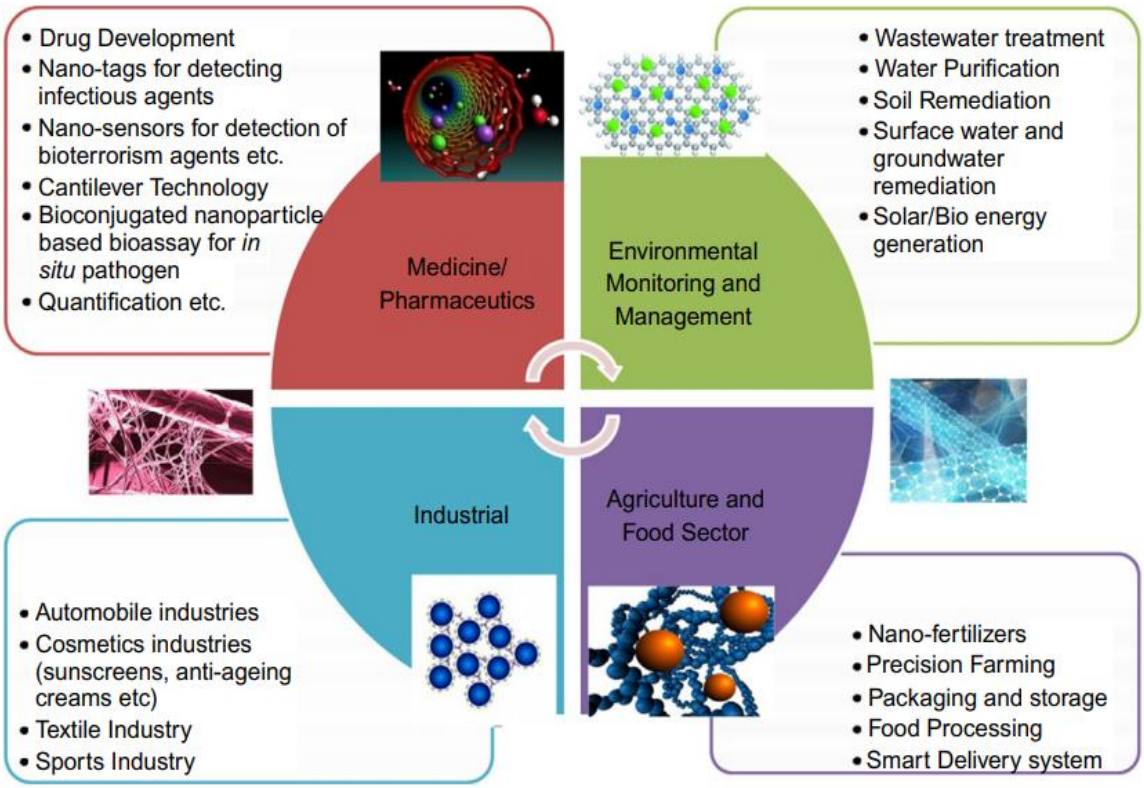


Fig 1. Application of nanotechnology in various sectors.

Nanomaterials (NMs) are fundamental to both nanoscience and nanotechnology. Nanostructure science and technology is a broad and interdisciplinary field of research and development that has exploded in popularity over the last few years all over the world (A. Alagarasi., 2013). NMs are materials with minimum dimension size of less than 100 nanometers (nm). This indicates that they are much smaller than microscale. NMs are usually one billionth of a meter in size, or 10^{-9} m (Kolahalam *et al*., 2019). NMs may be of different shapes like nanorods, nanoparticles (NPs), and nanosheets which can be characterized based on their dimensionality. NMs with zero-dimensional are nanoparticles (NPs), one dimensional is nanorods or nanotubes and two dimensional are generally films and layers type one (Kolahalam *et al*., 2019).

Dimensionality	**Type of Nanomaterials**
Zero dimensional	NPs, quantum dots
One Dimensional	Nanorods, nanotubes, nanowires
Two dimensional	Nanofilms, nanolayers
Three dimensional or Bulk NMs	Core shells, a bundle of nanowires, a bundle of nanotubes

NMs are classified according to their form, size, characteristics, and constituents (Table.1).

- **Carbon-based NMs**

  Carbon is the most important component in this sort of NMs. This category includes carbon nanotubes (CNTs) and fullerenes. Graphene sheets are inserted in the CNTs, which are then rolled into a tube. These are more durable than steel and can be used to improve structural integrity. There are two types of CNTs: single-walled and multi-walled (D. Vollath *et.al.*, 2008)

- **Metal- based NMs**

  Divalent and trivalent metal ions are the beginning ingredients for metal NMs. Metal NPs can be generated in a variety of ways, including chemical and photochemical processes. Metal ions are converted to metal NPs using reducing agents. These have a large surface area and are good in adsorbing small compounds. They're commonly employed in a variety of scientific fields, including environmental and bioimaging studies (Aydın *et. al.*, 2019)

  - **Semiconductor NMs**

  Metallic and non-metallic characteristics exist in semiconductor NPs. By changing it, they show different properties with broadband gaps. Photocatalysis and electronic gadgets both use them. For example, group II-VI semiconductor materials include zinc sulphide(ZnS), zinc oxide(ZnO),cadmium sulphide (CdS), cadmium selenide(CdSe), and cadmium telluride (CdTe) (Kolahalam *et al*., 2019).

  - **Nanocomposites**

  A nanocomposite is a polyphase solid substance with one, two, or three dimensions of less than 100 nm. Unlike traditional composites, nanocomposites have a high surface-to-volume ratio. The physicochemical qualities may differ depending on the size and shape (Ghosh *et.al.*, 2013).

Table.1: Types of NMs

Types of NMs	**Size (in nm)**	**Properties**	**Applications**	**References**
Carbon-based NPs	0.5-3 diameter & 20-1000 length	Unique electrical properties, higher elasticity, strong tensile strength	As carriers of gene delivery, semiconductors, sensors, peptide delivery	Fawole *et. al.*, 2016

Organic/polymeric NPs (starch, cellulose, dendrimers, micelles, liposomes, ferritin, etc.)	50-100	Natural, biodegradable and biocompatible, mechanical flexibility, less toxic	Drug delivery, therapeutic applications, used in water treatment	Lukowiak *et. al*., 2017
Inorganic/ metallic NPs (gold, silver, copper, aluminum, zinc, etc.)	<100	Very small in size & have large surface area, antimicrobial property, stable	Drug delivery, antimicrobial agents, bioremediation, water treatment	Geetha *et. al*., 2016
Quantum dots	2-9.5	Unique electrical & optical properties, semiconductors, bright fluorescence, high photo stability	Photodetector devices, immunoassay, labeling of cancer cells, DNA hybridization	Reshma *et. al*., 2019
Lipid- based NMs	10-100	Spherical, possess solid core made up of lipid	Drug delivery, RNA release in cancer therapy	Gujrati *et. al.*, 2014
Ceramics NPs	<100	Amorphous, polycrystalline, dense, porous or hollow forms	Catalysis, photo catalysis, photo degradation of dyes and imaging applications	Thomas *et. al.*, 2015
Metal-oxides based NPs	<100	They have modified properties and increased reactivity as compared to metal nanoparticles	Antimicrobial agents, anti-oxidative agents, disease diagnosis and therapy	Laad *et. al*., 2016
Fullerenes (C60)	8.2	It is a carbon molecule	In electronic devices, cancer	Ealias *et. al.*, 2017

In recent decades, nanoemulsion, an innovative and environment-friendly element of nanotechnology, has been extensively investigated in drug delivery systems, food, cosmetics, and pharmaceutical industries (Kadakia *et. al.,* 2019). Nanoemulsions are submicron emulsions that are being studied as medication carriers to improve therapeutic agent delivery. They are, by far, the most advanced nanoparticle systems for the systemic delivery of biologically active substances for targeted medication delivery. Nanoemulsions are a thermodynamically stable isotropic system in which two immiscible liquids (water and oil) are combined to produce a single-phase using appropriate surfactants or a droplet diameter of 0.5-100 µm. Droplet sizes in nanoemulsions typically vary from 20 to 200 nm, with narrow size distributions (Shah et. al., 2010). The size and form of particles dispersed in the continuous phase are the fundamental differences between emulsion and nanoemulsion. Their diameter ranges between 10 and 1,000 nm. These carriers are solid spheres with amorphous, lipophilic, and negatively charged surfaces. Site specificity can be improved using magnetic NPs. They improve the medicine's therapeutic efficacy while reducing side effects and hazardous reactions as a drug delivery mechanism. Treatment of reticuloendothelial system (RES) infections, liver enzyme replacement therapy, cancer treatment, and vaccination are only a few of the major applications (Jaiswal *et.al.,* 2015).

The frequency of drug-resistant (DR) bacterial strains has quadrupled globally in recent years, prompting the World Health Organization (WHO) to coin the phrase "Combat drug resistance: no action now, no cure tomorrow" to define the situation (Basak *et al*. 2016). Antimicrobial resistance is a huge danger to the treatment of bacterial illnesses since it leads to higher morbidity and death, as well as significant financial losses for patients and countries. The usage of broad-spectrum antibiotics, which increase the rate of DR bacterial infections and so create a negative loop, has been connected to the prevalence of certain DR bacteria. Furthermore, certain Gram-positive and Gram-negative bacterial strains have developed resistance to entire classes of antibiotics, not only frequently available medicines. Methicillin-resistant *Staphylococcus aureus* (MRSA) and extended-spectrum beta-lactamase (ESBL) producers, in particular, are resistant to tetracycline, aminoglycosides, macrolides, and chloramphenicol, in addition to methicillin and cephalosporin (Nikaido *et. al.,* 2009). The development of novel antibacterial agents to combat DR microorganisms is difficult but crucial and essential. Many scientists have attempted to create nanoemulsions with multiple potential targets, complex mechanisms, and desirable biological and physicochemical features. Charged lipids with high binding effectiveness and the capacity for electrostatic attraction are found in the bacterial cell

membrane (Matsuzaki *et. al.*,2010). Antibacterial nanodroplets are made up of nanoemulsions with charged lipids in their outer shell layers and a core component containing health-promoting bioactive chemicals. Nanoemulsions are colloids of nanoscale droplets with stable physicochemical properties over long periods of time (Balasubramani *et. al.*, 2017). Herbal plant oil nanoemulsions contain a wide range of biocidal properties against microorganisms. They disrupt bacteria's outer membranes and are a new class of antimicrobials for the treatment of Gram-positive and Gram-negative bacterial infections (Hamouda *et. al., 2000).*

REVIEW OF LITERATURE

Nanoemulsions are being studied extensively as drug carriers for enhancing drug delivery in general, and specifically for chemotherapeutics. Nanoemulsions are oil-in-water dispersions containing nanoscale phase droplets stabilized by a surface-active layer (McClements *et. al*., 2010). Oils, surfactants, cosurfactants, weighing agents, ripening inhibitors, thickeners, and gelling agents are some of the natural and synthetic components that can be used to make nanoemulsions (Wani *et. al*., 2018). The nanosize of the droplet prevents creaming or sedimentation from occurring on storage and droplet coalescence. Nanoemulsions provide a much larger oil-in-water contact area due to the nanosized droplet compared to classical emulsions, which facilitates drug release from the dispersed droplets. They are the most advanced nanoparticle systems for the systemic delivery of biologically active agents for controlled drug delivery and targeting. Nanoemulsion has shown great promise for the future of cosmetics, diagnostics, drug therapy, and the area of biotechnology (Sarke., 2005).

"In an emulsion, liquid droplets and/or liquid crystals are scattered in a liquid," according to the International Union of Pure and Applied Chemistry (IUPAC). Nano-emulsions are nanoscale particles created to improve the delivery of active medicinal components (Gutiérrez *et. al*., 2008). Nanoemulsion colloidal particulate system is defined as a thermodynamically and kinetically stable isotropic dispersion made up of two immiscible liquids, such as water and oil, that are stabilized by an interfacial film made up of surfactant and co-surfactant to form a single phase. High-pressure homogenization, micro fluidization, phase inversion, and spontaneous emulsification are some of the techniques used in the formulation of nanoemulsions (Gurpreet *et. al*., 2018).

Types of nanoemulsion

Emulsions are made up of two phases: a hydrophilic phase and a hydrophobic phase, each of which is spread through the other (Fig. 2). These emulsions are called oil-in-water (O/W) emulsions when small oil droplets are scattered through water, or water-in-oil (W/O) emulsions when water droplets are dispersed through oil. However, by encasing an emulsion within an emulsion, known as a double emulsion, extra complexity can be introduced to these simple systems, resulting in water-in-oil-in-water (W/O/W) or oil-in-water-in-oil (O/W/O) emulsions (Wilson *et. al*., 2021). This ‘nano-emulsion also refers to a mini emulsion which is fine oil/water or water/oil dispersion stabilized by an interfacial film of surfactant molecule having a droplet size range of 20–600 nm (Gurpreet *et. al*., 2018).

There are three types of nano-emulsion depending on the composition there are:

• Oil in water nano-emulsions (o/w) - oil droplets are dispersed in the continuous aqueous phase.

• Water in oil nano-emulsions (w/o) - water droplets are dispersed in the continuous oil phase.

• Bi-continuous nano-emulsions - microdomains of oil and water are inter-dispersed within the system.

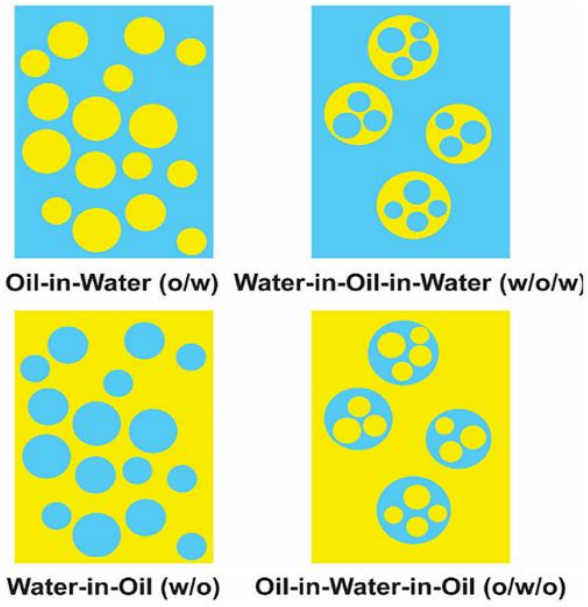


Fig. 2. Emulsion types are classified by their dispersed and continuous phases.

Properties of Nanoemulsion

Nanoemulsions exhibit unique features, such as small droplet size, high stability, transparency, and tunable rheology (Gupta *et. al*., 2016)**.** The physicochemical qualities of nanoemulsions, as well as the precise combination of their components, determine their production and stability (Barradas *et. al.*, 2021).

- Different from other emulsions that are thermodynamically unstable, naturally tending to physical separation, in nano-emulsions, the conditions of physical stability induced by the Brownian motion effects dominate over gravitational forces. In addition, the strength of attractive forces acting between droplets usually decreases with decreasing droplet diameters, reducing aggregation phenomena in nano-emulsions (McClements *et. al*., 2011).

- Nano-droplets have a huge surface area, which boosts their capacity to promote medication solubility and absorption in the intestinal epithelium, cornea, and skin (Singh *et. al*., 2017). Furthermore, even with significant amounts of oil in their composition, they have fascinating aesthetic non-greasy features, limiting organoleptic modification in the final formulation (Lohith Kumar DH *et. al.*, 2018).
- As droplet size decreases, also the biological activity of the lipophilic compounds encapsulated in nano-emulsions increases, because of the enhanced transport of active molecules through biological membranes, as well as the increased surface area/volume ratio, leading to improved reactivity (Salvia-Trujillo *et. al.*, 2015).
- Nanoemulsions, for instance, exhibit optical transparency for mean droplet sizes 40 nm, regardless of the oil fraction, but nanoemulsions appear hazy between 40 and 100 nm, with a strong dependency on the oil content, and appear white for sizes >100 due to consider multiple scattering (Mason *et. al.*, 2006).

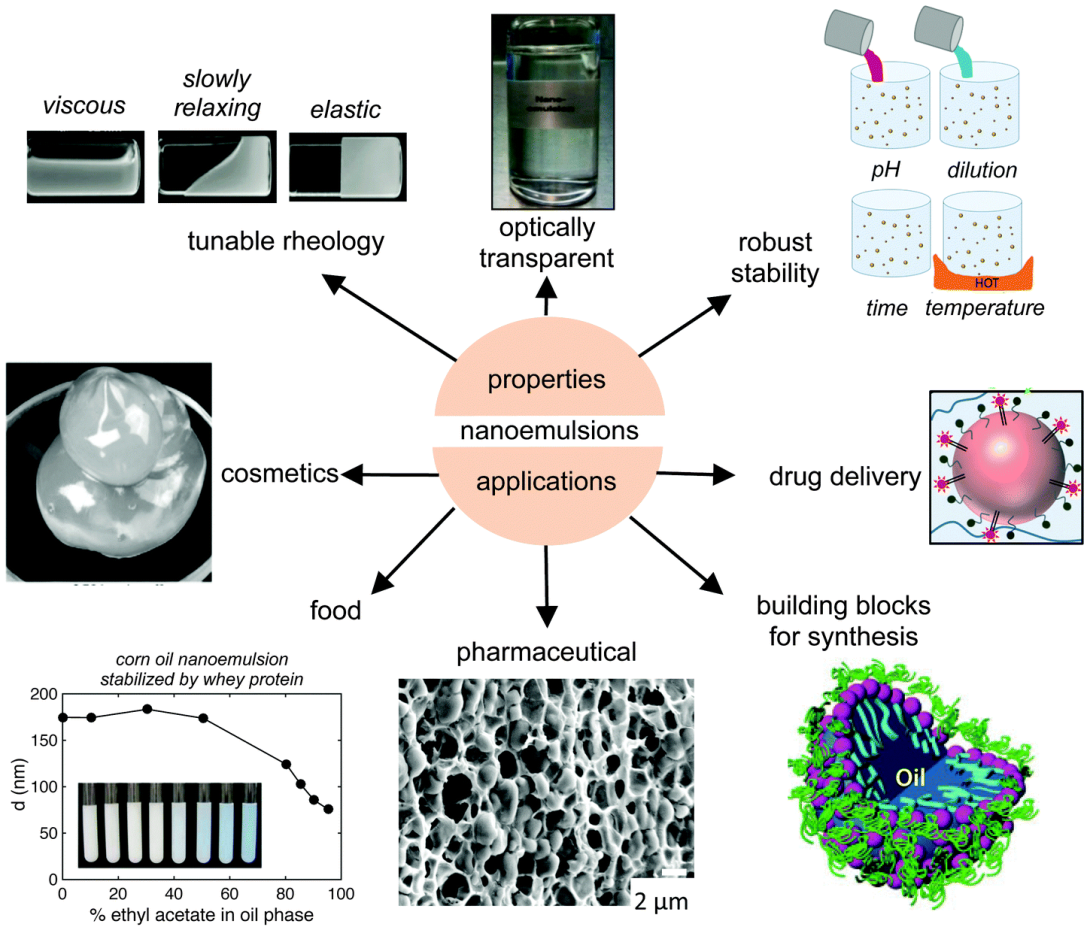


Fig-3 Schematic of various properties and applications of nanoemulsions (Gupta *et al*., 2016).

Components of Nanoemulsion

Nanoemulsion formulations typically consist of a dispersed phase and a dispersing phase, both of which are stabilized by a surfactant (Azeem *et. al.*, 2009).

Nano-emulsions contain three main components:

1. Oil or lipid phase
2. Emulsifying agent/surfactant
3. Aqueous phase/ water

Classification of surfactant

The surfactant should be able to microemulsify the oily process while also being able to solubilize hydrophobic chemical components. The choice of surfactant is critical when creating nanoemulsions. Surfactants with an HLB value less than 10 are hydrophobic (such as sorbitan monoesters) and form w/o nanoemulsions, whereas surfactants with an HLB value more than 10 are hydrophilic (such as polysorbate 80) and form o/w nanoemulsions (Jadhav *et al*., 2020). According to the Young–Laplace Theory, nanoemulsion requires a minimum energy input capable of producing droplet deformation (Tadros *et. al.*, 2004). To lower surface tension and stabilize the huge interfacial surface, substantial volumes of surfactants are necessary. As a result, the generation of nanoemulsions is a non-spontaneous process that necessitates the use of energy. Because the combination of high interfacial tension and small droplet size results in high Laplace pressures, minimizing the oil-water interfacial area results in a spherical shape (Mc Clements *et. al.*, 2011).

Types of surfactants

Surfactants such as phospholipids, proteins, and polysaccharides can act as ionic or non-ionic small-molecule synthetic and natural surfactants**.**

Three types of surfactants are used in the preparation of nanoemulsion to stabilize the system.

- N**on-ionic surfactants:** Fatty alcohols, Glycerol esters, Fatty acid esters, etc.
- **Anionic surfactant:** Carboxylate groups, Soaps, Sulfonates, Divalent ions, etc.
- **Cationic surfactants**: Amines and quaternary ammonium compounds such as cetyl trimethyl ammonium bromide.

Small compounds like sorbitan esters, like the Span and Tween series, and polymeric surfactants like polyoxyethylene ethers, like Brij®, and poly (ethylene oxide)-poly (propylene

oxide) copolymers are examples of non-ionic surfactants. Because of the fast adsorption on the surface of droplets, they can create small droplets (Jafari., 2017). Natural surfactants have high surface activity due to their small molecular size. Biodegradability and biocompatibility are important features of these surfactants (A. Salem and S. Ezzat., 2018).

Table 2. Natural and synthetic surfactants and their general aspects.

Type	Surfactant	Characteristic/Charge	General aspects	References
Natural	Phospholipids (ex: lecithin)	Zwitterionic	The mixture of natural polar phospholipids obtained from eggs or soybeans, amphoteric behavior.	Hoeller *et. al.* (2009), Klang and Valenta (2011), Khan and Krishnaraj (2014)
Natural	Polysaccharides (ex: gum arabic)	Negatively or positively charged	Provide good stability either by electrostatic or steric effects. Not suitable for low-energy emulsification methods.	Dickinson *et. al.* (1991), Bai *et. al.* (2016), Jafari *et. al.* (2017)
Natural	Amphiphilic proteins (casein-ate or whey protein isolate)	Charge depends on pH relative to isoelectric point	Provide good stability mainly by electrostatic ate or whey protein isolate) to isoelectric point repulsion and also steric effects under neutral pH. Not suitable for low-energy emulsification methods.	Dickinson *et. al.* (1998), McClements (2011), Kuhn and Cunha (2012), Sharma *et. al.* (2017)
Ionic surfactant	Sodium lauryl sulfate (SLS) or sodium drodecyl sulfate (SDS)	Negatively charged	Used in both low and high-energy methods	Rosety *et. al.* (2001), Bondi *et. al.* (2015), Lémery *et. al.* (2015), Tian *et. al.* (2016)
Non-Ionic surfactant	Sucrose monopalmitate Sorbitan monooleate	Sugar esters	Non-toxic, biodegradable and hydrophilic.	Strickley (2004), Rao and McClements (2011), Cerqueira-

				Coutinho *et. al.* (2015)
Non-Ionic surfactant	Brij	Polyoxyethylene alkyl esters	Relatively low toxicity used in low-energy methods	McClements (2011)
Non-Ionic surfactant	Tweens and Spans	Ethoxylated Sorbian esters	Used in high-energy methods. Spans aliphatic chain length varies, providing several types of Tween and Span. Ex: Tween 20, 40, and 60	Speranza *et. al.* 2013), Dias *et. al.* (2014), Salvia Trujillo *et. al.* (2015), PATHANIA *et. al.* (2018)

Preparation of nanoemulsion

For the preparation of nanoemulsions, there are two broad groups of techniques: (Fryd et al., 2012)

- High energy methods
- Low energy methods

1. **High energy methods:**

The process of obtaining nanoemulsions using high-energy technologies can be separated into two steps. In the first step, a homogenizer such as Turrax® or Politron® emulsifies the oil and aqueous phases, resulting in a coarse emulsion with droplet sizes of 500–1000 nm, depending on the equipment and operating conditions (Rao *et. al.,* 2011). The droplet diameter is then decreased to a minimum using high-pressure homogenizers, ultrasonicators, or microfluidizers, depending on the homogenizer's energy intensity, processing time, and sample composition (Salem MA *et. al.,* 2018).

- **High pressure homogenization**

  This approach, which employs numerous factors such as hydraulic shear, severe turbulence, and cavitation, is frequently utilized for the generation of nanoemulsions. To make nanoemulsions, two liquids containing surfactants and cosurfactants are pushed through a small orifice of a piston homogenizer at high pressure (500-5000 psi) (Setya S *et. al.*, 2014). To make nanoemulsions with extremely small particle sizes, this technology uses a high-pressure homogenizer/piston homogenizer (up to 1 nm). The size of the droplet is

determined by the number of homogenization cycles performed. Droplet size decreases as the number of homogenization cycles increases. The droplet size of the nanoemulsion produced by this approach diminishes as the ratio of dispersed and continuous phase viscosities is reduced to a specific level (0,05 <<5cDnn), where nD is the dispersed phase viscosity and nc is the continuous phase viscosity (Thakur *et. al.,* 2012).

- **Microfludization**

To make fine nanoemulsions, this mixing process uses a high-pressure displacement pump (3,45-137,89 MPa). Liquids (oil and water) from two opposing microchannels collide at a similar impingement location, resulting in severe shear and high pressure (Setya S *et. al.,* 2014). As the pressure of homogenization increases, or by increasing the number of passages through microchannel devices, increasing the concentration of surfactant, and decreasing the ratio of dispersed and continuous phase viscosities, the droplet size of the dispersed phase of nanoemulsions produced in microfluidizers decreases (Kentish S *et. al.,* 2004).

- **Sonication**

To make kinetically stable nanoemulsions, sonication or ultrasonic homogenization can be used. When the sonicator probe (of an ultrasonic homogenizer) comes into contact with a dispersion of liquids containing surfactants and cosurfactants, mechanical vibration and cavitation are generated, providing the energy input required for the creation of tiny droplets. In the small-scale manufacture of nanoemulsions, sonication or ultrasound processing is commonly used. However, shear-induced coalescence must be avoided at all costs (Delmas T *et. al.,* 2011). The particle size of the dispersed phase in sonicated nanoemulsions decreases as the duration of ultrasonic homogenization, power levels, and surfactant content increase (Kentish S et al., 2008). To produce a dispersed phase droplet size of 20 nm, it is important to optimize ultrasonic reaction chamber design, operating parameters, and product formulation (for example, surfactant concentration and type and content of the oil phase (Kumar *et. al.,* 2012).

- **High-amplitude ultrasonication method**

Ultrasonic cavitation causes an asymmetric and abrupt implosion of vacuum bubbles. Droplets are dispersed and broken up to nanoscale size using micro-nozzles. This technology has been effectively used in the manufacturing of medicinal nanoemulsions

and liposomes in modest quantities. Conventional ultrasonic technology processes work on the small quantity - high amplitude or high levels - low amplitude principles, with no way to combine processes like large amount - high amplitude. Despite its potential, the ultrasonic approach is only useful in the laboratory (Sowjanya et al., 2012)**.**

2. Low energy method:

In low energy methods, the smaller droplets are formed when the system undergoes a phase inversion in response to changes in composition or temperature, and passes through a state of low interfacial tension. These methods require significantly less input energy density ($\varepsilon \sim 10^3$-10^5 WKg^{-1}) as energy input can be achieved readily in a simple batch stirrer (Forgiarini et. al., 2001). To make nanoemulsions, low-energy approaches rely on internal physical features of the system, such as temperature or composition (Setya *et. al.,* 2014).

- **Phase inversion method**

The condensation method, also known as phase inversion, is based on the transition of phases during the emulsification process. These phase transitions are caused by changes in the surfactant's spontaneous curvature, which can be achieved in two ways: a) at constant composition, by changing the spontaneous curvature of nonionic surfactants with temperature (the well-known phase inversion temperature (PIT) method widely used in industry), or b) at constant temperature, by varying the system's composition using the emulsion inversion point (EIP) method. These approaches have the following drawbacks: intricacy, precise requirements, and the usage of a synthetic surfactant (Izquierdo et al., 2005). The phase inversion temperature method uses a mixture of oil, water, and nonionic surfactants that exhibit a positive curvature at room temperature. As the temperature rises, the polyethoxylated surfactant becomes lipophilic (due to dehydration) and is solubilized in the oily phase. The phase inversion occurs, and the O/W emulsion becomes a W/O emulsion with a negative curvature. It's worth noting that as the curvature approaches zero at intermediate temperatures, so-called HLB temperatures (when the surfactant's hydrophilic and lipophilic properties are balanced), highly unstable emulsions emerge. A rapid temperature change (25-30 o C rise or decrease in HLB temperature) avoids coalescence and produces stable nanoemulsions (Kumar *et. al.*,2014).

- **Spontaneous emulsification**

For the synthesis of nanoemulsions, spontaneous emulsification is a low-energy process. This approach, which does not require any extra equipment, may create nanoemulsions at ambient temperature. To make O/W nanoemulsions, water is gradually added to an oil and surfactant solution at a steady temperature while gently stirring. The interfacial tension, interfacial and bulk viscosity, phase transition region, surfactant structure, and concentration all have a role in the spontaneity of the emulsification process. (Bouchemal *et. al.*, 2004).

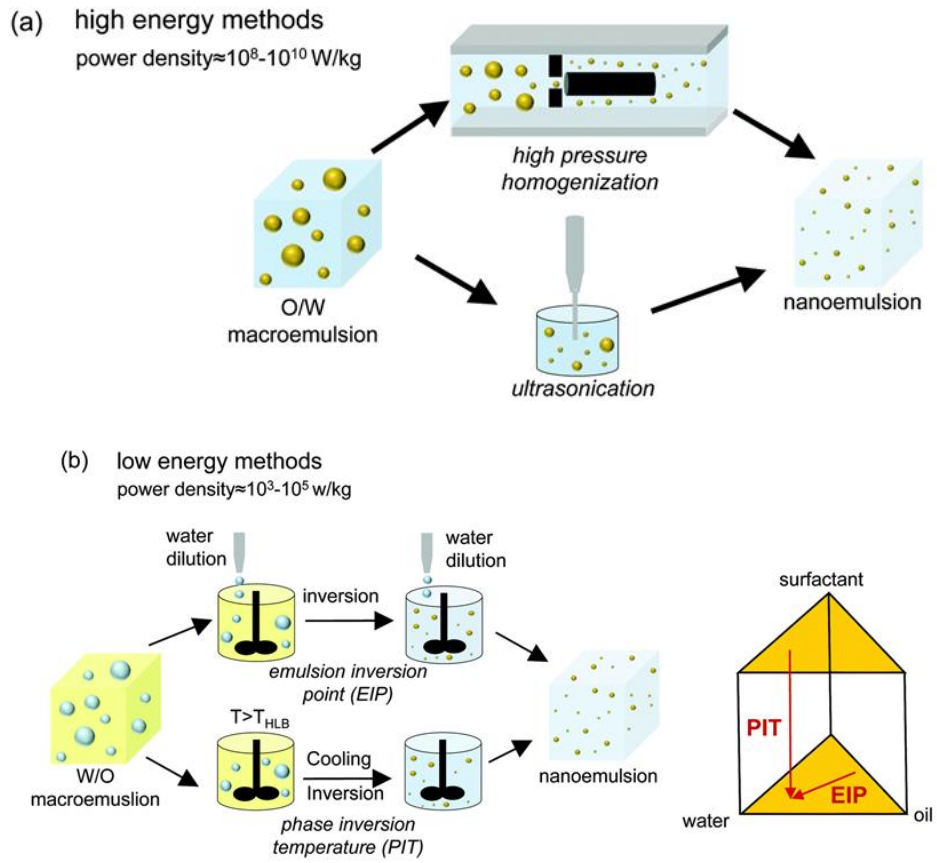


Fig.4: Schematic of various properties and applications of nanoemulsions. (a) High energy such as high-pressure homogenization (HPH) and ultrasonication break macroemulsion drops into smaller droplets. (b) Low energy methods start with W/O macroemulsions and break coarse emulsions into smaller droplets as they pass through a state of low interfacial tension during phase inversion. The emulsion inversion point (EIP) technique induces a phase inversion by water dilution whereas the Phase Inversion Temperature (PIT) approach induces a phase inversion on the cooling of the mixture. To prepare W/O nanoemulsions, one can simply reverse the continuous and dispersed phases (Piorkowski *et. al.*, 2014)

Table. 3 Synthesis of nanoemulsion by different techniques.

Technique	Formulation	Conclusions	Reference
High-pressure homogenization	Oral lipid nanoemulsion (primaquine)	Enhanced oral bioavailability, 10-200 nm particle size	Singh *et. al.* (2008)
Pseudoternary phase diagram+spontaneous emulsification method	Ramipril nanoemulsion	Increased bioavailability, droplet size 80.9 nm	Shafiq S *et. al.* (2007)
High-pressure homogenization	O/W nanoemulsions	Improved skin hydration and elasticity	Yilmaz E *et. al.* (2006)
Spontaneous emulsification	O/W nanoemulsion (aceclofenac)	Nanoemulsion with potential for transdermal delivery of aceclofenac	Shakeel F *et. al.* (2007)
Spontaneous emulsification	Celecoxib nanoemulsion	Enhanced physical and chemical stability of celecoxib in nanoemulsion	Shakeel F *et. al.* (2008)
High-pressure homogenization	Lecithin-based nanoemulsions (progesterone)	Improved permeation rates of progesterone with long-term stability	Klang V *et. al.* (2010)
High-pressure homogenization	Prednicarbate nanoemulsion	Increased chemical stability of the drug in formulation	Baspinar Y *et. al.* (2010)
Phase-inversion temperature method	Acyclovir-loaded multiple W/O/W nanoemulsions	Excellent physicochemical stability for 6 mo at RT, mean droplet size of 100 nm	Schwarz JC *et. al.* (2012)
Spontaneous nanoemulsification method	Clotrimazole nanoemulsion	Improved solubility of clotrimazole, mean globule size <25 nm	Borhade V *et. al.* (2012)
Ultrasonic emulsification method	Basil oil nanoemulsion	Nanoemulsions with droplet size of 29.6 nm, for food preservation	Ghosh V *et. al.* (2013)
Phase-inversion composition method	Efavirenz nanoemulsion	Enhanced bioavailability, globule size < 30nm	Kotta S *et. al.* (.2014)
High-pressure homogenizer	Dimethyl silicone dry nanoemulsion inhalation	Effective in acute lung injury, particle size of 19.8 nm	Zhu L *et. al.* (2015)
High-pressure homogenizer	Parenteral lecithin-based nanoemulsions (risperidone)	Enhanced brain availability of risperidone with a mean particle size of 160 nm	Baboota S *et. al.* (2008)
Microfluidization method	Pitavastatin-containing nanoemulsions	Enhanced permeation	Başpınar Y *et. al.* (2015)
High-pressure homogenization+ultrasound	Nanoemulsion	Reduced energy demand for emulsification, low particle	Calligaris *et. al.* (2018)

		dimensions and higher stability	
Sonication method	Saponin-stabilized quercetin-loaded o/w nanoemulsion	Stable for 45 d at RT, mean particle size of 52±10 nm	Kaur K *et. al*. (2016)
High-pressure homogenization	Paclitaxel-baicalein nanoemulsion	Strategy to overcome multidrug resistance	Meng L *et. al*. (2016)
Nanoemulsion templating	PLGA nanoparticles	Imaging agents for biomedical purposes	Fornaguera C *et. al*. (2016)
Spontaneous emulsification method	Chitosan films with cinnamaldehyde nanoemulsions	Good UV barrier properties	Chen H *et. al*. (2016)

Characterization of nanoemulsion

Nanoemulsions can be characterized by physical and chemical testing which includes analysis of material uniformity, pH, composition, viscosity, density, conductivity, zeta potential, size, surface tension, etc. (Mandal *et. al.,* 2012). Several techniques can be used for the complete characterization of nanoemulsion systems.

Dynamic light scattering (DLS): This technique has been used traditionally to analyze morphology, droplet size, and size distribution of the particles in the nanoemulsion. DLS can also be used to determine the shelf life of nanoemulsion.

Differential Scanning Calorimetry (DSC): Interaction between different components of nanoemulsion can be studied by using Differential Scanning Calorimetry (DSC).

Polarization microscopy: It is used with crossed polarizers to determine the isotropic of the formulation (Chiesa M et al., 2008).

Diluted atomic force microscopy (AFM): It can provide valuable details about the size and morphology of emulsion nanodroplets (Dario *et. al.,* 2016; Galvão, Vicente, and Sobral, 2018).

Viscometer: Viscosity can be measured by using a viscometer.

Electron praramagnetic resonance (EPR): EPR is a spectroscopic method for determining microwave absorption by paramagnetic centres with one or more unpaired electrons. Understanding molecular structure close to entities with unpaired electrons requires the use of EPR spectroscopy (Demisli *et. al.,* 2020)**.**

Nuclear magnetic resonance (NMR): The study of compounds in either the liquid or solid state is possible with the help of the potent and sophisticated analytical technique known as NMR, which is equally useful for quantitative and structural investigation. It is effective at obtaining structural details about molecular molecules. It can be employed as an additional technique to mass spectrometry and optical spectroscopy, providing accurate information on the structural formula, stereochemistry, the favoured 25 nm, and repeating distances in partially ordered systems of up to 150 nm. The size, shape, and structure of macromolecules can be determined from the scattering patterns (Silva *et. al.,* 2012).

Table.4: Comparison of thermodynamic stability and physicochemical properties of emulsion, nanoemulsion, and microemulsion (McClements & Rao, 2011)

S.No.	System	Droplet Radius	Stability	Surface-to-Mass Ratio (m^2/g particles)
1.	Emulsion	100 nm–100 µm	Unstable	0.07-70
2.	Nanoemulsion	10-100nm	Unstable	70-330
3.	Microemulsion	2-100nm	Stable	330-1300

Antimicrobial activity of essential oils

Antibiotic overuse has contributed to the emergence and spread of antibiotic-resistant microorganisms. In addition, when bacteria build biofilms, their resistance to antimicrobial treatments rises. A biofilm is a system composed of surface-attached bacteria exposed by their extracellular polymer matrix, which is the most common bacterial lifestyle (Hamayel *et. al.,* 2019). Furthermore, resistant bacteria are linked to two key pathogenicity factors: adhesion and biofilm development (Kuhn *et. al*., 2002). Biofilm formation on the surface of tissues, organs, or medical devices is linked to approximately 65 % of clinical infections (Kojic *et al*., 2004).

Plants may create a wide range of chemicals naturally, particularly secondary metabolites, which have been shown to defend plants from infections due to their biological features (Hancock *et al.,* 2015) More than 3000 essential oils (EO), which are complex mixes primarily composed of secondary metabolites, have been found and are known among these compounds. (Bassolé *et al*., 2012). EOs are typically abundant in diverse compounds, containing 20 to 60 active chemicals, and can be distinguished by up to three primary components, which are

present in relatively high concentrations when compared to other compounds present in trace levels (Bakkal *et. al.*, 2008). For example, Linalool (68%) is found in Coriander sativum EO, limonene (54%) and, carvacrol (65%) and thymol (15%) in *Origanum heracleoticum* EO, and menthol (59%) and menthone (19%) in *Mentha* x *Piperita* EO (Amri *et. al*., 2012).

The major components found in EOs are often responsible for their biological properties and can be gathered into two main groups-

- Terpene hydrocarbons constituted of monoterpenes and sesquiterpenes. Monoterpenes represent 80% of the EO's composition (Turek *et. al*., 2012).
- Oxygenated compounds constituted mostly of alcohols, phenols, aldehydes, and esters. The aromatic and oxygenated compounds occur less in EO than in terpenes but are yet frequent (Buckle *et. al*.,2015).

Essential oils possess several properties such as antioxidant, antimicrobial, and anti-inflammatory activities and are hence widely used in traditional medicines, cosmetics, and food industries (Raveau *et. al.,* 2020). Mihajilov-Krstev *et. al.,* (2009) found that *Satureja hortensis* L. oil showed significant antimicrobial activity against a wide spectrum of Gram-negative bacteria (*E. coli* ATCC 8739, *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella enteritidis* ATCC 13076, and *Erwinia amylovora* NCPPB 595) and Gram-positive bacteria (*Bacillus subtilis* ATCC 6633, *Clostridium perfringens* ATCC 19404, *Micrococcus flavus* ATCC 40240, *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 6538 and *Sarcina lutea* ATCC 9341), as well as against fungal strains (*Aspergillus niger* ATCC 16404, *Candida albicans* ATCC 10231 and *Saccharomyces cerevisiae* 112).

Table. 5: Antimicrobial activity of Essential oil against pathogenic bacteria.

S.No.	**Medicinal Aromatic Plants**	**Part used**	**Major chemical compounds**	**Inhibited microorganisms**	**References**
1.	*Achillea clavennae*	Leaves and flowers	camphor, myrcene, 1,8-cineole, β-caryophyllene, linalool, geranyl acetate	*K. pneumonia, S. pneumonia, Haemophilus influenzae, P. aeruginosa*	M. Skocibu *et. al.* (2004)
2.	*Achillea fragrantissima*	Aerial parts	Yomogi alcohol, 1,8-cineole,	*S. aureus, S. epidermidis, E. coli*	G. S. G. Zeedan *et. al.* (2014)

			artemisia alcohol, thujone		
3.	*Coriandrum sativum*	Leaves	2E-Decenal, decanal, 2E-decen-1-ol, n-decanol	*S. aureus, Bacillus spp., E. coli, Salmonella typhi, K. pneumonia, Proteus mirabilis, P. aeruginosa*	J. C. Matasyoh et al. (2009); A. F. Begnami *et. al.* (2010)
4.	*Cuminum cyminum*	Leaves	γ-Terpin-7-al, γ-terpinene, β-pinene, cuminaldehyde	*S. typhimurium, E. coli*	D. S. Bisht *et. al.* (2014)
5.	*Juniperus phoenicea*	Arial part	α-Pinene, β-phellandrene, α-terpinyl acetate	*S. aureus, L. monocytogenes, L. monocytogenes, E. faecium, S. Enteritidis, E. coli, P. aeruginosa*	A. Ait-Ouazzou *et. al.* (2012)
6.	*Mentha pulegium*	Arial part	Piperitone, piperitenone, α-terpineol, pulegone	*S. aureus, S. epidermidis, B. cereus, L. monocytogenes, E. coli, S. typhimurium, V. cholera, L. monocytogenes, E. faecium, S. Enteritidis*	A. Bejaoui. (2013)
7.	*Origanum vulgare*	Leaves, Arial part	Carvacrol, thymol, γ-terpinene, trans-sabinene hydrate, cis-piperitol, borneol, terpinen-4-ol, linalool	*Clostridium botulinum, C. perfringens, L. monocytogenes, E. coli, S. choleraesuis, S. typhimurium, S. aureus, B. subtilis, P. aeruginosa, Shigella sonnei, Sarcina lutea, M. flavus, K.*	D. Runyoro *et. al.* (2010); J. C. Matasyoh *et. al.* (2009)

				pneumoniae, K. oxytoca	
8.	*Pogostemon cablin*	Leaves	Patchoulol, δ-guaieno; gurjunene-α, α-guaiene, aromadendrene, β-patchoulene	*K. pneumonia, H. pylori, E. coli, B. subtilis, S. aureus, P. aeruginosa, E. faecalis*	M. K. Swamy and U. R. Sinniah. (2015)
9.	*Rosmarinus officinalis*	Leaves, flower	Camphor, camphene, limonene, geraniol, myrcene, linalool benzoylacetate, linalool, α-pinene, α-terpinolene, bornyl acetate, borneol	*E. coli, S. typhimurium, B. cereus, Bacillus subtilis, S. aureus, S. agalactiae, S. epidermidis, S. aureus, P. vulgaris, P. aeruginosa, K. pneumonia, E. faecalis, B. thermosphacta, L. innocua, L. monocytogenes, P. putida, S. typhimurium, S. putrefaciens, M. smegmatis*	B. Teixeira *et. al.* (2013); C. R. Flores. (2014)

Antimicrobial activity of nanoemulsion

Nanoemulsion has antimicrobial properties and is also effective anti-biofilm agents. Antimicrobial nanoemulsions are emulsified mixture of detergent, oil and water which have been shown to have broad antimicrobial activity against bacteria, enveloped viruses, and fungi at concentrations that are nontoxic in animals (Hamouda *et al*., 2001). Unlike antibiotics, the antimicrobial activity of nanoemulsion is nonspecific, thus allowing broad-spectrum activity while limiting the capacity for the generation of resistance. These features make nanoemulsion a suitable candidate for both wound treatment and surface decontamination (Hemmila *et. al*., 2010). Nanoemulsions have broad biocide efficacy against bacteria, enveloped viruses, and fungi by disruption of their outer membranes (Karthikeyan *et al*., 2011). The nanoemulsion particles are thermodynamically driven to fuse with lipid-containing organisms. When enough nanoparticles fuse with the pathogens, they release part of the energy trapped within the

emulsion. Both the active ingredient and the energy released destabilize the pathogen lipid membrane, resulting in cell lysis and death (Majeed *et al*., 2015; Singh *et al*., 2015). Nanoemulsion is attracted to pathogens by opposite charge and causes ruptures of the lipid membrane, cell lysis, and finally cell death. It can be used in long term as the use of antibiotics has many side effects (Majeed *et al*., 2015). For the antimicrobial activity of nanoemulsions various reports are available, Marei *et. al.,* (2017) had concluded that the conversion of citral oil into nanoemulsion enhanced its antibacterial activity against an important plant pathogenic bacterium *Erwinia carotovora* and fungi *A. niger* and *Rhizobium stolonifera*. Prakash *et. al.,* (2019) has formulated Linalool nanoemulsion using an ultrasonic emulsification method and showed antibacterial and antibiofilm activity against *S. Typhimurium*. Liang *et. al.,* (2012) have synthesized peppermint oil nanoemulsion, showing antimicrobial against gram-positive bacteria *Listeria monocytogenes Scott A* and *S. aureus*. Moghimi *et. al.,* (2016) converted *Thymus daenensis* essential oil into nanoemulsion enhancing ten times its antibacterial activity against an important food-borne pathogenic bacteria *E. coli*. Moraes- Shahavi et. al., (2015) formulated stable nanoemulsion of Clove oil in water with suitable droplets size as nano pesticides were prepared. One advantage of using them is the low risk of developing resistance in long-term usage. Moradi *et. al.,* (2019) nanoemulsion obtained great attention in pharmaceutical, food industry, cosmetics, and drug delivery system they formulated thyme and rosemary oil nanoemulsions that showed antibacterial activity against *E. coli*. Yazgan *et. al.,* (2020) the sage essential oil displayed a strong inhibitory effect against *K. pneumonia*, and *Salmonella paratyphi*.

Table.6 Nanoemulsions and their antimicrobial effect

S.No.	Nanoemulsion	Antimicrobial activity	References
1.	*Eucalyptus globulus* oil nanoemulsion	antibiofilm activities against *Candida albicans, C. tropicalis,* and *C. glabrata*	Quatrin *et. al.,* 2017
2.	oregano and thyme oil nanoemulsion	Oregano exhibited the strongest anti-biofilm effect against *S. epidermidis* and inhibit *P. acnes and* Thymol showed antibacterial activity against both *P. acnes* and *S. epidermidis*	Taleb, M. H., *et. al.,2018*

3.	Eucalyptus oil nanoemulsion	*Proteus mirabilis*	Saranya S. *et. al., 2012*
4.	Soybean oil nanoemulsion	*S. mutans, L. casei*, Actinomyces viscosus	Karthikeyan R. *et. al.*, 2012
5.	BCTP nanoemulsion	*Listeria monocytogenes*	Paula C. Teixeira *et. al., 2007*

Application of nanoemulsion

Nano-emulsions have advantages in many technical applications due to their small droplet size, excellent kinetic stability, and optical transparency when compared to traditional emulsions. The vast majority of publications on nano-emulsion applications are concerned with the synthesis of polymeric nanoparticles employing a monomer as the disperse phase (the so-called mini-emulsion polymerization method) (Pandya *et. al.,* 2015). In contrast to emulsion and microemulsion polymerization, droplet cleation is reported to be the primary mechanism in nano-emulsion polymerization, allowing for the preservation of droplet size and composition during the creation of latex particles (J.M. Asua *et. al.,* 2002). As a result, nano-emulsion droplets can be thought of as little nanoreactors. Nanoemulsions are also used in cosmetics, as a preventative measure in bioterrorism attacks, as mucosal vaccines (under trial), as a non-toxic disinfecting cleanser, and in cell culture technologies (Pandya *et. al.,* 2015).

MATERIALS AND METHODS

Objective- Synthesis and characterization of the herbal nanoemulsion for controlling pathogenic bacteria.

Place of work-

The present study entitled **"Interactive Role of Nanoemulsion against Pathogenic Bacteria"** was conducted at the Division of Plant-Microbe Interaction, National Botanical Research Institute, Lucknow, under the supervision of Dr. Aradhana Mishra, from March to June 2022.

Procurement of materials-

Bacteria isolates: Bacteria isolates that are used in this work were procured from the lab repository, Division of Microbial Technology, NBRI, Lucknow.

O_9NE- O_9NE is the lab's oil coding.

Media and other chemicals-

Media and other chemicals were obtained from Sigma, Himedia, Qualigens, CDH, and MerckChemicals.

Composition of Nutrient Agar

S.No.	components	Amount g/l
1	Peptone	5
2	Beef extract	10
3	Sodium chloride	5
5	Agar agar type 1	15

Composition of Nutrient Broth

S.No	Component	Amount g/l
1.	Yeast extract	2.0
2.	Peptone	5.0
4.	Sodium chloride	5.0

Screening of essential oils

Crude oil extracts from different plants have antimicrobial properties.

9 different essential oils that were taken to be screened for antimicrobial activity were as follows:

Table 8: 9 different oils used in screening

S.No.	Essential oil
O_1	CO
O_2	LO
O_3	EO
O_4	OO
O_5	HO
O_6	CO
O_7	PO
O_8	GO
O_9	YO

- The antimicrobial activity of different essential oils was tested against four different pathogens i.e., Bacteria 1, Bacteria 2, and Bacteria 3.
- The antibacterial activity was assessed by disk diffusion.
- NA was prepared in a flask. After autoclave NA plates were prepared.
- To study the antimicrobial effects of silver nanoparticles on pathogens, 100 µl of overnight grown bacterial cultures were spread uniformly on nutrient agar plates. Pre sterilized cotton of $1cm^2$ was placed on the centre of the plates and pipetted with 150 µl of essential oil and allowed to air dry.
- The plates were incubated at 37˚C for 24 h, after which the zone of inhibition was observed.

Synthesis of nanoemulsion-

- Essential oil extracted from different plants was used to synthesize a stable antimicrobial nanoemulsion. For oil-in-water (O/W) nanoemulsion synthesis, a proper conc. of oil: emulsifier/surfactant: dispersant is required.

- Mix the different volumes of oil and surfactant, then add dispersant slowly to the oil phase with a constant rate of agitation.
- Vortex the sample to mix the contents before sonication.
- After vortexing sonicate the sample for 20 minutes with 10 seconds on/off interval at 30% amplitude.

Fig.5: ultrasonic-homogenizer

Characterization of synthesized nanoemulsion

- **Particle size distribution and zeta potential-**

  To confirm the nanoemulsion synthesis, size and zeta potential of nanoemulsion were confirmed by the Dynamic Light Scattering process. The particle size distribution of the synthesized nanoemulsion was determined by particle size analyzer (Malvern, nanoseries zetasizer). All measurements were made at a fixed scattering angle of 90° and a temperature of 25.0 ± 0.1 °C. The light source of the particle size analyser is a solid-state laser operating at 658 nm with 30 Mw power, and the signals were detected by a high-sensitivity avalanche photodiode detector. To avoid multiple scattering effects, emulsion was first diluted 100 times with deionized water and stirred continuously before the measurements to ensure the sample was homogenous.

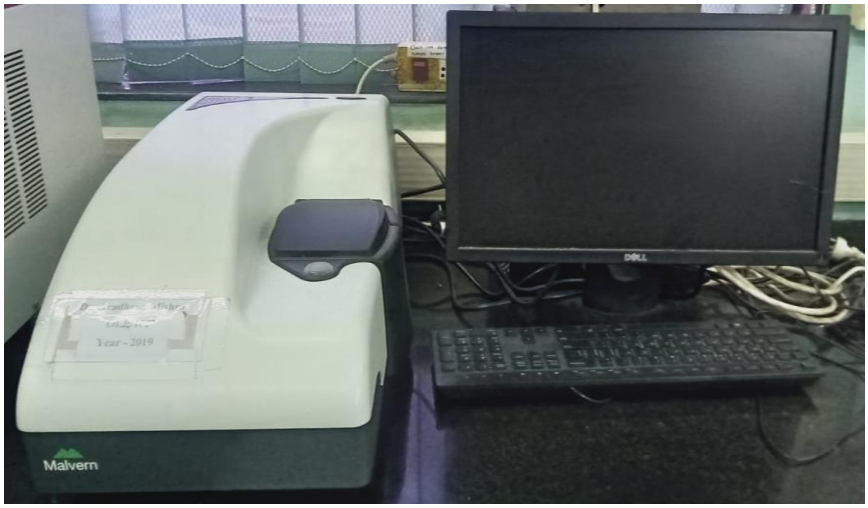


Fig.6: Particle size analyzer

- **Antimicrobial test of Synthesized Nanoemulsion:**

  To examine the antibacterial activity, the pathogenic bacteria i.e., Bacteria 1, Bacteria 2 and Bacteria 3 were procured from laboratory and were cultured under specific environment. The cultures of the bacteria were maintained at 4°C throughout the study and were used as stock cultures.

  Antimicrobial activity of different synthesized nanoemulsion was evaluated against Bacteria 1, Bacteria 2 and bacteria 3 through cotton immobilization assay.

  Diameter of zone of inhibition was measured in cm (kumari *et. al.,* 2017).

- **Determination of Minimum Inhibitory Concentration (MIC) of Nanoemulsion:**

  The MIC of synthesized nanoemulsion of O_9NE5 against pathogenic bacteria 1 was evaluated *in vitro* using micro-broth dilution method.

  The final concentrations of antimicrobial agents in culture media were varied from control to 6%. The samples were inoculated with 1% of a microbial suspension (10^6 CFU/ml) and incubated for 24 h at 37^{o}C. The MIC value was determined as the lowest concentration of the antimicrobial agent that inhibited the growth of the tested microorganism (Severino *et. al.,* 2015).

RESULTS AND DISCUSSION

Screening of essential oils

Antimicrobial potential of different essential oils against all three pathogenic bacteria wasv evaluated as shown in Fig. 7 (a) and (b). Among all 9 tested essential oils, O_9 essential oil showed significant highest antimicrobial activity against all tested bacteria.

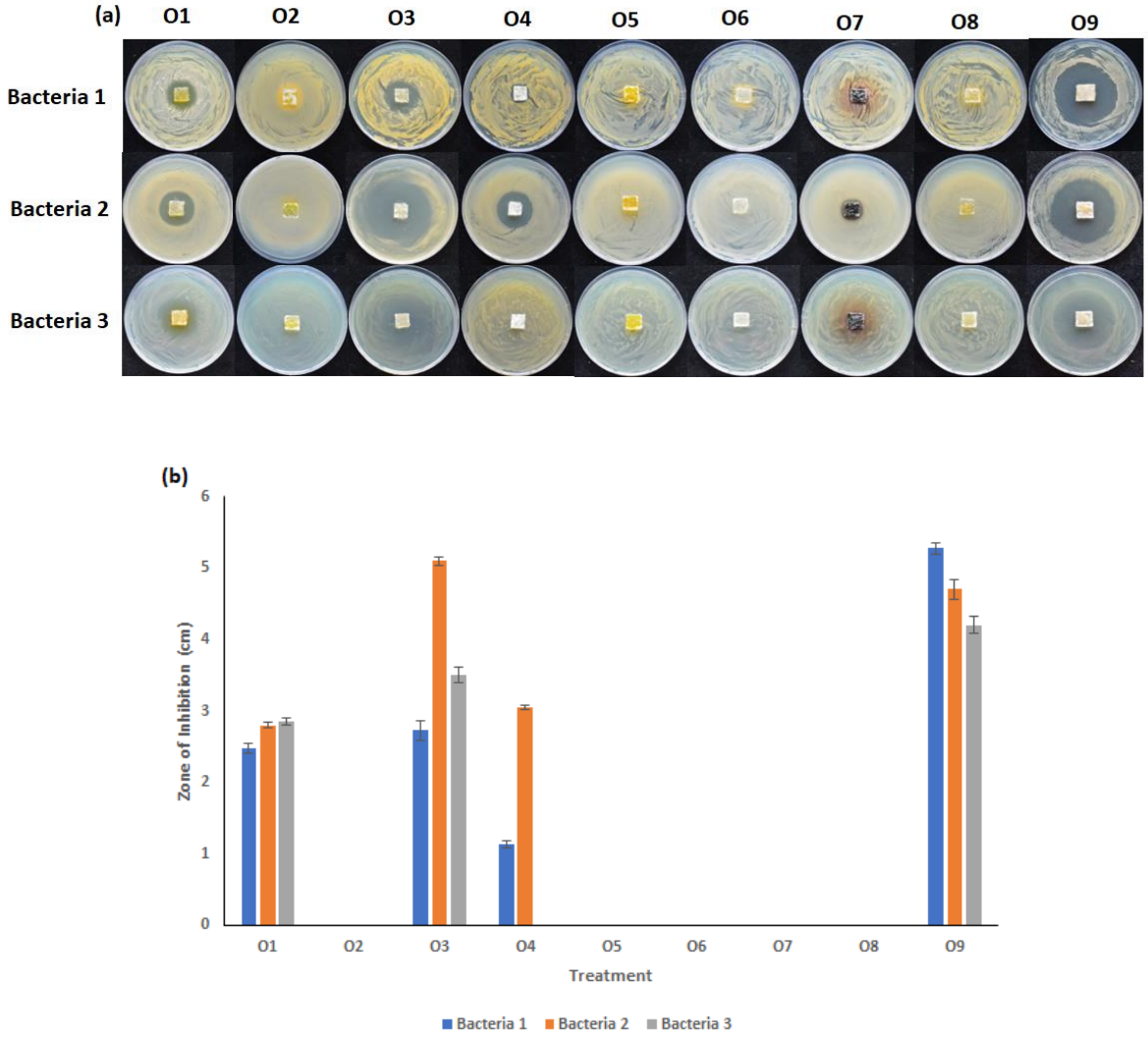


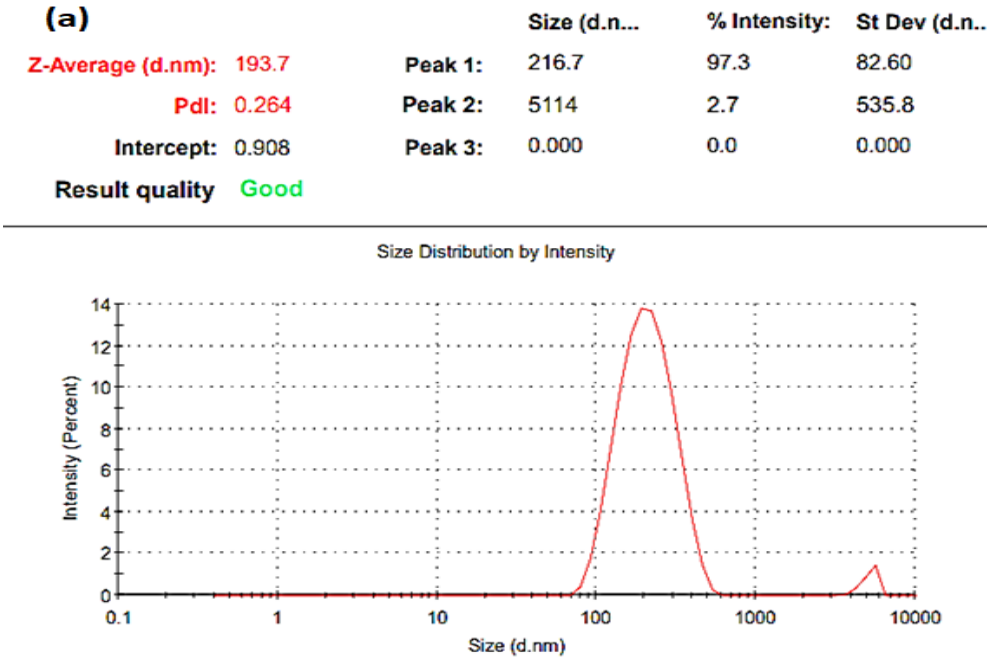
Fig.7 Antimicrobial activity of different Essential Oils against Bacteria 1, Bacteria 2 and Bacteria 3 (a) Screening of different Essential Oils (b) Zone of Inhibition (in cm)

Preparation of nanoemulsion: After the screening test a high energy method was used to prepare nanoemulsion. In our study, the nanoemulsion are prepared with different concentration of O_9 essential oil i.e., NE1, NE2, NE3, NE4, NE5 and NE6 have been synthesized by using high energy sonication method, the basic component of this instrument is a probe that imposes high mechanical energy that disrupts and combine the immiscible phase of oil and water and forms small droplets with increased surface area (Cerqueira, M.A. *et. al.*, 2014). The homogenous mixture was initially prepared by mixing different concentration of

crude oil, surfactant and rest volume was made up by adding dispersant (distilled water). After sonication the synthesised different O_9NE was stable at room temperature.

Characterization of nanoemulsion

- **Particle size distribution and zeta potential**: The particle size distribution of prepared emulsions was determined by particle size analyzer (Malvern, nanoseries zetasizer). Dynamic light scattering is a reliable technique which provides valuable information on the size distribution of nanoemulsions. In our study the size of droplets present in homogenized nanoemulsion (O_9NE5) were 193.7 nm as shown in Fig.8 (a). Moreover, poly dispersity index (PDI) of nanoemulsion was 0.264 (Fig.8 (a)), showing the homogeneity nature of nanoemulsion (Rinaldi et al., 2017). Zeta potential of prepared O_9NE5 was -57.3 as shown in Fig.8 (b). The zeta potential is a method for measuring surface charge of particles when it is placed in liquid and it can be used for predicting the stability of the nanoemulsion. The greater zeta potential values correspond to the greater is the stability of the nanoemulsion (Anarjan *et. al.,* 2012). Zeta potential can be used to evaluate the potential stability of the nanoemulsion system as it provides the electrostatic repulsion between the similarly charged dispersed droplets (Sharif *et. al.*, 2017).



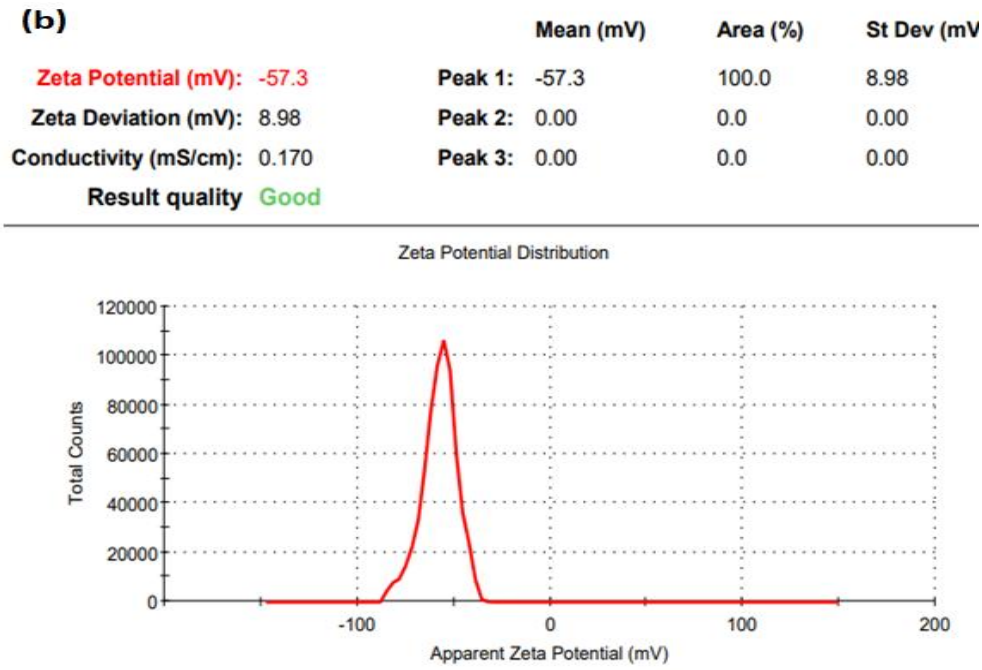
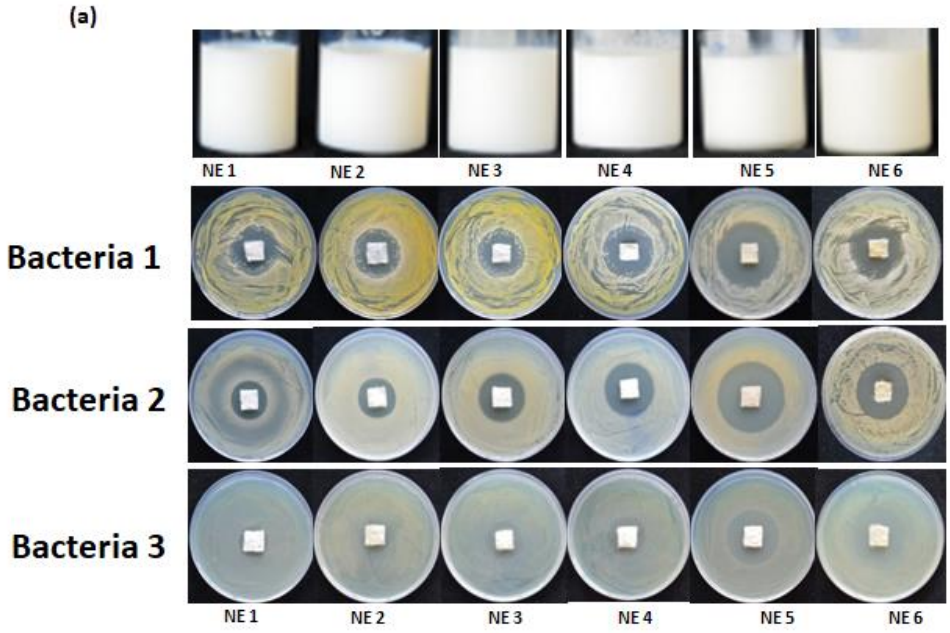


Fig.8: Characterization of O_9 NE5 nanoemulsion (a) Particle size distribution and (b) zeta potential

Antimicrobial activity of Synthesized Nanoemulsion:

O_9 essential showed highest antimicrobial activity against all tested bacteria. So, the O_9 essential oil was used to prepare different nanoemulsions. The antimicrobial activity of different nanoemulsions was evaluated against all three bacteria (Fig. 9 (a) and (b)). O_9 NE5 showed the highest antimicrobial activity against all three tested bacteria.



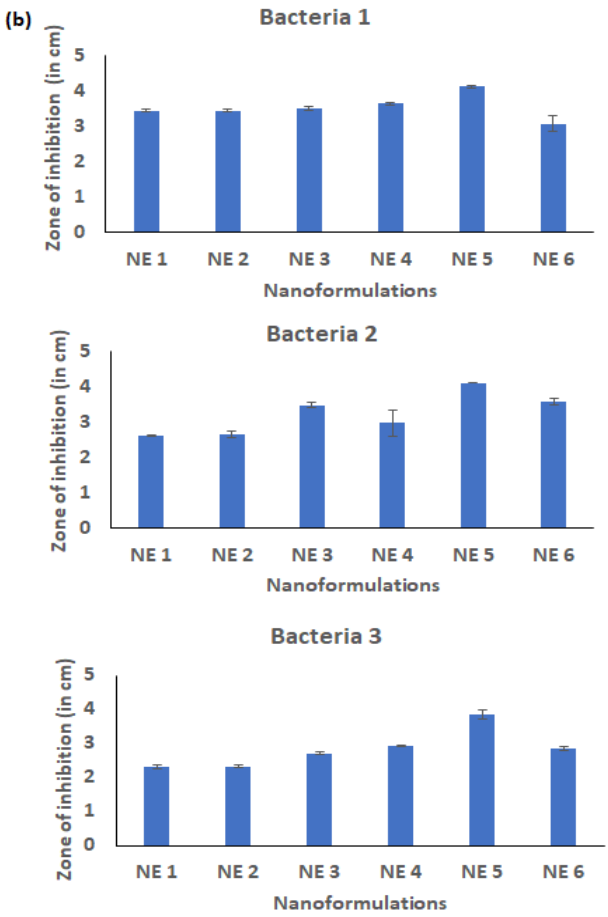


Fig.9: Antibacterial activity of different nanoemulsions synthesized with different concentrations of O_9 essential oil against all three tested bacteria (a) antibacterial activity and (b) diameter of zone of inhibition (in cm)

MIC determination-

MIC values of O_9NE5 nanoemulsion of O_9EOs, measured against bacteria 1. O_9NE5 nanoemulsion showed a MIC value, for bacteria 1, was 1% as shown in Fig.10.

Based on the obtained data, O_9NE5 was considered the most effective of the antimicrobial agents against all three tested bacteria.

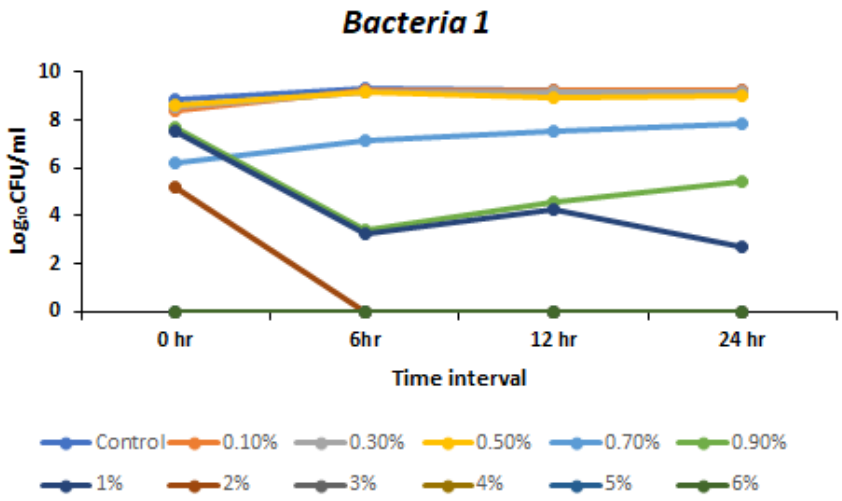


Fig.10: MIC of O_9NE5 against Bacteria 1 at different concentration and different time intervals.

Conclusion

Among all tested essential oils, O_9 was found to have the most potent antibacterial activity against all tested bacterial cultures. Then O9 is used to prepare different nanoemulsions i.e., NE1, NE2, NE3, NE4, NE5 and NE6 and screened for antibacterial activity against all three bacteria. O_9NE5 was 193.7 nm in size having 0.264 PDI with the -57.3 zeta potential. Among all prepared nanomeulsions, O_9NE5 showed highest antibacterial activity against all three bacteria. In broth assay complete inhibition of bacteria 1 was at 1% concentration of O_9NE5.

Most microbes including bacteria, fungi, protozoans and others have developed resistance to antibiotics, and therefore investigations for new alternatives and novel strategies are gaining importance in today's world. The use of antimicrobial nanoemulsions may exert synergistic antimicrobial effects and would be a novel approach.

Future Perspective

Nanotechnology has held a prominent position for human welfare in a very short span of time through its multifactorial functions (Javed *et. al.*, 2017; Kumari *et. al.*, 2017). Nanoemulsions can be used as an alternative to the chemical agents which are highly toxic, costly and also causes adverse environmental effects. Nanoemulsions have a number of advantages as compared to larger-scale emulsions. These can be stabilized for enhancing the time before creaming takes place. These are transparent or translucent with a greater surface area owing to the small particle size. These are presently not only exploited in commercially available cosmetics but also have been employed toward imaging as well as delivery of poorly soluble

drugs. Nanoemulsions are effective systems for encapsulating lipophilic active components as the decrease of droplet size enhances their solubility, stability and can increase their biological activity. Hence nanoemulsions are being widely used in pharmaceutical, cosmetics, agriculture and food industries.

- **Antimicrobial nanoemulsions**- Antimicrobial nanoemulsions are o/w droplets that range from 200-600 nm. They are made of oil and water and are stabilized by surfactants. They have a broad spectrum of activity against bacteria like *E. coli*, *salmonella*, *S. aureus*, enveloped viruses like HIV and fungi like Candida, etc. The nanoemulsions particles are thermodynamically driven to fuse with lipid-containing microorganisms. This fusion is enhanced by the electrostatic attraction between the cationic charge of the emulsion and the anionic charge on the pathogen. When enough nanoparticles fuse with the pathogens, they release part of the energy trapped within the emulsion. Both the active ingredient and the energy released destabilize the pathogen lipid membrane, resulting in cell lysis and death.
- **Nanoemulsions in targeted drug delivery-** Nanoemulsion formulations can be used for the controlled drug delivery and targeting (Wang L, Li X, Zhang G, Dong J, Eastoe J, 2007). Because of their submicron size, they can easily be targeted to the tumor area. They have been recently received increasing attention as colloidal carriers for targeted delivery of various anticancer drugs, photosensitizers, neutron capture therapy agents, or diagnostic agents. The development of magnetic nanoemulsions is an innovative approach for cancer therapy.
- **Nanoemulsions in vaccines delivery**- There is recent evidence that HIV can infect the mucosal immune system. Therefore, developing mucosal immunity through the use of nanoemulsions may become very important in the future fight against HIV and they can be used to vaccinate against human immunodeficiency virus (HIV).
- **Prophylactic in bio-terrorism attack-** Due to their antimicrobial activity, research has begun on use of nanoemulsions as a prophylactic medicated dosage form, a human protective treatment, to prevent the people exposed to bio-attack such as Anthrax and Ebola. The nanoemulsions can be formulated into a cream, foam, liquid and spray to decontaminate a large number of materials, which is marketed as NANOSTAT™ (Nanobio Corp) (Charles L, Attama AA, 2011).
- **Nanoemulsions as non-toxic disinfectant cleaner**- Nanoemulsions can be employed as a disinfectant cleaner. A nontoxic disinfectant cleaner for use in routine markets that

include healthcare, travel, food processing and military applications has been developed by EnviroSystems. They have been found to kill tuberculosis and a large spectrum of viruses, bacteria and fungi within 5 to 10 min without any of the hazards posed by other categories of disinfectants.

- **Applications in cosmetics**- Nanoemulsions are embedded in many hair care products as well, such as hair creams, lotions, and gels. The nanoemulsions, which are comprised of vitamins or phospholipids, are patented. Particularly the L'Oréal group has recorded many patents in this field (Koroleva and Yurtov, 2012). Recently importance of nanoemulsions have become increasing as good vehicles for the controlled delivery of cosmetics. The small-sized droplet with its high surface area permit effective delivery of the active to the skin. Nanoemulsions are also acceptable in cosmetics because there is no chance of creaming, sedimentation, flocculation or coalescence, which is observed within microemulsions. Also, nanoemulsions have shown incredible effects on dry hair. After treating hair with cationic nanoemulsions, they became less greasy and shinier (Patravale and Mandawgade, 2008).

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