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NANOEMULSION BASED ESSENTIAL OILS (EUCALYPTUS AND OREGANO) AGAINST PHYTOPATHOGENIC FUNGI

Raviswamy G. H. Math*[1], T. B. Suneetha[1], S. Jothi Prabha[2], Alifia C. Jafer[3], N. Preveena[4]

[1*]Acharya Institute of Technology, Soladevanahalli, Karnataka, India.
[2]PSG College of Arts and Science, Coimbatore, Tamil Nadu, India.
[3]Amrita Vishwa Vidyapeetham, Amaravati, Andhra Pradesh, India.
[4]CPGS, Jain University, Bengalore, Karnataka, India.

ABSTRACT

The deduction in the crop yield due to phytopathogenic fungi is increasing these years. Among which *Mucor sp.,* causes a variety of diseases in crops. Although the botanical substances such as plant extract and essential oils impart high insecticidal and pesticidal activities, their application is not widespread due to its poor physicochemical properties. In order to overcome this problem, nano based synergistic essential oil (eucalyptus and oregano) technology has been deployed. In this study, a non-ionic surfactant was used to create a nanoemulsion from Eucalyptus globus and Origanum vulgare oil utilising a high energy emulsification technique. The Z-average diameter of the nanoemulsion droplets was discovered to be 19nm, and fluorescence microscopy imaging verified the spherical droplet shape of the nanoemulsion. When stored at room temperature (22°C), the nanoemulsion's size was shown to be physically stable for up to one month. The nanoemulsion's minimum inhibitory concentration (MIC) against Mucor sp. was 0.151mg/ml. Scanning electron microscopy and sodium dodecyl sulphate polyacrylamide gel electrophoresis were used to demonstrate how the nanoemulsion's ability to break the membrane of Mucor sp. The disease in chilli plants was significantly reduced by the use of synergistic nanoemulsion.

KEYWORDS

Eucalyptus oil, Oregano oil, Nanoemulsion and Bio-pesticide.

Author for Correspondence:

Raviswamy G. H. Math,
Acharya Institute of Technology,
Soladevanahalli, Karnataka, India.

Email: ras93gh@gmail.com

INTRODUCTON

Plant pathogens affect a wide range of crops and cause severe losses in economic and agricultural sectors. The world-wide annual production tonnage percentage loss due to microbial diseases estimated in the 21st century is found to be 16% (Moore *et al*, 2019)[1]. The world fungal diseases are caused due to 19,000 fungal species. In India, the reduction in crop productivity caused by pests, diseases and

weeds is estimated to account for between 10 and 30 percent of production. (Kumar, 2014)[2]. Phytopathogens can penetrate plant surfaces either through natural openings or can penetrate through wounds (Abdulkhair *et al*, 2016)[3]. Most fungal phytopathogens belong to the family of Ascomycetes and the Basidiomycetes. Fungi were responsible for the widespread symptoms such as leaf spots, wilts, blights, cankers, and fruit rots. Anthracnose, Botrytis rots, Downy mildews, Fusarium rots, Powdery mildews, Rusts, Rhizoctonia rots, Sclerotinia rots, Septoria leaf spot, Powdery mildew, Cedar-apple rust, and Armillaria root rot are only a few of the diseases that are caused by fungi.

The present methods of combating this terrible disease mainly rely on synthetic fungicides, such as systemic fungicides, which are absorbed by plants before having an impact on fungi, and contact fungicides, which take effect when sprayed on the affected area. Though synthetic fungicides stabilise crop output and market quality, their widespread usage has led to toxicity toward non-target organisms, adverse environmental consequences, and the emergence of pathogen strains that are resistant to them. Scientists are looking for alternatives to synthetic fungicides in order to manage fungal diseases due to the drawbacks of these chemicals and the public's growing concern for environmental protection. (Sharma *et al*, 2018)[4].

Studies show that the toxic waste of chemical fungicide remains on the plant and fruit after treatment as it is consumed in a short time. The National Academy of Sciences (NAS) has demonstrated the carcinogenic nature of fungicide residues in food products which can be considered as hazardous to humans. However, mutant strains that are resistant to various fungicides often arise and the fungicide causes high environmental impact since they are not readily biodegradable and appear to remain in the ecosystem for many years. Fungicides account for around 20% of the overall cost of production. Furthermore, throat irritation, sneezing, and coughing may be brought on by inhaling spray mist or dust from these fungicides. Even low levels of fungicide exposure over an extended period of time can have negative health effects. Most human fungicide poisonings are caused by eating seed grains (Lorenz, 2017)[5].

The use of essential oils generated from plants is one of these such strategies. Essential oils (EOs) obtained from plants are a vastly developing bio resource that can be used to protect crops. The potential of essential oils to damage cell membranes, result in cell death, or prevent the sporulation and germination of fungi accounts for their antibacterial or antifungal properties. Several *in-vitro* studies reveal that terpenes and terpenoids have less antibacterial effect when taken individually as opposed to the entire EO (Nazzaro *et al*, 2017)[6]. Eucalyptus and oregano essential oil chiefly contain terpenes as a major compound and they are considered as eco-friendly, easily available and cost effective when compared to other essential oils. While maintaining the intended antifungal effect, the use of a synergistic combination of bioactive EOs (Eucalyptus and Oregano) in a formulation lowers the concentration of individual oils and lowers the price of the product (Sharma *et al*, 2018)[4].

Nanoemulsion of EOs is an emerging technology in case of nanotechnology. Nano modification of EOs, which is hydroimmisible in nature, significantly improves plant growth and also they can be effectively used as fungicides. Oil droplets are dispersed in an aqueous media to form nanoemulsions, which are colloidal dispersions of two immiscible liquids that are stabilised by surfactant molecules (Chavda *et al*, 2019)[7]. Currently the studies of nanoemulsion have shown most interest with plant based oils due to its bioavailability and biocompatibility. (Espitia *et al*, 2018)[8]. Nanoemulsions are transparent or translucent, thermodynamically stable nanosized dispersions of oil in water (o/w) or water in oil (w/o). They are interfacial films of surfactant and cosurfactant molecules with droplet sizes 20–200nm. Numerous methods, including high-pressure homogenization and low-pressure homogenization, can be used to create nano emulsions (Chavda *et al*, 2019)[7].

As a result, the present study makes use of the benefits of nanoemulsion-based Eos against phytopathogenic fungi over conventionally used synthetic fungicides because it is easy to prepare, has higher thermodynamic stability, low production costs, a larger surface area for interaction, and also has the potential for industrial production (Echeverria *et al*, 2019)[9].

METHODS SECTION

Materials used

The selected EOs (Oregano and Eucalyptus) were procured from Katyani exports, New Delhi. Potato dextrose broth, Agar- agar type 1 from HiMedia Laboratories available at DRDO BU-CLS, Tween 20 (Surfactant), Polyethylene Glycol Diethyl Ether (Co- Surfactant), Ethylene Glycol (Anti-Freezing Agent) available at DRDO BU-CLS.

Sample Collection

The infected leaf samples (Scientific Name: *Capsicum annuum*; Common Name: Chilli and Scientific Name: *Lablab purpureus*; Common Name: Beans) were collected aseptically in a sterile leather bags from the local farms of Madampatti 10.9698°N, 76.8598°E, Coimbatore, Tamil Nadu.

Isolation of Fungal pathogens

Small fragments of the infected chilli and beans that had been collected had been processed. The pathogenic fungi showing the obvious signs can be seen after once cleaning the area with sterile distilled water. The next step is to chop the contaminated tissues into little (2-5mm squares) pieces and transfer them with flame sterilised forceps to the sterile PDA plates. The plates were then kept at room temperature for 5-7 days to allow the fungi to fully develop (Thilagam *et al*, 2018)[10]. To obtain the isolated colonies, the cultures are repeatedly sub-cultured once the fungus have grown to maturity. The cultural traits outlined by Gilman (1957)[11], Barnett and Hunter (1972)[12] and Nelson *et al*, (1982)[13] as well as microscopic observations were used to identify the fungi.

Identification of Fungal Pathogens

The fungal organisms were identified based on colony and morphological characteristics by performing KOH staining and lactophenol cotton blue staining

Preparation of Nanoemulsion

High energy emulsification was used to create an oil-in-water nanoemulsion. The non-ionic surfactant tween-20 was combined with the co-surfactant polyethylene diethyl ether, the anti-freezing agent ethylene glycol, and the EOs eucalyptus oil, oregano oil, and a blend of eucalyptus and oregano oil until a homogenous mixture was achieved. After that, the aqueous phase was gradually introduced via the oily phase while being stirred magnetically at 37°C (750rpm) for 30 minutes. The mixture was then allowed to equilibrate at 25°C for 24 hours. This combination was ultrasonically processed for 20 minutes at 400 watts (Abhishek Sharma *et al*, 2018)[4]. Distilled water makes up 80% (w/w) of the final mass, followed by ethylene glycol at 5% (w/w), a combination of ORE and EU at 1%, and surfactants or surfactant mixture at 10% (w/w) (S-mix). Table No.1 contains a list of the nanoemulsion's ingredients.

Characterisation of Nanoemulsion

The nanoemulsion was characterised using a number of physicochemical factors, including shape, particle size and distribution, zeta potential, FTIR analysis, stability tests, dye test and pH test.

Morphology of Nanoemulsion

Inverted microscope (EVOS FLC inverted fluorescent microscope) was used to observe the morphology and structure of the nanoemulsion.

Stability Studies

The stability examination was carried out by centrifuging the nanoemulsions prepared as stated in Table No.1 at 3500rpm for 30 min. They were then stored for up to a month at both room temperature (25°C) and at 7°C in the refrigerator. These tests' goal allows us to choose a formulation with stable physicochemical qualities, a nanoemulsion droplet, and low surfactant levels (Abd-Elsalam and Khokhlov, 2015)[14].

Dye solubility test

The nanoemulsion (1ml) was mixed with a few drops of water soluble dye, methylene blue. The homogenized nanoemulsion was viewed under a microscope.

Particle Size and Particle charge analysis

To ascertain the size distribution of the nanoparticles/nanoemulsion, dynamic light scattering was used. By pumping 2ml of deionized water into the internal flow cell, the remaining particles that were present in the flow cell were removed. To establish a baseline scattering intensity, the light scattering data were captured for 2 min. A 1ml syringe containing 0.8ml of the sample solution was injected into the flow cell at the conclusion of the baseline intensity. To investigate the scattering intensity within the detector limit, only 0.7ml of the 0.8ml solution was pumped into the flow cell. The flow rate was stopped once the 0.7ml of solution had entered the flow cell and a measurement of light scattering was made.

The zeta potential of the particle charge was assessed by measuring the electrophoretic mobility of the nanoemulsion in an electric field. The measurement was taken in triplicate using Zeta sizer. The analysis was carried out in values that typically range from +100mV to -100mV.

pH Determination Test

The portable pH metre was used to measure the pH of the nanoemulsion. The electrode was then immersed directly in 1ml of nanoemulsion that had been homogenised with 9ml of deionized water after the pH metre had been calibrated with standard buffer solutions prior to use (Gurpreet and Singh, 2018)[15].

Fourier-Transform Infrared Spectroscopy (FTIR) analysis

A tool for examination is Fourier Transform Infrared (FTIR) spectroscopy, which can be used to identify bonding details and find functional groups. The FTIR instrument operates under the assumption that infrared radiation of around 10,000 to 100cm-1 is passed through a sample, with some of the radiation being absorbed and some being passed through. The sample molecules transform the absorbed radiation into rotational and/or vibrational energy. The resulting signal, which appears as a spectrum in the detector and typically ranges from 4000cm-1 to 400cm-1, represents the sample's molecular fingerprint.

Determination of Minimal Inhibitory Concentration (MIC) OF EU and ORE Nanoemulsion alone and in combination

By means of the sterile 96-microwell plate method, the MIC of EU, ORE, and synergistic EU-ORE nanoemulsions were determined (Sharma *et al*, 2017)[16]. In a plate with 2-9 columns, 100 l of the essential oil dilution nanoemulsion was put into the well of the first column, followed with 100 l of sterile PDB. Columns 1 through 9 were serially diluted, and the surplus sterile PDB (100 l) was thrown from the 9th column's wells. A 200ml inoculum suspension was added to each well, for a total amount of 100ml. The inoculum suspension and sterile PDB were each 100 l in the growth control wells. In the positive control wells, 100 l of sterile PDB and 100 l of inoculum were used, while 180 l of sterile PDB and 20 l of nanoemulsion were used in the negative control wells. Each well's contents were combined before being left to incubate for 48 hours at room temperature.

Antifungal assay by agar well diffusion method

Formulated nanoemulsions were chosen for testing utilising a well diffusion approach against the fungus Mucor sp. based on their stability and lowest particle size (Lima *et al*, 2019)[17]. Pure cultures were subcultured in PDA broth for three to four days at room temperature. Using sterile cotton swabs, each strain was uniformly swabbed onto the individual sterilised PDA plates. Next, 5mm-diameter wells were created using well piercing. 100 l of the essential oils (eucalyptus and oregano) nanoemulsion are placed, both separately and together, into a sterile micropipette. The zone of inhibition was then evaluated after the plates had been incubated at room temperature for 3-4 days.

Membrane Disruption and loss of UV- absorbing cell constituents and cellular proteins

A UV-VIS spectrophotometer was used to measure the amount of proteins and cell components that leaked into the supernatant (Sharma *et al*, 2018)[4]. Mycelia of Mucor sp. were extracted from a six-day-old PDB by centrifugation at 4000g for 20 min. The cell pellet that was produced was then washed three times with phosphate buffer saline (PBS, pH 7.2) and then it was re-dissolved in 50ml of PBS.

The cell suspensions were split in half, with one half receiving no treatment (the control) and the other receiving the MIC of the nanoemulsion. 2ml of each sample were removed after 1 hour of incubation at 28 degrees Celsius with agitation, and they were centrifuged at 12,000g for 30 minutes. Each sample's supernatant was carefully removed and the absorbance at 260nm (for protein contents) and 280nm (for nucleic acid contents) was determined. Finally, to examine the proteins released as a result of membrane disruption, each of these samples was also run on a 12 percent SDS polyacrylamide gel (SDS-PAGE).

RESULTS

Observation of infected leaves

Bean and chilli leaf samples that had been gathered were examined for the presence of fungal colonies on their surface. On bean leaf samples (2-20mm in diameter), the colonies appeared as brown circular or angular-irregular shaped necrotic lesions and on chilli leaf samples, the colonies appeared as a diffused yellowish halo and fungus development.

Fungal Isolation

There were spots with mycelium that was white, black, green, and salmon pink, as well as colonies made from spores grown in medium PDA that had scattered colouring.

Fungal Identification

The fungal colonies were subcultured from the central part of the leaf spot on the fully grown culture media PDA was isolated and individual colonies were obtained (Figure No.1a). The cultural characteristics and microscopic observation of the isolated fungal spores by KOH staining and lactophenol cotton blue staining (Figure No.1b) was identified to be *Mucor sp*.

Preparation of Nanoemulsion

Eucalyptus and oregano essential oils were combined and produced individually and at various concentrations as nanoemulsions using the high energy method (high pressure homogenization and ultrasonication). A murky or opaque-looking clear white solution was produced during formulation. (Figure No.2).

Characterisation of Nanoemulsion

Morphology of Nanoemulsion

Figure No.3 shows the photomicrographs taken using an EVOS FLC inverted fluorescent microscope and a nanoemulsion comprising 20-30% (w/w) oils. The emulsions clearly displayed the huge globular droplets that were of a similar size to those determined using an optical light scattering microscope. The outcome demonstrates the rapid Brownian motion of oil droplets with a size of less than one micron. Furthermore, there was a strong correlation between droplet size and particle size analyzer measurements. Even after being reduced to less than 100nm in size, the results showed that the droplets were spherical in shape.

Stability Studies

After centrifugation at 3500rpm for 30 minutes up to one month at room temperature (25°C) and refrigerator temperature (7°C), it was discovered that the size of the nanoemulsions was physically stable. A certain concentration of the oil phase, surfactant, and water is used to create nanoemulsions, which are proven to be kinetically stable for one month. This demonstrates that the synthesised nanoemulsions withstood the stability tests.

Dye solubility test

When 1ml of a nanoemulsion is combined with the water-soluble dye methylene blue, the result is a continuous phase that appears blue under a light microscope because water is the exterior phase and the dye dissolves in it to produce the colour (Figure No.4). According to tests for identifying emulsion types conducted by Sharma *et al*, (2010)[18], the formed nanoemulsion type is O/W because methylene blue serves as the continuous phase and water acts as the exterior phase.

Particle Size and Particle charge analysis

The size distribution of the nanoemulsion was determined using dynamic light scattering (DLS). The maximal size of the nanoemulsions was revealed by the DLS observation. The nanoemulsions' maximum average size ranged from 15 to 120nm (Figure No.5a), and ultrasonication produced obvious droplet-size distributions that resulted in nanoemulsions with small droplet sizes.

To identify the surface charges that essential oil nanoemulsions gained, zeta potential analysis was used. The produced nanoemulsion's zeta potential was discovered to be -16 mV (Figure 5b), which suggests that the nanoemulsions are generally stable in nature.

pH Determination Test

pH of the oil was checked before and after formulation of the Nanoemulsion in a portable pH meter. The pH value before and after formulation of nanoemulsion was listed in the Table No.2. It is found that the pH of nanoemulsion before and after formulation varies significantly and the pH of nanoemulsion usually ranges between 4.9 to 5.5, (Morsi *et al*, 2014)[19], thus claiming it to be adequate.

Fourier-Transform Infrared Spectroscopy (FTIR) analysis

The mixture of eucalyptus and oregano essential oils and their nanoemulsion both had their FTIR spectra captured. Figure 6a depicts the FTIR spectrum of an oregano essential oil nanoemulsion. The strong, broad peaks for O-H stretching of alcohol were observed at 3502.73cm-1; a robust peak for O=C=O stretching of carbon dioxide was observed at 2353.16cm-1; a weak peak for S-H of thiol groups and C-H bending of aromatic compounds was observed at 2600.04cm-1. Figure 6b displays the FTIR spectrum of an oregano and eucalyptus essential oil nanoemulsion. A strong peak was seen at 2353.16cm-1 for O=C=O stretching of carbon dioxide, a weak peak at 2229.71cm-1 for CN of nitrile groups and a weak peak at 2229.71cm-1 for CC of alkyne groups. The strong, broad peaks were observed at 3502.73cm-1 for O-H stretching of alcohol, 2954.95cm-1 for O-H stretching of carboxylic acid, and 2522.89cm. These reports matched the data from earlier studies quite well.

Determination of minimal inhibitory concentration of nanoemulsion of EU and ORE alone and in combination

Our investigations used the Minimal Inhibitory Concentration (MIC) of EU and ORE nanoemulsion alone and in combination to assess the antifungal efficacy of essential oils based on nanoemulsions. The A3 formulation of essential oils based on nanoemulsions was selected for the research based on shape, particle size, and stability. The microtiter plate reader was used to calculate the Minimal Inhibitory Concentrations (MICs) against the isolated phytopathogen (Mucor sp.). The table below shows the essential oils based nanoemulsions' lowest inhibitory concentration (Table No.3). The nanoemulsion of eucalyptus and oregano essential oil suppressed the development of the microorganism with the lowest least inhibitory concentration of 0.520mg/ml and 0.532mg/ml against Mucor sp., respectively. The minimal inhibitory concentration was found to be very effective against *Mucor sp.,* (0.163mg/ml).

Antifungal assay by agar well diffusion method

To investigate the zone of inhibition displayed by the nanoemulsion of essential oils (eucalyptus and oregano) alone and in combination against the isolated microorganism, an antifungal experiment using the agar well diffusion method was carried out (Figure No.7). The zone of inhibition exhibited against the phytopathogen (*Mucor sp.*,) was tabulated in the table (Table No.4). The zone of clearance of nanoemulsion of oregano oil and combination of oil against *Mucor sp.,* was very effective showing maximum inhibition of 16mm.

Membrane disruption and loss of UV absorbing cellular constituents

Using spectrometric measurement for the cell constituents (proteins and nucleic acids) at 260nm and 280nm, the MIC of the nanoemulsion treated with phytopathogenic fungi for one hour was detected for the membrane breakdown (Figure No.8) and the release of cellular constituents. According to the aforementioned results (MIC and agar well diffusion), the nanoemulsion of the two oils (eucalyptus and oregano) performed better than each oil used alone. Thus, the MIC of nanoemulsion of combination of essential oil was selected for the study of membrane disruption of cellular constituents (Figure No.9). The readings were tabulated in the below table (Table No.5). The treated sample shows an increase in protein band which is confirmed by SDS PAGE (Figure No.10).

DISCUSSION

The morphology, particle size, and dispersion of a nanoemulsion made from eucalyptus oil, oregano oil, and a mixture of eucalyptus and oregano oil were all characterised in the current work. The high energy ultrasonic method used to create the nanoemulsions included varying ratios of water, oil, and surfactants; as a result, the increased solubility of oil components in the aqueous phase in the presence of surfactants led to increased interaction between the antifungal and microorganisms (Elsalam *et al*, 2015)[14]. The synthesised essential oil nanoemulsion was stable for up to a month without phase separation, according to stability experiments. Due to the nanosized particles' higher bioavailability against plant pathogens, the nanoemulsion's efficiency is increased by their smaller particle size (19nm).

Evaluation of the produced nanoemulsions' antifungal properties When compared to the individual nanoemulsions of the oils, it was revealed that the synergistic combination of eucalyptus and oregano essential oils inhibited more effectively, lowering the cost of the product without compromising the desired antifungal result (Sharma *et al*, 2018)[4].

It is evident that combining essential oils produced outcomes that were just as effective as using each oil alone in a nanoemulsion. The results of the spectrometric measurement also revealed that when the microorganisms were treated with the nanoemulsion, the release of cellular components rose dramatically at both 260nm (for proteins) and 280nm (for nucleic acids).As shown by the enlarged band in the SDS PAGE analysis due to cellular protein release, the increase in OD 260 nm and OD 280 nm suggests that total proteins and nucleic acids were released from the cell component due to membrane breakdown. As a result, the nanoemulsion of the essential oil mixture (eucalyptus and oregano) plays a significant role in the physical destruction of fungus and the facilitation of its usage as an efficient synergistic antifungal agent. It is necessary to conduct more research on the mechanisms of cytotoxicity in plants and plant pathogens.

Table No.1: Lists the ingredients used to make the synergistic EU-ORE nanoemulsions and EU-ORE and ORE nanoemulsions using Tween 20, PEG diethyl ether, ethylene glycol, and water

Sample	Eu (ml)	Ore (ml)	Eu+Ore (ml)	Ethylene glycol (ml)	Water (ml)	Propylene Diethyl Ether (S1) (ml)	Tween 20 (S2) (ml)
A1	5			5	80	10	
		5		5	80	10	
			5	5	80	10	
A2	5			5	80		10
		5		5	80		10
			5	5	80		10
A3	5			5	80	5	5
		5		5	80	5	5
			5	5	80	5	5
A4	5			5	80	6	4
		5		5	80	6	4
			5	5	80	6	4

*Eu- Eucalyptus oil; *Ore- Oregano oil

Table No.2: pH of the essential oils (Eucalyptus and Oregano) alone and in combination before and after formulation of Nanoemulsion

S.No	Sample	pH of the oil	pH of the Nanoemulsion
1	EU	5.56±0.15	4.67±0.5
2	ORE	9.46±0.05	4.7±0.34
3	CO	6.53±0.05	4.9±0.09

EU- Eucalyptus; ORE – Oregano; CO – Combination

Table No.3: Antifungal susceptibility testing (Minimal Inhibitory Concentration) of Nanoemulsion based essential oils alone and in combination against *Mucor sp*

S.No	Nanoemulsion	MIC (mg/ml)
1	EU	0.548±0.01
2	ORE	0.52±0.02
3	CO	0.137±0.03

MIC- Minimal Inhibitory Concentration; EU- Eucalyptus; ORE – Oregano; CO – Combination

Table No.4: Antifungal assay (Zone of inhibition by Agar Well Diffusion) of Nanoemulsion based essential oils alone and in combination against *Mucor sp*

S.No	Nanoemulsion	Zone of Inhibition (mm)
1	EU	1.2±0.2
2	ORE	9.16±0.28
3	CO	15.33±0.76

*EU- Eucalyptus; *ORE – Oregano; *CO – Combination

Table No.5: UV-VIS readings of the samples before and after treatment of nanoemulsion

S.No	Sample	UV - Spectroscopy readings (280nm)	UV - Spectroscopy readings (260nm)
1	Control	0.206±0.005	0.96±0.02
2	Combination	0.812±0.009	4.53±0.29

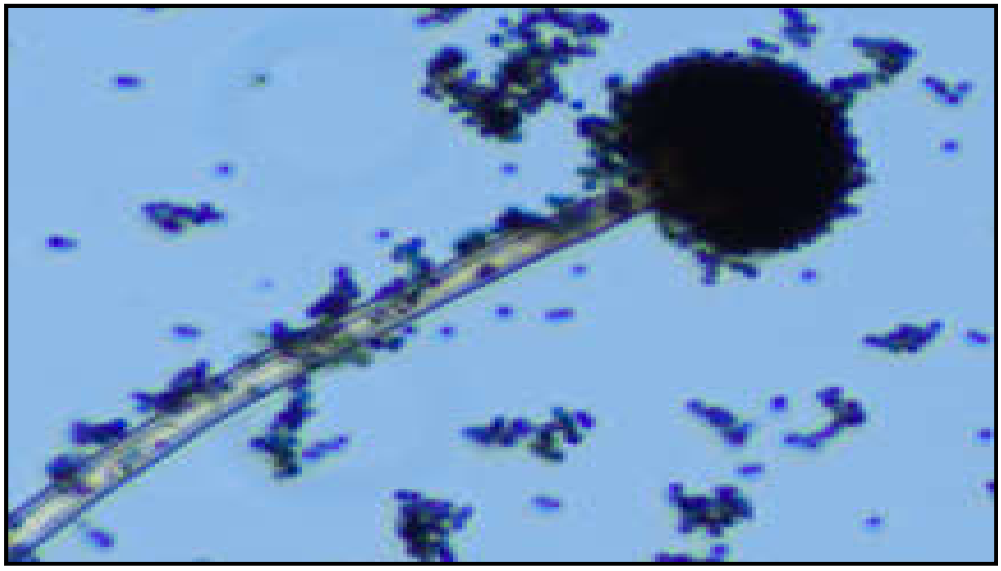


Figure No.1a: Isolated colonies on PDA

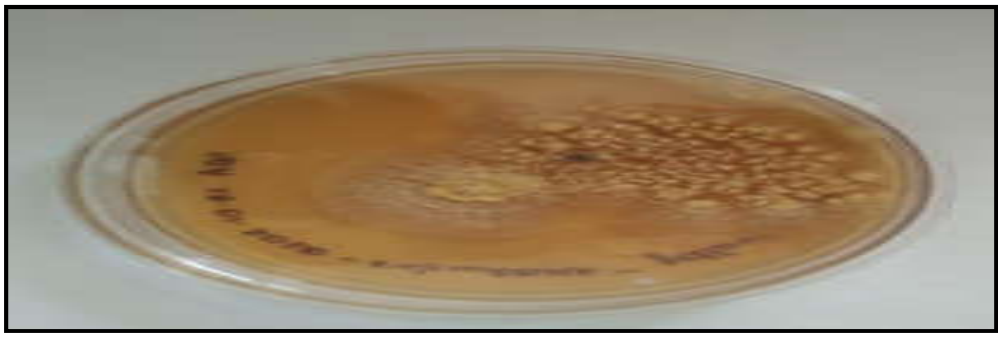


Figure No.1b: Microscopic observation of *Mucor sp*

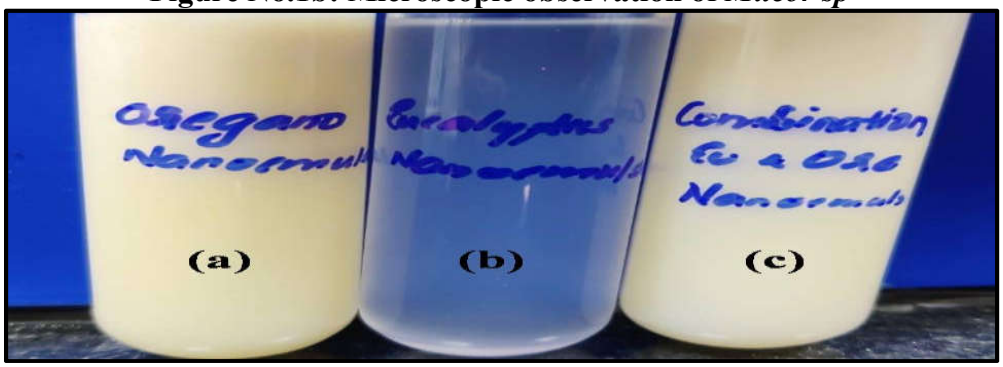


Figure No.2: a) Nanoemulsion of combination of Eucalyptus and Oregano oil; b) Nanoemulsion of Eucalyptus oil; c) Nanoemulsion of Oregano oil

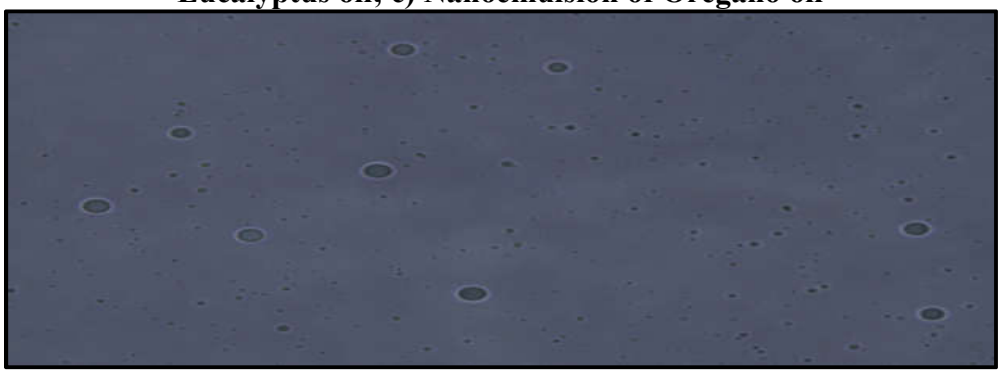


Figure No.3: Photomicrograph of inverted Fluorescent microscope

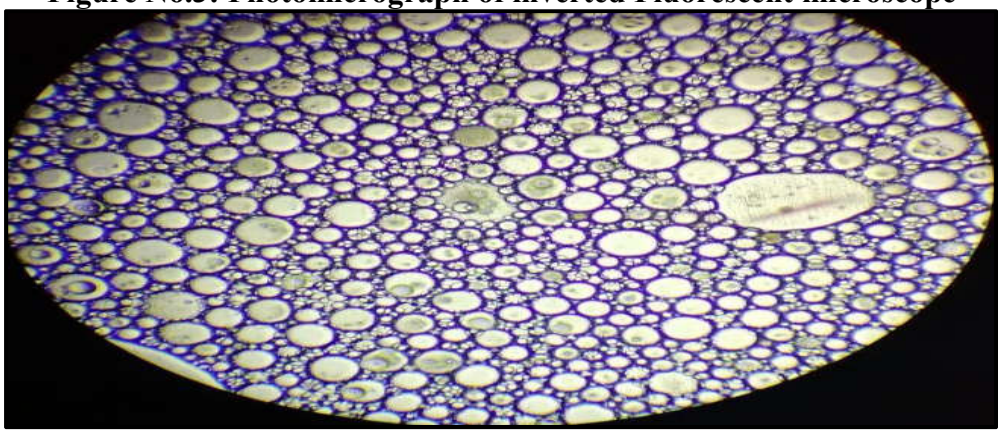


Figure No.4: O/W Nanoemulsion in which water acts as the external phase with methylene blue as continuous phase

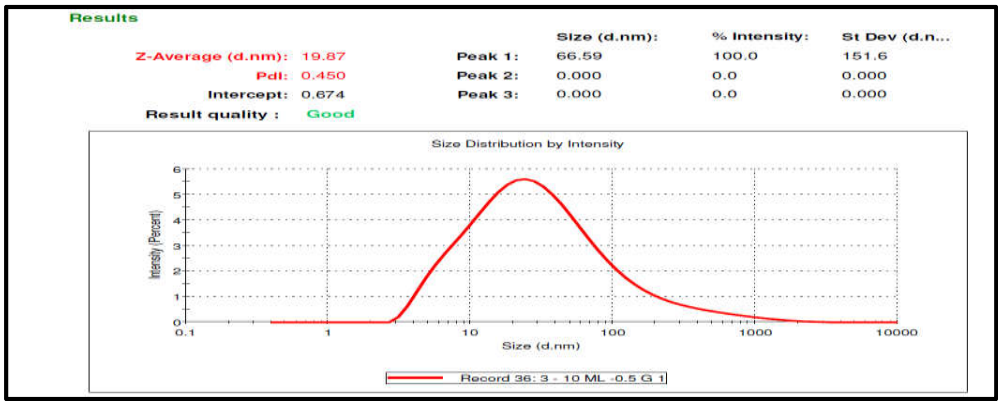


Figure No.5a: It shows that the average nanoemulsion particle size distribution is 19.87nm

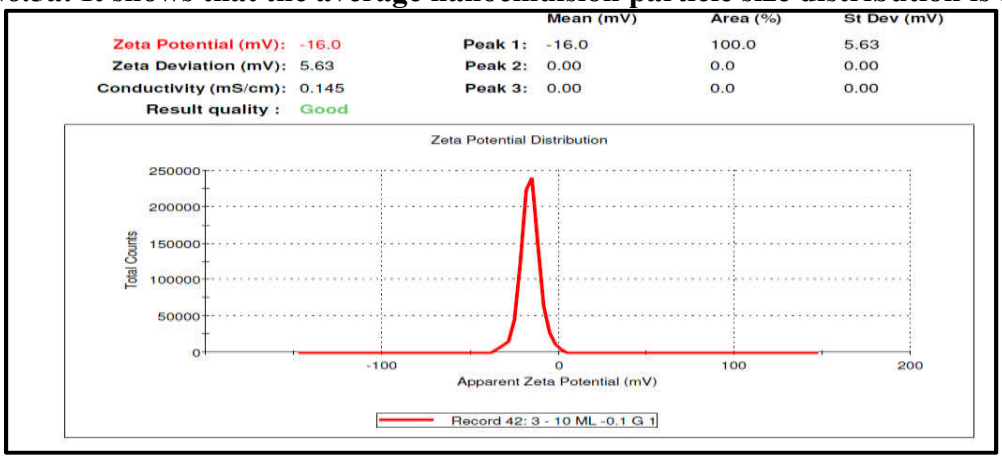


Figure No.5b: The surface charge acquired by nanoemulsion was found to be -16mV

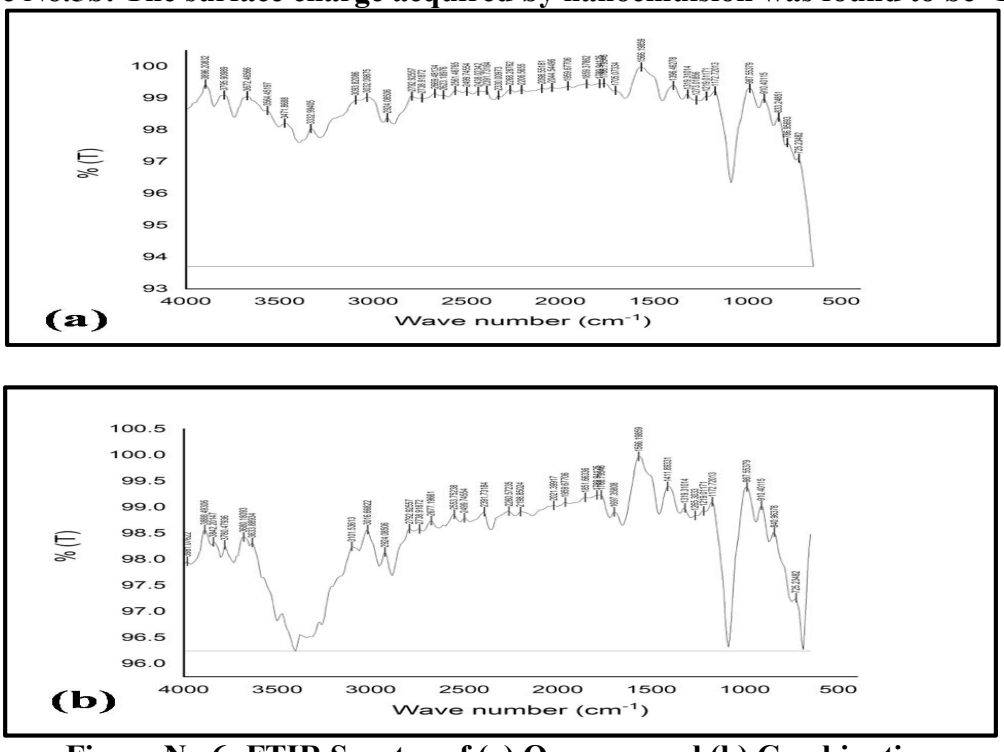
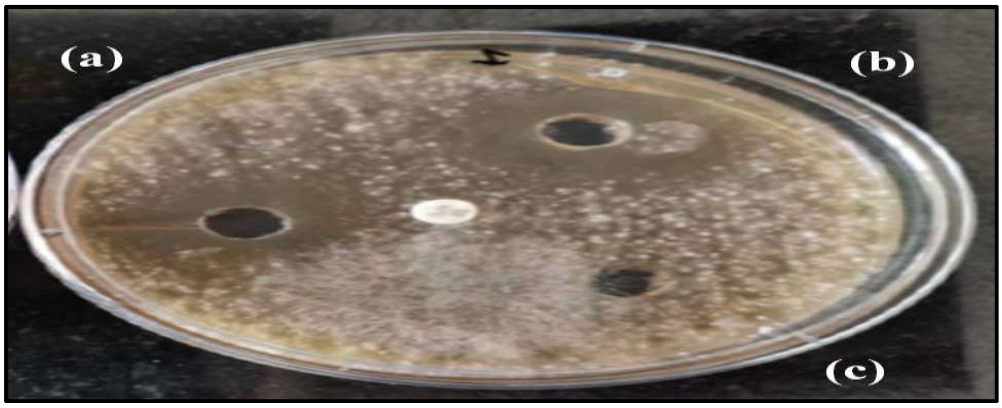


Figure No.6: FTIR Spectra of (a) Oregano and (b) Combination



a) Sample; b) Positive control; c) Negative control

Figure No.7: Zone of Inhibition by Agar well diffusion method

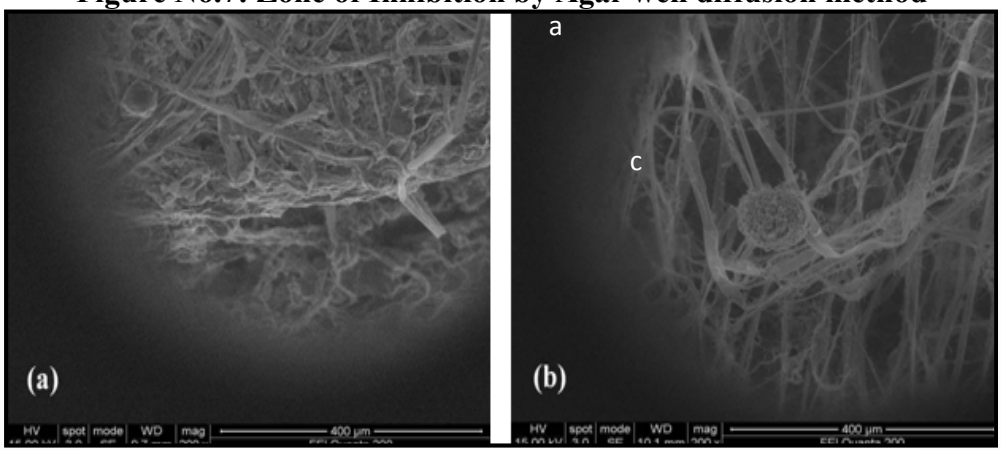


Figure No.8: a) Control; b) MIC Treated hyphae of *Mucor sp*

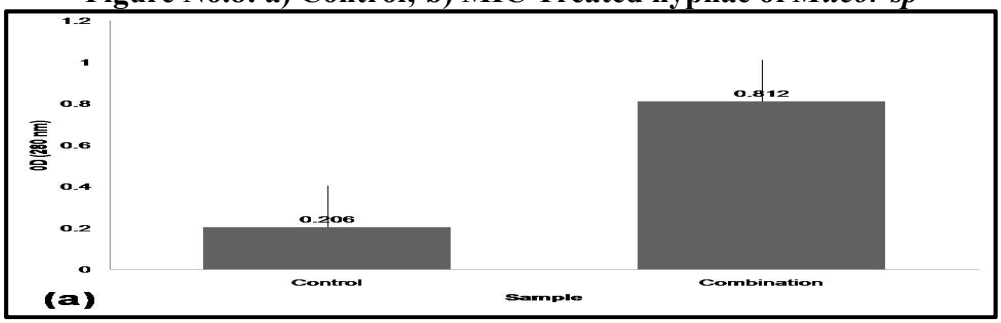


Figure No.9a: Results of nanoemulsion (MIC) on Protein release at OD - 280nm

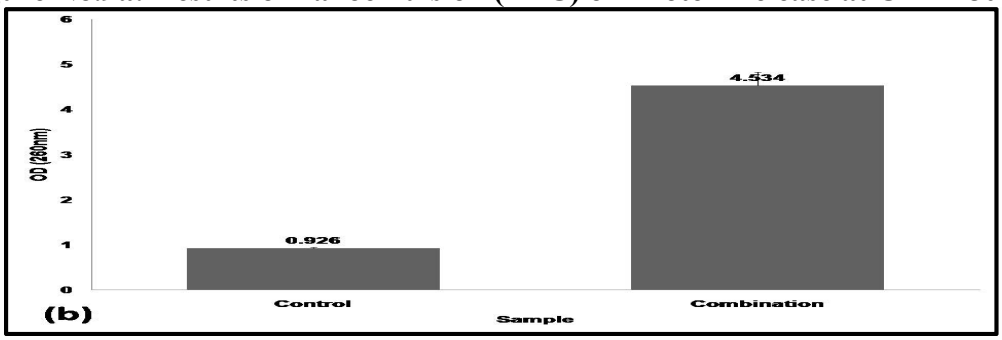
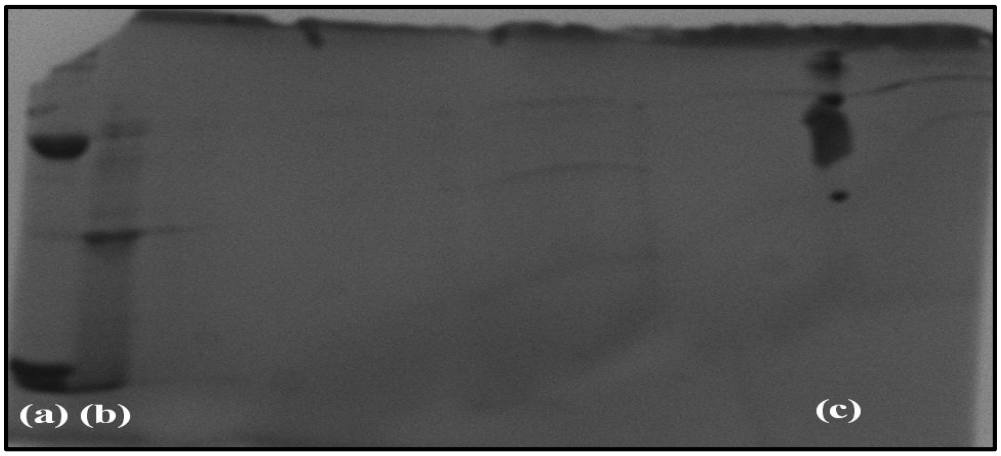


Figure No.9b: Results of nanoemulsion (MIC) on DNA release at OD - 260nm



(a) Standard; (b) Sample treated cell suspension; (c) Control
Figure No.10: SDS – PAGE

CONCLUSION

The current work concentrated on the ability of essential oils derived from nanoemulsions (Eucalyptus and Oregano) to be more effective against the phytopathogenic fungi Mucor sp. when used together than when used separately, as shown by the experiments on membrane disruption. These discoveries might offer a synthetic fungicide substitute. To understand the toxicity mechanism in plant diseases and higher plant species, more research is required. The promising oil and water nanoemulsion technology can be used to regulate fungicide among mixed surfactants since it is green, biosafe, and environmentally beneficial.

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ABBREVIATIONS

EU: Eucalyptus oil, FTIR: Fourier-Transform Infrared Spectroscopy, MIC: Minimal Inhibitory Concentration, ORE: Oregano oil, PBS: Phosphate buffer saline, PDA: Potato Dextrose Agar, PDB: Potato Dextrose Broth, S1: Surfactant 1, S2: Surfactant 2, SDS PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication.

BIBLIOGRAPHY

1. David Moore, Geoffrey D. Robson and Anthony P. J. Trinci, 21st Century Guidebook to Fungi, 2nd Edition, 2019, 610.
2. Kumar R. Research methodology: A Step-by-step guide for beginners, *Sage Publications Ltd, London,* 4th Edition, 2014, 432.
3. Abdulkhair W M, Alghuthaymi M A. Plant pathogens, *Plant Growth,* 2016, 49.
4. Sharma A, Sharma N K, Srivastava A, Kataria A, Dubey S, Sharma S, Kundu B. Clove and lemongrass oil based non-ionic nanoemulsion for suppressing the growth of plant pathogenic Fusarium oxysporum f. sp. Lycopersici, *Industrial Crops and Products,* 123, 2018, 353-362.
5. Eric S. Lorenz S E. Potential health effects of pesticides, *Pennstate Extension,* 2017.
6. Nazzaro F, Fratianni F, Coppola R, Feo V D. Essential oils and antifungal activity, *Pharmaceuticals,* 10(4), 2017, 86.

7. Chavda V P. Nanobased nano drug delivery: A comprehensive review, *Applications of Targeted Nano Drugs and Delivery Systems,* 2019, 69-92.
8. Espitia P J, Fuenmayor C A, Otoni C G. Nanoemulsions: Synthesis, characterization, and application in bio-based active food packaging, *Comprehensive Reviews in Food Science and Food Safety,* 18(1), 2019, 264-285.
9. Gloria V Echeverria, Zhongqi Ge, Sahil Seth. Resistance to neoadjuvant chemotherapy in triple-negative breast cancer mediated by a reversible drug-tolerant state, *Sci Transl Med,* 11(488), 2019, eaav0936.
10. Thilagam R, Kalaivani G, Hemalatha N. Isolation and identification of phytopathogenic fungi from infected plant parts, *Intl J Curr Pharmaceut Res,* 10(1), 2018, 26-28.
11. Gilman J C. A manual of soil fungi, *Lowa State College Press, Anes, Iowa USA,* 1957, 392.
12. Barnett H L, Hunter B B. Illustrated genera of imperfect fungi, *Burgess Publishing Co, Minneapolis,* 3rd Edition, 1972, 241.
13. Nelson P E, Toussoun T A, Marasas W F O. Fusarium spp: An illustrated manual for identification, *The Pennsylvania University, Parl, USA,* 1983, 198.
14. Abd-Elsalam K A, Khokhlov A R. Eugenol oil nanoemulsion: Antifungal activity against Fusarium oxysporum f. sp. vasinfectum and phytotoxicity on cottonseeds, *Applied Nanoscience,* 5(2), 2015, 255-265.
15. Gurpreet K, Singh S K. Review of nanoemulsion formulation and characterization techniques, *Indian Journal of Pharmaceutical Sciences,* 80(5), 2018, 781-789.
16. Sharma M S, Choudhary P R. Effect of fenugreek seeds powder (Trigonella foenum-graecum L.) on experimental induced hyperlipidemia in rabbits, *J. Diet. Suppl,* 14(1), 2017, 1-8.
17. Lima S L, Colombo A L, De Almeida Junior J N. Fungal cell wall: emerging antifungals and drug resistance, *Frontiers in Microbiology,* 10, 2019, 2573.
18. Sharma N, Bansal M, Visht S, Sharma P K, Kulkarni G T. Nanoemulsion: A new concept of delivery system, *Chronicles of Young Scientists,* 1(2), 2010, 2.
19. Morsi N M, Abdelbary G A, Ahmed M A. Silver sulfadiazine based cubosome hydrogels for topical treatment of burns: Development and *in vitro/in vivo* characterization, *European Journal of Pharmaceutics and Biopharmaceutics,* 86(2), 2014, 178-189.
20. Basak S, Guha P. Betel leaf (Piper betle L.) essential oil microemulsion: Characterization and antifungal activity on growth and apparent lag time of Aspergillus flavus in tomato paste, *Lebensmittel-Wissenschaft + [i.e. und] Technologie,* 75, 2017, 616-623.
21. Batish D R, Singh H P, Kohli R K, Kaur S. Eucalyptus essential oil as a natural pesticide, *Forest Ecology and Management,* 256(12), 2008, 2166-2174.
22. Bernardi D S, Pereira T A, Macie N R, Bortoloto J, Viera G S, Oliveira G C. Formation and stability of oil-in-water nanoemulsions containing rice bran oil: *In vitro* and *in vivo* assessments, *Journal of Nanobiotechnology,* 9(44), 2011, 44.
23. Calvert G M. Health effects from pesticide exposure, *American Family Physician,* 69(7), 2004, 1613. Chime S A, Kenechukwu F C, Attama A A. Nanoemulsions-advances in formulation, characterization and applications in drug delivery, *Application of Nanotechnology in Drug Delivery,* 3, 2014, 1-50.
24. Chu W, McLean R J. Quorum signal inhibitors and their potential use against fish diseases, *Journal of Aquatic Animal Health,* 28(2), 2016, 91-96.
25. Dorman H J D, Deans S G. Antimicrobial agents from plants: Antibacterial activity of plant volatile oils, *Journal of Applied Microbiology,* 88(2), 2000, 308-316.

26. Echeverria J, Albuquerque R D D G D. Nanoemulsions of essential oils: New tool for control of vector-Borne diseases and *in vitro* effects on some parasitic agents, *Medicines,* 6(2), 2019, 42.
27. Ferhat M A, Meklati B Y, Chemat F. Comparison of different isolation methods of essential oil from Citrus fruits: Cold pressing, hydrodistillation and microwave 'dry' distillation, *Flavour and Fragrance Journal,* 22(6), 2007, 494-504.
28. Figueredo A C, Barroso J G, Pedro L G, Scheffer J J C. Factors affecting secondary metabolite production in plants: Volatile and essential oils, *Flavour Fragr. J,* 23(4), 2008, 213-226.
29. Forgiarini A, Esquena J, Gonzalez C, Solans C. Studies of the relation between phase behavior and emulsification methods with nanoemulsion formation, In Trends in colloid and interface science XIV, *Springer, Berlin, Heidelberg,* 115, 2000, 36-39.
30. Hazra D K. Nano-formulations: High definition liquid engineering of pesticides for advanced crop protection in agriculture, *Adv Plants Agric Res,* 6(3), 2017, 56-57.
31. Hubert T, Tiebe C, Banach U. Electronic noses for the quality control of spices, in electronic noses and tongues in food science, *Academic Press,* 2016, 115-124.
32. Kaufmann B, Christen P. Recent extraction techniques for natural products: microwave-assisted extraction and pressurised solvent extraction, Phytochemical Analysis: *An International Journal of Plant Chemical and Biochemical Techniques,* 13(2), 2002, 105-113.
33. Leyva-Lopez N, Gutiérrez-Grijalva E P, Vazquez-Olivo G, Heredia J B. Essential oils of oregano: Biological activity beyond their antimicrobial properties, *Molecules,* 22(6), 2017, 989.
34. Mishra P, Balaji A P B, Mukherjee A, Chandrasekaran N. Bio-based nanoemulsions: An eco-safe approach towards the eco-toxicity problem, *Handbook of Ecomaterials; Springer: Singapore,* 2018, 1-23.
35. Mishra P, Balaji A P B, Tyagi B K, Mukherjee A, Chandrasekaran N. Nanopesticides: A boon towards the control of dreadful vectors of lymphatic filariasis, *In Lymphatic Filariasis, Springer, Singapore,* 2018, 247-257.
36. Naveed R, Hussain I, Tawab A, Tariq M, Rahman M, Hameed S, Iqbal M. Antimicrobial activity of the bioactive components of essential oils from Pakistani spices against Salmonella and other multi-drug resistant bacteria, *BMC Complementary and Alternative Medicine,* 13(1), 2013, 265.
37. Nguefack J, Budde B B, Jakobsen M. Five essential oils from aromatic plants of Cameroon: their antibacterial activity and ability to permeabilize the cytoplasmic membrane of Listeria innocua examined by flow cytometry, *Letters in Applied Microbiology,* 39(5), 2004, 395-400.
38. O'Bryan C A, Pendleton S J, Crandall P G, Ricke S C. Potential of plant essential oils and their components in animal agriculture-*in vitro* studies on antibacterial mode of action, *Frontiers in Veterinary Science,* 2, 2015, 35.
39. Patel A, Kadiya K, Ghosh S. The effect of emulsifier type on the formation and stability of nanoemulsion gels, *Lipid Technology,* 29(11-12), 2017, 111-114.
40. Pieterse C M, Van Loon L C. NPR1: The spider in the web of induced resistance signaling pathways, *Current Opinion in Plant Biology,* 7(4), 2004, 456-464.
41. Reza K H. Nanoemulsion as a novel transdermal drug delivery system, *International Journal of Pharmaceutical Sciences and Research,* 2(8), 2011, 1938-1946.
42. Salager J L, Anton R E, Anderez J M, Aubry J M. Formulation des micro-emulsions par la methode HLD, *Techniques De l'Ingenieur,* 2001, 157.
43. Sharifi-Rad J, Sureda A, Tenore G C, Daglia M, Sharifi-Rad M, Valussi M, Sharifi-Rad R.

Biological activities of essential oils: From plant chemoecology to traditional healing systems, *Molecules,* 22(1), 2017, 70.

44. Solans C, Izquierdo P, Nolla J, Azemar N, Garcia-Celma M J. Nanoemulsions, *Current Opinion in Colloid and Interface Science,* 10(3-4), 2005, 102-110.
45. Tisserand R, Young R. Essential oil Safety-E-Book: A guide for health care professionals, *Elsevier Health Sciences,* 2nd Edition, 2013, 800.
46. Trombetta D, Castelli F, Sarpietro M G, Venuti V, Cristani M, Daniele C, Bisignano G. Mechanisms of antibacterial action of three monoterpenes, *Antimicrobial Agents and Chemotherapy,* 49(6), 2005, 2474-2478.
47. Ultee A, Bennik M H J, Moezelaar R J A E M. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen Bacillus cereus, *Appl. Environ. Microbiol,* 68(4), 2002, 1561-1568.
48. Wang L, Li X, Zhang G, Dong J, Eastoe J. Oil-in-water nanoemulsions for pesticide formulations, *Journal of Colloid and Interface Science,* 314(1), 2007, 230-235.
49. Yang J, Hsiang T, Bhadauria V, Chen X L, Li G. Plant fungal pathogenesis, *Bio Med Research International,* 2017, Article ID 9724283, 2017, 2.
50. Zitzelsberger C, Buchbauer G. Essential oils as "a cry for help" A review, *Natural Product Communications,* 10(6), 2015, 1-12.

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