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Development of basil oil nanoemulsions and emulsion filled hydrogels

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ABSTRACT

The indiscriminate use of antibiotics has led to the emergence of drug-resistant bacteria which has become one of the biggest challenges of the twenty-first century for the researchers to combat and in turn search for novel targets which could lead to the development of effective and sustainable therapies. Inhibition of virulence of microbial pathogens is an emerging approach to address the challenges related to microbial infections. To suppress the virulence micro organism, we developed stable nanoemulsion filled hydrogel of Basil oil (*Ocimum basilicum*). Basil oil based nanoemulsion were formulated by ultrasonic emulsification method. Increase in surfactant concentration and emulsification time decreased emulsion droplet size but increased viscosity and optical transparency of emulsion system. Nanometric emulsion droplet size was determined by dynamic light scattering technique using a particle size analyzer. SEM images confirmed the spherical morphology of emulsion droplets. Basil oil nanoemulsion (BNE-2) exhibited smallest droplet size and greater kinetic stability. Hence, this BNE-2 formulation was selected for application studies. FormulatedBNE-2 hydrogel was evaluated for bactericidal activity against bacteria and fungi by dose and time dependent killing experiment. Alteration in membrane permeability was confirmed by quantifying leakage of 260 nm absorbing substances. Optical microscopic analysis suggested that hydrogel treatment resulted in alteration of permeability and surface features of bacterial cell membrane which lead to lysis and cell death.

Keywords: Basil oil, Nanoemulsion, Hydrogel, Anti microbial activity.

MATERIALS

Basil oil *(Ocimum basilicum) is procured from* Yarrow chemicals, Mumbai, Span 20, Span 80, Carragenan, Calcium chloride were *procured from* SD Fine chemicals, Mumbai, Nutrient agar medium, Sabourad dextrose agar, Muller Hintor agar, Sabourrad dextrose broth were obtained from Hi Media, Mumbai [1].

METHODOLOGY

PREPARATION OF BASIL OIL NANOEMULSION

Nanoemulsion was formulated using basil oil, non-ionic surfactant Span 60 and water. Span 60 was preferred to be used as surfactant because it has a high hydrophilicnbalance value, which is equal to 15. Span 60 is non-ionic in nature and stabilizes emulsion droplets stearic stabilization. Concentration of basil oil (6%v/v) 1:1, 1:2, 1:3, and 1:4, followed by the addition of water. Then, the coarse emulsion was subjected to ultrasonic emulsification using a 20 kHz Sonicator with a maximum power output of 750W. The temperature difference between initial coarse emulsions to final nanoemulsion was less than 10 °C. Heat is generated during the prolonged process of emulsification by high energy method like ultrasonic emulsification. This heat is nullified by placing them temperature difference was minimized. Then the formulated nanoemulsion was characterized and also stability of the emulsion was investigated. All the characterization process was done in room temperature [2].

Preparation of Gels and Nanoemulsion –Filled Gels

Preparation of Carrageenan Gels

Pure Carrageenan gels were prepared by adding the powder (0.25%w/w) with stirring to de ionized water on the magnetic hotplate stirrer at 350 rpm, and then incubated in an 85°C water bath for 1 hr. The dispersion was magnetically stirred for 30s at 10min intervals, and the last 10 min of incubation coincided with the gradual addition of CaCl2 powder to 0.025 M in the final dispersion Next, the hot dispersion were each poured into cylindrical container. They were then left at room temperature (25°C) to cool down. Screw caps were used to seal the containers, and gelation was then facilitated by overnight storage in a refrigerator at 4°C

The nanoemulsion with the smallest particle size and highest stability with storage time and temperature was chosen as the best system and used for the preparation of the filled gel samples. Considering the final gel samples. Considering the final concentration of the nanoemulsion and dispersions of biopolymers in the filled hydrogel sample [3].

PREFORMULATION STUDY

Solubility study, Boiling point, Refractive index, Specific gravity, Fourier transform infra-red (FTIR) spectroscopy, Fourier transform infra-red (FTIR) spectroscopy, GC-MS analysis were formed

Solubility study

A semi quantitative determination of the solubility was made by adding solvent in small incremental amount to a test tube containing fixed quantity of solute of vice versa. After each addition, the system is vigorously shaken and examined visually for any un dissolved solute particles. The solubility is expressed in terms of ratio of solute and solvent. The results are shown in results and discussion [4].

Boiling point

A small quantity of oil was placed into a fusion tube. That tube is placed in the boiling point apparatus containing mineral oil. The temperature of the mineral oil was gradual increased automatically and read the temperature at which powder started to melt and the temperature when all the powder gets melted was recorded. This was performed in triplets and average value was reported. The results are shown in results and discussion

Refractive index

Abbe's refract meter is used to measure the refractive index of the given Basil oil. Using a particular monochromatic light source, the apparatus is calibrated with water as the liquid. Adjust the micrometer screw to focus the boundary between the bright and dark regions. Adjust the refract meter scale to place the cross wire of the telescope exactly on the boundary between the bright and dark regions. Temperature of the sample can be varied by using the slider [5].

Specific gravity

Specific gravity of basil oil was measured by pycnometer. A pycnometer is simply a bottle which can be precisely filled to a specific, but not necessarily accurately known volume. Placed upon a balance of some sort it will exert a force where using the mass of the bottle filled with the product being tested and the mass of the bottle full of water the specific gravity can be calculated.

Fourier transform infra-red (FTIR) spectroscopy

Infrared spectrum of basil oil, span 60, span 80 and carragenan was determined on Fourier Transform Infrared spectrophotometer (8400S Shimadzu) using KBr dispersion method. The base line correction was done using dried potassium bromide. Then the spectrum of dried mixture of drug and potassium bromide was run followed by drug with excipients in the wavelength region 4000 and 400 cm-1.

GC-MS analysis

Basil oils were analyzed by GC–MS (JEOL GCMATE II). Carrier gas used was helium gas at a flow rate of 1 ml/min. The samples were injected with a split ratio of 1:10. Injector temperature was 80 °C and detector temperature was 275 °C. Mass spectra was recorded over 40–400 amu range with 70 eV of ionization energy and ion source temperature 240 °C. Identification of components of oil was done by matching the obtained mass spectra data with NIST MS search version on Wiley library.

EVALUATION OF NANO EMULSION

Thermal Stability Analysis

To investigate the stability of the nanoemulsion with thermal stress, they were heated from 20 to either 65 or 85°C. The samples were kept at the specified temperatures for 30 min, and were then cooled back down to 20°C by transferring them to a 20°C water bath. To confirm the stability of the emulsion, the turbidity of each sample was determined after each cycle.

Centrifugation

The formulated nanoemulsions were centrifuged at 3000 rpm for 30 min and observed for phase separation if any. Stable samples were further analysed for heating cooling and freeze-thaw cycle.

Heating-Cooling cycle

This was performed by keeping formulated nano emulsion at 40°C and 4°C, alternating each temperature for 48 h. The cycle was repeated thrice. The stability of nanoemulsion was checked at varying temperature.

Freeze-Thaw stress

This was carried out by keeping the nanoemulsion alternatively at -4°C and 25°C for 48 h at each temperature. The cycle was repeated twice. The experiment was performed in triplicates. The formulations that passed the thermodynamic stress tests were taken for further characterization studies.

pH measurement

The pH values of the emulsion system were determined at regular intervals for every 5 min sonication at room temperature with a pH meter.

Turbidity Measurement

The turbidity of the emulsion was assessed via absorbance at 600 nm to study the optical properties of the emulsion system using UV-Vis Spectrophotometer. The sample was diluted in 1:10 ratio with double distilled water at room temperature.

Viscosity Determination

The viscosity of the formulated nanoemulsion was measured as such without dilution using a viscometer (Brookfield Viscometer, Model LVF 69726). A 20 ml of the formulation was used for viscosity determination. The speed of the spindle was adjusted to 60 rpm, and a single run was performed at a temperature of 25±0.5°C.

Particle size analysis

The optimized formulation analysed for their particle size and particle size distribution by photon correlation spectroscopy using a Zeta sizer Nano ZS (Malvern, UK) at 25°C. Samples were diluted with freshly distilled water 1:100 (v/v) to diminish opalescence. The obtained polydispersity index (PDI) values represent the particle size distribution within the formulations. PDI values below 0.2 indicate a narrow size distribution; this indicates good long-term stability due to reduction of degradation processes like Ostwald ripening. The parameters of interest were measured immediately after preparation of the formulations.

Particle surface charge (zeta potential) analysis

The particle surface charge of the formulation was determined by laser Doppler electrophoresis using a Zeta sizer Nano ZS (Malvern, UK). Zeta

potential (ZP) values of the formulations were determined at 25 ◦C. Samples were diluted with distilled water (1:100, v/v) containing sodium chloride (0.01 mmol) in order to ensure constant conductivity below 0.05 mS/cm. As distilled water alone might lead to fluctuating conductivity as solvent, addition of electrolytes ensures reproducible measurement conditions (Yilmaz and Borchert, 2005). The ZP roughly characterises the surface charge of the emulsion particles. High absolute values lead to repulsive forces between particles which might improve the stability of multiphase systems. Absolute values higher than 30mV generally indicate good long-term stability (Mueller, 1996). Zeta potential values were measured immediately after preparation of the formulations.

Scanning electron microscopy (SEM) analysis

The optimised nanoemulsion samples particle size conformation microstructure of the formulations was analysed by SEM. The samples were dissolved (1:10, v/v) in distilled water (pH 6.7); then a drop of each solution was placed on a SEM copper grid covered with a perforated carbon film and blotted with a filter paper to form a thin liquid film of the sample. The Vitrified specimens was examined in a Philips T12 scanning electron microscope (Philips) operating at an accelerating voltage of 10-20 kV using an Oxford CT3500 (Oxford Instruments). Images were recorded digitally on a cooled Gatan Bio Scan CCD camera (Gatan) using the Digital Micrograph 3.4 software (Gatan) in low-dose imaging mode to minimise beam exposure and electron beam radiation damage

ANTI MICROBIAL ACTIVITY

Culture media

Mueller Hinton Agar was used for bacteria and Sabouraud Dextrose Broth for fungi. For the agar well diffusion experiments, Sabouraud Dextrose Agar was employed. The Mueller Hinton Agar (MHA) medium was used for well diffusion assay and Mueller Hinton broth and supplemented with 10% glucose was used for the minimal inhibition concentration (MIC).

Test Microorganisms

The test microorganisms used in the study gram-positive bacteria such as *Bacillus subtilis* (NCIM 2458), *Staphylococcus aureus* (ATCC 8013), gram-negative bacteria *Escherichiae coli* (ATCC 15830) *Klebsiella pneumoniae* (ATCC11229) *Salmonella* (ATCC 871 3). The fungi used for Antifungal study are *Aspergillus niger* (NCIM 1207), *Candida albicans* (NCIM 3484).

Preparation of 24 hours pure culture

A loop full of each microorganism was suspended in about 10ml of physiological saline in a Roux bottle. Each of these was streaked on to the appropriate culture slants and was incubated at 37ºC for 24 hours except for *Candida albicans* which was incubated at 25ºC for 24-48 hours.

Standardization of microorganisms

The suspension was standardized by adjusting the optical density to 0.1 at 600 nm (ELICO SL-244 spectrophotometer). One hundred micro litres (100 µl) of cell suspension with approximately 106-108cells per millilitre was placed in petri dishes dispersed over agar.

Zone of Inhibition determination by Agar well diffusion assay

Antibacterial assay

Antimicrobial activities of the optimized formulation were first screened for their zone of inhibition by the agar well-diffusion method. Briefly, optimized formulationBHG2 and Basil oil was prepared concentration of 5 and 10µg/ml with solvent. The Mueller Hinton Agar (MHA) medium (Hi Media) was prepared and sterilised at 121°C 15 lp/sq for 20 min the autoclave. Thirty millilitres of this sterilised agar medium (MHA) were poured into each 9 cm sterile petridishes under aseptic conditions and allowed to settle. In the following, a well was prepared in the plates with the help of a sterile stainless steel-borer (8 mm diameter) two holes per plates were made into the set agar containing the bacterial culture. Each well received 200 µl of the BHG-2 and Basil oil at the various concentrations. For each bacterial strain controls were maintained where pure solvents, instead of extract as a negative control. The BHG-2 and Basil oil to diffuse for 1 h into the plates and then incubated at 37°C for 18h in inverted position. The results were recorded by measuring the zone of growth inhibition (mm) surrounding the wells. Each assay was performed in triplicates and

repeated twice. Diameters of inhibition zone less than 9 mm were recorded as nonactive.

Antifungal assay

Sabouraud dextrose agar medium (SDA) was prepared and 25ml of each was poured in to sterile universals. The universals with the broth were inoculated with different species of fungus and incubated at 28° C overnight. A total of 25ml of medium was poured into each sterile universal. Each universal was inoculated with 200 µl of different fungal species spread well and allows to set. Using a sterile cork borer 8 mm diameter, four holes per plate were made into the set medium containing fungal culture. Each well received 200 µl of the BHG-2 and Basil oil at the various concentrations and one containing distilled water. The plates were incubated overnight for 36 to 48 hr and the diameter of the zone of inhibition was then recorded if greater than 9 mm.

Determinations of Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC), which is considered as the lowest concentration of the sample which inhibits the visible growth of a microbe was determined by the micro broth dilution method. The MIC method was performed as described below on extracts that showed their high efficacy against microorganisms the well diffusion method.

Preparation of inocula

The microorganisms were sub-cultured on MHA or SDA for bacteria and fungi, respectively, follow by incubation for 24h at 37°C. Inocula were prepared by transferring several colonies of microorganisms to sterile distilled water (5ml). The suspensions were diluted in sterile distilled water were made to obtain the required working suspensions (1-5x105 CFU/ml).

Preparation of Tube

The test was performed in sterile test tubes. All the tubes received 100 µl of Mueller Hinton broth (for bacteria) or Sabouraud broth (for fungus) supplemented with 10% glucose. The 1ml of the working solution (1-10 µg/ml) of BHG-2 and Basil oil were added into the test tubes. By using a multichannel pipette, 9 ml medium was transferred and the contents of the tubes were mixed well. The 100 µl of the inoculums suspension was added to the all the tubes. One tube served as drug free controls. Each tube was covered and incubated for 24h at 37°C. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the sample which prevents visible growth.

Membrane integrity assessment

The membrane integrity of the treated culture was assessed according to the method of Hou et al (2007) with a slight modification. To explain briefly, over night bacterial culture grown at 37ºC was harvested, washed, re suspended and adjusted to1 × 108 CFU/mL with PBS. 0.5 mL of the bacterial suspension diluted with 9.5 mL of different concentrations of basil oil, BHG-2 (10µg/ml) was considered as the test sample. The bacterial suspension (0.5 mL) diluted with sodium benzoate (9.5 ml) at a concentration of 500 mg/L was used as a positive control. The suspension diluted with PBS (9.5 mL) was used as the negative control. All the samples were incubated at room temperature for 1h. The samples were centrifuged at 6000 rpm for 10 min to release the cytoplasmic contents from the bacterial cells. The supernatant was used for measuring the absorbance at 260 nm using UV-Vis Spectrophotometer. The absorbance obtained was directly proportional to the leakage of cytoplasmic contents

Optical microscopy imaging

The morphological changes in the bacterial cells after interaction with basil oil and basil oil hydrogel observed using microscope. The bacterial cell, after being treated with BHG-2, basil oil for a minute, was harvested by centrifugation for 10 min at 5000 rpm. The pellet coated onto glass piece the surface morphology of the coated sample was observed by an optical microscope at X-40 magnification.

STABILITY STUDY OF THE FORMULATION

The optimized formulations (BHG-2) was selected and subjected to the stability testing for 90 days. Formulations were kept at 40°C, 25°C & room temperature for 90 days & evaluated for following parameters

i. Physical stability: The basil oil gel formulations were evaluated in terms of physical character like phase separation appearance and colour changes. Physical stability testing was done by visual inspection of the formulation at 30 days interval for 3 months.

ii. Chemical stability: The optimized gel formulations were evaluated in terms of chemical character like pH for 3 month at 30 days interval.

RESULTS AND DISCUSSION

Solubility studies

Solubility studies for basil oil were carried out at room temperature. The equilibrium solubility performed in various solvents at the indicate was shown in the.

Boiling Point of basil oil

The Boling point of basil oil was determined by capillary method using boiling point apparatus and found to be 213.33 °C which correlates with that of standard melting

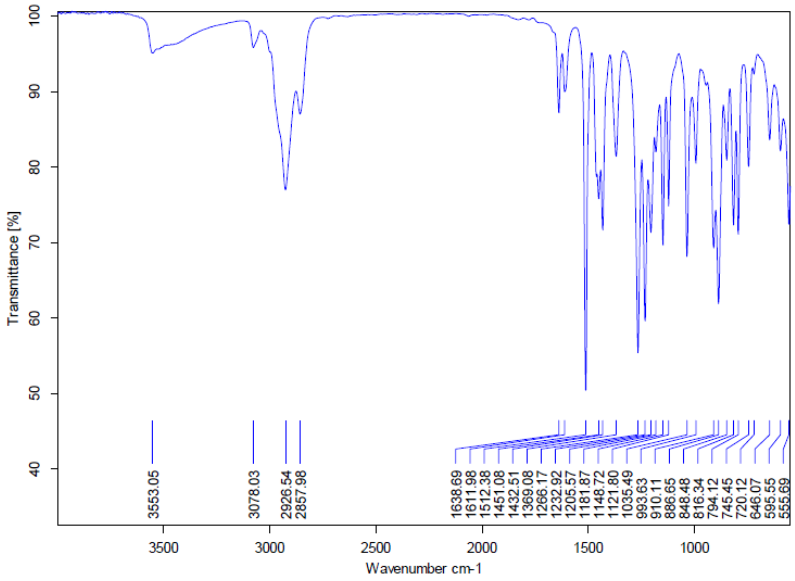
point range of basil oil.

Refractive index of basil oil

The Refractive index of basil oil was determined by Abbe's refracto meter and found to be 1.47235 which correlates with that of standard Refractive index range of basil oil.

Specific gravity of basil oil

The Specific gravity of basil oil was found determined by pycnometer and found to be0.9111 at 25° C which correlates with that of standard Specific gravity range of basil oil.



FTIR Studies Basil oil and other Excipients

Infrared band assessment of Basil oil

S.No	Functional groups	Wave number (Cm^{-1})
1	Alcoholic-OH	3650-3590
2	Alkane $-CH_3$	2972-2953

GC-MS analysis

Qualitative analysis of the basil oil, was done using GC-MS. The GC-MS chromatograph of basil oil and all the components identified in the basil oil by GCMS analysis. Estragole (p-allyl anisole) was found to be the major component of the oil with 21 % of total peak area. Other important peaks were found to be phenol-2-methoxy-6-(2-propenyl) - (Trans-Isoeugenol) (8.81 %), linalyl acetate (7.55%), eucalyptol (5.6 %), Caryophyllene (7.52 %), Levomenol (6.20 %).

SCREENING OF BASIL OILS FOR NANOEMULSION FORMATION

The formation of emulsion with a stable droplet size is mainly based on the type of oil (cloud and flavour oil) used during the emulsification process. Oil extracted from vegetables is highly viscous and crude hence termed as cloud oils whereas, oil extracted from aromatic plants exhibit less viscosity and are highly refined and so called as flavour oils. Formulation of nanoemulsion with highly viscous cloud oil resulted in unstable emulsion with a large droplet size. Therefore, cloud oils were not chosen for the study. The less viscous flavour basil oils resulted in kinetically stable formulation with droplets in the size. Hence, the flavour oils were used for nanoemulsion preparation.

NANOEMULSION FORMULATION

The nanoemulsion was prepared by using basil oil along with the surfactants (Span 60, Span 80) and water. The turbidity of the nano emulsions were measured immediately after mixing with the stirrer, whereas visual observation of emulsion were made after they are allowed to stand overnight for 24 h. The increase in the surfactant concentration from 3% to 24%resulted in the decrease in turbidity and optical properties of basil oils nanoemulsion (**Figure4.7-4.8**). The basil formulations BNE-1 to BNE-5 containing Span 60 as a surfactant exhibited good ted transmittance and appeared transparent in nature. The formulation BNE-2 with less surfactant concentration (1:1 ratio) resulted in a milky white. When using Span 80, all the formulations (BNE-6 to BNE-10) appeared milky white, and the turbidity of the sample was low transmittance.

THERMODYNAMIC STABILITY STUDY

The formulations prepared by spontaneous emulsification method were subjected to different stress tests such as centrifugation, heating-cooling and freeze-thaw cycle. The basil oil nanoemulsion (BNE-1 to BNE-5) formulated with Span60 as a surfactant, was found to be stable except the BNE-1formulation. The nanoemulsion formulations (BNE-6 to BNE-10) containing Span 80exhibited unstable emulsion. Based on these results, the stable nanoemulsion formulations of basil oil (BNE-2) containing reduced surfactant concentration (1:1) were chosen for size characterization studies.

EFFECT OF SURFACTANT CONCENTRATION ON PH AND VISCOSITY, TURBIDITY

The pH of Basil oil nanoemulsion formulations (BNE-1 to BNE-5) was increasing with the increase in surfactant concentration from 3% to 24%. The pH of the basil oil nanoemulsions formulated with the 1:0.5 1:1, 1:2, 1:3, and 1:4 ratios of surfactant span 80 and the constant oil concentration of 6% v/v were 3.17, 3.36, 3.48, 3.79 and 3.85 respectively.

The viscosity values for basil oil nanoemulsion prepared with the 1:05 1:1, 1:2, 1:3 and 1:4 ratio of surfactant span 60 were 4.12 cP, 5.51cP, 7.98cP, 11.23 cP and 14.12 cP, respectively. The increase in surfactant concentrations from 3% to 24% resulted in an increase in the viscosity of basil oil nanoemulsion. A similar trend was observed in the basil nanoemulsion prepared with surfactant span 80. Turbidity of the emulsion was expressed as absorbance at 600 nm. Absorbance was reduced with the increased surfactant concentration. With increase in surfactant, turbidity of basil oil emulsion reduced. With 6 % of basil oil and 6 % of span 60, emulsion turbidity (abs 600 nm) was 1.1610. As the surfactant increased to 12 %, 18 %, and 24 %, abs 600 nm value reduced to 0.6321, 0.0561, and 0.01201.

PARTICLE SIZE

The droplet size distribution of stable, optimized formulations of basil oil nano emulsion are given in (.The Monomodal type of size distributions was observed for optimized formulations of basil oil nanoemulsion (BNE-2). The average diameter of nano emulsions was 49.2nm. The polydispersity index for the both the nanoemulsions were 0.404, which indicates the relative homogeneity of the nanoemulsion.

PARTICLE SURFACE CHARGES ZETA POTENTIAL

The zeta potential of BNE 2 was found to be -33 mV. As far as zeta potential is concern, the particles in suspension have a large positive zeta potential, then they will tend to repel each other and there will be fewer tendencies for the particles to come together and aggregate. The particles in suspension with zeta potentials more positive than +30mV or more negative than -30mV are considered stable. However, if the particles in emulsion have low zeta potential values, then there will be no force to prevent the particles coming together and aggregating reported that the zeta potential value of BNE 2 was -33 mV, which was found to be highly stable.

MICROSCOPIC ANALYSIS OF SELECTED NANOEMULSION FORMULATION

Morphological analysis of the emulsion droplets was performed by scanning electron microscopy (SEM). The nano emulsion was subjected to ultramicroscopic analysis for detailed size characterisation. The morphology of the nanoemulsion droplets appeared spherical shape with intact structure.

ANTI BACTERIAL ACTIVITY

The antimicrobial efficacy of Basil oil and basil oil hydrogel was evaluated by agar well diffusion method. The selected basil oil hydrogel formulation (BHG) exhibited enhanced antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. From it can be observed that the (BHG) demonstrated a higher zone of inhibition (mm) than basil oil for all the strains tested. The Gram-positive bacteria (*S.aureus*) and fungi *C. albicans* were highly susceptible with zone size of 22 and 19 mm respectively to basil oil hydrogel than the Gram-negative bacteria, *E*. coli, which showed a zone of 18 mm. The basil oil showed significant activity against the tested gram-positive bacteria compared to gram-negative, bacteria, and fungus.

MINIMAL INHIBITORY CONCENTRATION

The minimal inhibitory concentration (MIC) of selected microorganism *Bacillus subtilis*, *Staphylococcus aureus, Escherichia coli* and *Candida albicans* was recorded by tube dilution method for 24 h.In BHG no growth was observed for higher concentrations of nanoemulsion (10 µL/mL), during 24 h of incubation. Among these bacteria, *Bacillus subtilis* can cause septicaemia, wound and burn infections, ear infections. *Staphylococcus aureus* can cause infective endocarditis as well as osteo articular infections. *Escherichia coli* can cause serious such as diarrhoea, dysentery, typhoid fever and other intestinal diseases to the human beings.

The *Candida albicans* leads to the formation of a bio film which is resistant to the penetration of antifungal agents. Based on total activity However, BHG was found to be active against the above Gram-positive bacteria (*B.subtillis*04µg/ml and *S.aureus*02µg/ml). The BHG act exhibit appreciable activity against Gram-negative bacterial strains.

MEMBRANE PERMEABILITY

The bactericidal activity and fungicidal activity of basil oil hydrogels evidenced by the leakage of cytoplasmic cell contents from the microorganism was expressed in terms of percentage units.An increase in the release of cytoplasmic contents was observed for all four microbial isolates. The cytoplasmic leakage was studied during the time range of 0 to 60 min for control, basil oil and BHG (10 fold) treated bacteria. The leakage of cell contents was higher for BHG treated microorganism (*Bacillus subtillis* 78% ,*Staphylococcus aureus* 81 %, *Escherichia coli* 68% and *Candida albicans* 71%) for the interaction period of 0-60 min when compared to control microorganism, (*Bacillus subtillis* 6%,*Staphylococcus aureus* 5 %, *Escherichia coli* 4 % and *Candida albicans* 4%).

CELL DAMAGE STUDY BY OPTICAL MICROSCOPE

The microscopic analysis of untreated and basil oil and BHG treated *Bacillus subtillis* was studied by optical microscopy at X40. The control *Bacillus*

subtillis cells were rod shaped bacteria that occurs short chain small clumps, or single cells and remained intact. The nano emulsion basil oil and BHG treated cells of *Bacillus subtillis* showed morphological changes with disintegration of cell structure.The microscopic analysis of untreated and basil oil and BHG treated *Staphylococcus aureus* was studied by optical microscopy at X40 . The control cells were spherical and remained undamaged. The BHG treated cells of *S. aureus* showed rigorous morphological changes with disintegration of cell structure when compare with basil oil treatment. Irregular shape and integrity of the cells were distorted in the cells exposed to the basil oil hydrogel

The ultramicroscopic analysis of untreated and basil oil and BHG treated *Escherichia coli* was studied by optical microscopy at X40 The control cells were rod shaped and remained intact. The BHG treated ells of *Escherichia coli* showed cell wall damages with breakdown of cell structure when compared with basil oil treatment. Irregular shape and integrity of the cells were distorted in the cells exposed to the basil oil hydrogel.

DISCUSSION

The two immiscible liquids, oil and water along with surfactant resulted in emulsion. The dispersed oil phase plays an important role in the formation of nanoemulsion and its stability (McClements, 2011). Oil in water (O/W) nano emulsion was formulated using basil oil by ultrasonic emulsification. Rao and McClements (2012) reported that the flavour oil contains citral which enhances the stability of nanoemulsions than cloud oils (e.g. vegetable oils). The impact of oil type on nanoemulsion formation may be due to the low viscous nature of oil. The oil in water nanoemulsions was formulated using plant based oils such as basil oil by ultrasonic emulsification method. The nanoemulsion was optimized for different process parameters such as oil type, surfactant type, surfactant concentration, oil surfactant mixing ratio, and optical properties. The Span60 based basil oil nanoemulsion milky white, highly transparent and less viscous. The viscosity of the nanoemulsion formulation was low as expected for O/W emulsion. This might be due to the high HLB value of surfactant present in the formulation that favours O/W type of emulsion. The steric effect of the non-ionic surfactant plays a vital role in the stabilization of nanoemulsion.

As the surfactant concentration increased from 3% v/v to 24% v/v, viscosity increased from 4.1 cP to 14.12. At elevated surfactant concentration, the water molecules were entrapped in cross-linking molecules of surfactants and increase the viscosity of the emulsion system. Tween 60 is non-ionic and tend to produce droplets that are stable over a wide range of pH and in varying ionic strength. Nanoemulsions were formulated by applying high energy with the use of less surfactant, and it is observed to be highly stable. With an emulsification time of 10 min, using 6 % of basil oil, and increasing concentrations of surfactants (Span 60 and

Span 80) from 6% to 24%, corresponding decrease thermal stability. Thermodynamic stability of the formulated emulsions was investigated by subjecting to centrifugation, freeze-thaw cycle, heating-cooling cycle. The kinetically stable emulsion was selected by checking droplet size. The basil oil nanoemulsionBNE-2 demonstrated highly stable emulsion formation with a reduced droplet size of 49.2nm. Testability of emulsions to coalesce and flocculate decreases as the droplet size reduces, because the strength of the attractive forces diminishes more rapidly than the strength of the repulsive forces . Basil oil based fine emulsion was turbid in nature, which is due to the lower concentration of surfactant. After subjecting to ultrasonic emulsification, the emulsion appears to be transparent with an absorbance at 600 nm. The turbidity of the basil oil emulsion reduced from 1.2301 to 0.0120 as by the sonication. Even at low surfactant concentration, sonication contributes to droplet size minimization and optical transparency of nanoemulsion. The morphology of the droplets in the formulation observed under the microscope indicates the presence of particles or aggregation and is comparatively more sensitive than laser diffraction. Droplets in the basil oil nanoemulsion were spherical in morphology in the range of 49.2 nm. The formation of spherical droplets in the emulsion is due to high Laplace pressure and curvature at the oil/water interface. The basil oil with an oil-surfactant (Span 60) mixing ratio of 1:1 v/v was used for delivery of effective antimicrobial agent. The plant oil nanoemulsion demonstrated lower droplet size and higher stability even at lower concentration of surfactant with the use of

high-energy ultrasonication method. The reduction in particle size increases the surface area that may result in greater interaction of nanoemulsion with the bacterial membrane, thereby; resulting in the enhanced antibacterial activity due to the activation of passive mechanisms of cell absorption. Our investigation based on the influence of the nano droplets size on antimicrobial activity is similar with the previous reports Antibacterial activity of basil hydrogel is due to the active ingredients present in essential oil and the reduced droplet size. Complete loss of bacterial viability was observed within 60 min of interaction with both diluted hydrogels. Several authors reported antimicrobial nano emulsion against bacteria and fungi. Upon treatment with nanoemulsion, the bacterial membrane is damaged with leakage of intracellular components which can be quantified at 260 nm. A gradual increase in absorbance was observed with increase in leakage when *B.subtilis, S. aureus E. coli, and C.albicns* cells were treated with basil oil hydrogel. It is interesting to observe that 10-fold dilution of the hydrogels treatment caused the rapid release of UVabsorbing substances. The release of UV-absorbing substances was in agreement with the killing kinetics of bacteria by hydrogels. The cell membrane upon interaction with antimicrobials frequently caused damage to both structure and function of bacteria membrane. The basil oil hydrogel fuse with the lipid bilayer cell membrane of bacteria and this fusion destabilizes the membrane integrity and function, resulting in lysis and death of the pathogen. On treatment with Basil oil Hydrogel, the bacterial cells were damaged which was confirmed by microscopic techniques. Oil in water based nanoemulsion system is attractive for topical application studies due to their biocompatibility between water and tissue. As the water evaporates, the emulsion droplets form a continuous film, thereby, promoting healing activity. By virtue of the antibacterial property of basil oil and its nanoformulation may be the reason for faster wound healing activity and non-irritancy to the skin.

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