International Journal of Scientific Research in Engineering and Management (IJSREM)
Volume: 05 Issue: 06 | June - 2021 ISSN: 2582-3930

FABRICATION AND CHARACTERIZATION OF MAGNETIC NANOEMULSION FOR BIOFILM INHIBITION

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ABSTRACT

The main objective of the present study was to elucidate the antibiofilm potential of the synthesized magnetic nanoemulsion. Biofilms are a collective of various (heterogeneous) sorts of microorganisms that can grow on completely different surfaces. Microorganisms that make biofilms comprise of bacteria, fungi and protists. The Nano-biotechnology has been widely used against pathogenic infections by using nanoparticles. In this present study, the bacterial biofilms were isolated and the formed biofilm community was inhibited by Magnetic nanoemulsion. The first step involved was the synthesis of magnetic Graphene oxide (GO-Fe3O4) nanoparticles from charcoal and it was characterized by Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM) techniques. Then, it has been conjugated with the essential oil extracted from the leaves of *Mentha arvensis*. Thus, the magnetic nanoemulsion was fabricated which has the potential of bacterial biofilm inhibition. The leaves of *Mentha arvensis* was chosen because the MIC value ranged between **3.125µl/ml to 12.5 µl/ml** and also has the anti-biofilm property. Essential oil of *Mentha arvensis* has shown good antibiofilm activity at low concentrations. The crystal violet assay was performed with essential oil, magnetic nanoparticles and also with the magnetic nanoemulsion to quantify the biofilm. From this study, it is shown that the biofilm has been highly inhibited by the employment of the magnetic nanoemulsion. The minimum biofilm inhibition concentration (BIC_{50}) was achieved at **300 µl/ml** while using the magnetic nanoemulsion.

Key Words: Magnetic nanoemulsion, *Mentha arvensis,*
Anti-biofilm, Bacterial Biofilm, Biofilm inhibition, Graphene oxide, Crystal violet assay.

1.INTRODUCTION

Biofilms are the main cause for the 80% of infections in humans such as cystic fibrosis, endocarditis and osteomyelitis. Recent development in nanotechnology has become a robust tool for dealing with biofilm infections. The plants with antibiofilm properties have been widely used nowadays to eradicate the biofilm forming organisms. Bacteria are mostly involved in the formation of biofilms. There are two different types of bacteria namely: Gram-positive Bacterium such as *Bacillus sp*., *Listeria monocytogenes*, *Staphylococcus sp*. and Lactic acid bacteria like *Lactobacillus plantarum* and *Lactococcus lactis*. Gram-negative Bacterium such as *Escherichia coli* (or) *Pseudomonas aeruginosa* are involved in the formation of biofilm. Bacterial biofilms are formed by communicating with another bacterial cells by Quorum Sensing (QS) which is a process of signaling or communication between each bacteria which results in a bacterial biofilm which is an effective infection. Biofilms can be mostly developed on the interiors of water distribution systems which is defined by the term called "Biofouling" means the microbial growth represents a contamination that may cause a threat to human health. Chlorination is the usual method employed to control biofilm growth. Super chlorination is the method in which chlorine levels are raised temporarily, and used when biofilm causes water quality problems. Although water treatment operators face a huge battle against biofilms, but also these bacterial communities can also be used to improve water quality.

Mentha arvensis commonly known as pudina (menthol mint, corn mint or wild mint) is widely cultivated in Bangladesh, Nepal, India, Srilanka, Thailand and Japan. Mint essential oil has been used to prevent the microbial growth and prolong the shelf-life of food, and also it has possible antibacterial mechanism and anti-biofilm activity against *Staphylococcus aureus*. Moreover, a crystal violet quantification assay was used to evaluate the effects of mint essential oil on inhibiting and inactivating *S. aureus* biofilms. In addition, mint essential oil significantly inhibited the formation of biofilm, and inactivated mature biofilm formed by *S. aureus*. So, this has the potential to control planktonic *S. aureus* and its biofilm in food processing environments. Graphene oxide (GO) is a promising and surprising nanomaterial that shows antimicrobial action because of its particular

surface–interface collaborations. In the current work, interestingly, we have detailed the antibiofilm property of GO-coated surfaces arranged by two distinct strategies against bacterial biofilm development. Essential oil of *Mentha arvensis* has been used to prevent the microbial growth and also it has possible antibacterial mechanism and anti-biofilm activity against *Staphylococcus aureus* (*S. aureus*). Moreover, a crystal violet quantification assay was used to evaluate the effects of mint essential oil on inhibiting and inactivating *S. aureus* biofilms. So, this has the potential to control *S. aureus* and its biofilm in the water processing environments. *Mentha arvensis* essential oil has been combined along with the synthesized magnetic graphene oxide nanoparticles (i.e., Magnetic Nanoemulsion) was an instance methodology introduced for inhibiting the growth of bacterial biofilm. The nanoemulsion produced by green synthesis of lavender essential oil and iron oxide nanoparticles will helps in controlling the development of bacterial biofilm by attaching over the cell wall and disturb their growth and development. Generally, nanomaterials have the ability to penetrate the bacterial cell membrane and eradicate bacterial biofilm.

2.MATERIALS AND METHODS

Sample Collection and Essential oil extraction

The fresh leaves of *Mentha arvensis* were collected from Thirunindravur (13.1181°N,80.0336°E), Chennai. The collected leaves were washed twice with distilled water to remove the dust particles. Washed leaves were shade dried for a period of one week, in order to prevent the denaturation of phytochemicals due to heat. Dried leaves were crisp and were able to be easily powdered using a commercial blender. The obtained dried powder was about 100 g and stored in an air tight container at 4℃ until further use. The extraction method was based on standard Soxhlet method for extraction. The use of solvent like petroleum ether gives a better yield of essential oil.

Fig.1 Collection of leaves of *Mentha arvensis*

Soxhlet Extraction

In the Soxhlet method of extraction, the sample containing some of the desired compound was placed inside the thimble made from thick filter paper, which was loaded into the main chamber of the Soxhlet extractor. The main chamber was placed on to the round bottom flask set at 100℃ containing petroleum ether (as extraction solvent). The plant extract was found to be 60ml approximately and transferred into a sterile container and kept at 4℃ until further use.

Thin Layer Chromatography (TLC)

This method is used to identify the purity of the extracted essential oil. A TLC sheet was taken and the line was marked at the top and bottom of 1 cm. At one end of the TLC sheet, a drop of the oil sample was placed with the help of capillary tube and kept in an TLC chamber which contains a solvent and left undisturbed for few minutes. Once the solvent reaches 3/4th of the TLC sheet, it was then removed from the chamber and observed under UV chamber to determine the purity of the oil.

Sample Collection for the Synthesis of Magnetic Nanoparticles

For the synthesis of graphene oxide nanoparticles, fully burnt charcoal was collected at nearby local areas, Chennai, Tamil Nadu and brought to the laboratory.

Fig.2 Collection of Charcoal

Synthesis of Graphene oxide Nanoparticles from Charcoal

The collected charcoal was then crushed into a powder by using mortar and pestle. A total of 0.5 g of charcoal was mixed with 11.5mL of 98% sulfuric acid (H_2SO_4) and 0.25 g of sodium nitrate ($NaNO_3$). Furthermore, the mixture was conditioned in an ice bath (0–10 ◦C) with stirring using a stirrer for 1hr. After that 15 g of potassium permanganate ($KMnO_4$) was added slowly for 2h and the temperature remains controlled below 10 ◦C. The suspension temperature is then raised to 35 ± 3 ◦C for 30min by keeping it in a heating mantle, while still stirring followed by the addition of 23mL of distilled water and then allowed to stand for 15min. The oxidation reaction was terminated by the addition of 70mL of distilled water and 1mL of 37% hydrogen peroxide (H_2O_2) solution and continued with rest for 24 hours in the oven.

Synthesis of Magnetic material Graphene oxide (GO-Fe_3O_4)

The procedure for making GO-Fe_3O_4 was given as follows: Graphene oxide (GO) of 0.025 g was dispersed in 50mL of distilled water under ultrasonic vibrations for 15min. The solution of 100mL containing Fe (II)/Fe (III) (2mmol:4mmol) in the form of $FeSO_4 \cdot 7H_2O$ (0.556 g) and $FeCl_3$ (0.602 g) salts were prepared and stirred constantly for 30min at 40 ◦C followed by 1 M NaOH was added to reach pH 4. The sonicated GO solution was then added to

the mixture of Fe (II)/Fe (III) and stirred for 30min. The solution was adjusted to pH 10 with 1 M NaOH and then allowed to stand for 30min. The resulting solution was poured into a column and then the precipitate was separated. Then the black precipitate was poured into petri dish and it was kept in a hot plate at 10 ◦C for 15mins.Once after the water gets evaporated, the petri dish was cooled. The magnetic graphene oxide nanoparticles were appeared as black precipitate. The powdered form of graphene oxide nanoparticles was scrubbed using a sterile blade and collected. The magnetic property of the synthesized nanoparticles was checked using a magnet.

Characterization of Graphene Oxide Nanoparticles

The synthesized magnetic nanoparticles were characterized by two techniques such as:

- Fourier-Transform Infrared Spectroscopy (FTIR)
- Scanning Electron Microscope (SEM)

Fourier Transform-Infrared Spectroscopy (FT-IR)

Fourier Transform Infrared Spectroscopy is a characterization method in which the presence of surface functional groups can be identified. This analysis was performed at Anna University, Chennai. The analysis was performed in a range of 500 cm^{-1} to 4000 cm^{-1} . Therefore, different peak values were obtained. The values were compared with the standard IR spectral chart and the corresponding functional groups of graphene oxide nanoparticles were identified.

Scanning Electron Microscope (SEM)

Scanning Electron Microscopy is a characterization method in which the three-dimensional surface image of graphene oxide nanoparticle can be obtained. The SEM analysis was performed at Anna University, Chennai using Hitachi S-4500 SEM machine. The graphene oxide nanoparticle was visualized and the image was captured at different magnification levels such as 1.00kx to 10.0kx in a micro-meter to nano-meter scale.

Fabrication of Magnetic Nano emulsion

The conjugation of extracted essential oil along with the synthesized magnetic nanoparticles forms the magnetic nano-emulsion. The magnetic nano-emulsion are becoming a widespread bacterial biofilm treating agent from the field of Nano-technology. In an Eppendorf, around 20 mg of magnetic nanoparticles were taken and to that 2 ml of essential oil was added and mixed gently, which gives us magnetic nano-emulsion.

Collection of Biofilm forming bacteria

Biofilm forming bacteria was collected from the water tank in the local areas of Pallavaram (12.96°N,80.14°E), Tamil Nadu, India. The biofilm has been collected with a sterile eppendorf tubes. The collected sample was then brought to the laboratory and stored at 4 °C before use. Furthermore, the collected water tank samples have been cultured for purpose of isolation of pure colonies.

Fig.3 Collection of water tank sample

Serial dilution and spread plating

Initially, the collected biofilm sample was serially diluted from 10^{-1} to 10^{-9} concentrations including control, followed by the LB (Luria Bertani) media was prepared and sterilized in an autoclave. Once after the media gets solidified, spread plating was performed by inoculating the 10^{-3},10^{-5} and 10^{-7} concentrations into 3 petri plates and it was incubated for 24 hours in an incubator. Luria Bertani media was preferred mostly because only our desired bacteria would grow in the plate.

Streaking of isolated colonies

On the next day, the formed bacterial colonies were isolated and inoculated in the three test tubes each containing 3 ml of Nutrient Broth. It was then incubated for 24 hours. Turbidity was obtained in the inoculated tubes. With this culture, plates were streaked (Quadrant streaking) in the nutrient agar and kept in an incubator for 1 day.

Slant culture preparation

Followed by slant culture was prepared with the nutrient agar in tubes for obtaining the pure colonies of bacteria. The isolated colonies were then inoculated into Luria Bertani broth where the biofilm formation was seen very clear in the tubes.

Formation of biofilm

Fresh culture was used for the bacterial biofilm formation. To the test tube, 3ml of LB broth was prepared and sterilized in an autoclave at 121° C for 15 mins. 100 µl of the existing biofilm culture was added to it and kept in an incubator for 24 hrs.

Crystal Violet Assay

Crystal violet assay is a quantitative protocol which is used in quantification of biofilm mass and bacterial viability in biofilm.

METHOD 1: Using Essential oil

For the inoculation of test samples, the essential oil was prepared in the three different concentrations such as 30µl, 90µl, 270µl and these were added as triplets sets in tubes.

- 30µl of oil, 270µl of ethanol was added and mixed

gently until there is no separation of two layers. To that, 600µl of PBS (Phosphate Buffer Saline) was added.

- 90µl of oil, 210µl of ethanol was added and mixed gently. To that, 600µl of PBS was added and mixed gently.
- 30µl of oil,270µl of ethanol was added and mixed gently. To that, 600µl of PBS was added.

STEP 1: Inoculation

Initially, 12 test tubes were taken and labelled as control, negative control 1&2, and the remaining were test samples which were labelled as triplets sets. Control has only 3ml of LB broth whereas negative control 1&2 has broth along with 100µl of culture. In the remaining test tubes, different concentrations of prepared oil concentrations were added as triplets into the tubes. These test tubes were kept in a shaker for 2 hours and after that it was incubated in an incubator for 2 days. After 48 hours of incubation, the biofilm formation was clearly visible.

STEP 2: Staining the biofilm

After incubation, the cells were removed from the tubes by pipetting it out without disturbing the biofilm. The tubes were kept inverted to remove out the excessive cells present in it. They were air dried for 15 mins. After that, crystal violet was added to all the test tubes at the level above the biofilm formation. It was kept undisturbed for about 30 mins to stain the biofilm. Once after the biofilm has been stained with the crystal violet, it was the removed and the tubes were kept inverted.

STEP 3: Quantifying the Biofilm

Then, 30% of glacial acetic acid was added to all the test tubes at the level above the biofilm. The formed biofilm gets dissolved during the addition of glacial acetic acid. The absorbance readings were taken at 570 nm under the UV visible Spectrophotometer.

METHOD 2: Using Magnetic Graphene oxide nanoparticles

20 mg of magnetic nanoparticles were weighed and to that 2ml of PBS was added and mixed well. The test samples used were magnetic nanoparticles. They were added to the triplets sets at different concentrations such as 100µl ,200µl ,300µl. From this, different concentrations were added into triplets sets. The above procedure was followed to perform the assay. But here, the test sample was the magnetic nanoparticles. The assay was performed as given above and the OD values were noted. The biofilm inhibition was quantified by this assay.

METHOD 3: Using Magnetic nanoemulsion

The nanoemulsion was prepared by combining 20 mg of the magnetic nanoparticles and 2ml of the essential oil and mixed gently until there is no separation of oil layer. The test samples were the magnetic nanoemulsion. They were added to the triplets sets at different concentrations such as 100µl ,200µl ,300µl.The same procedure was repeated but the only difference is that the test sample varies i.e., Magnetic nanoemulsion. The absorbance readings were noted and the biofilm inhibition was quantified.

On comparing all above three assay procedures, the biofilm was highly inhibited by the use of the Magnetic nanoemulsion i.e., Magnetic graphene oxide nanoemulsion.

3.RESULTS

Soxhlet Extraction

The shade dried leaves of ***Mentha arvensis*** was powdered and it weighed about 100g which was used for the extraction process by Soxhlet apparatus using Petroleum ether as solvent. (Hot Percolation method of extraction). The following figure 4 shows the Soxhlet extraction process.

Fig.4 Extraction process using Soxhlet Apparatus

Extraction of Essential oil

Once after the completion of extraction process, the obtained extract was collected and placed in a hot plate until the excessive solvent gets evaporated. The essential oil was collected. Figure 5 shows the extraction of essential oil.

Fig.5 Essential oil extraction

Thin Layer Chromatography

The extracted essential oil was 99% pure which was determined by performing Thin layer chromatography.

Synthesis of Graphene oxide Nanoparticles

The Graphene oxide nanoparticles were synthesized from charcoal and the precipitate obtained was separated using the separating funnel. Only the graphene oxide nanoparticles will settle at the bottom and which was collected.

Synthesis of Magnetic material Graphene oxide (GO-Fe3O4)

The following Fig.6 represents the magnetic graphene oxide nanoparticles. The dark black colored spots were identified as the magnetic graphene oxide nanoparticles and the remaining were unreacted Ferric chloride. Magnetic separation was performed with the help of the magnet. Only the particles which were magnetic moves along with the magnet which gets separated easily. Thus, the powdered form of magnetic GO-Fe3O4 nanoparticles were separated.

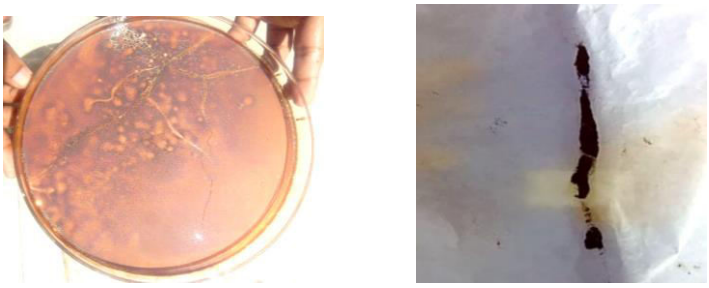


Fig.6 Magnetic GO-Fe3O4 nanoparticles

Characterization of Graphene Oxide Nanoparticles

- ✓ Scanning Electron Microscopy (SEM) and
- ✓ Fourier Transform Infra-Red Spectroscopy (FT-IR)

Fourier Transform Infrared Spectroscopy (FT-IR) of Graphene oxide nanoparticles

The functional groups present at the surface of the nanoparticles were identified at various transmittance peaks. The following Fig.7 represents the FTIR data of the graphene oxide magnetic nanoparticles.

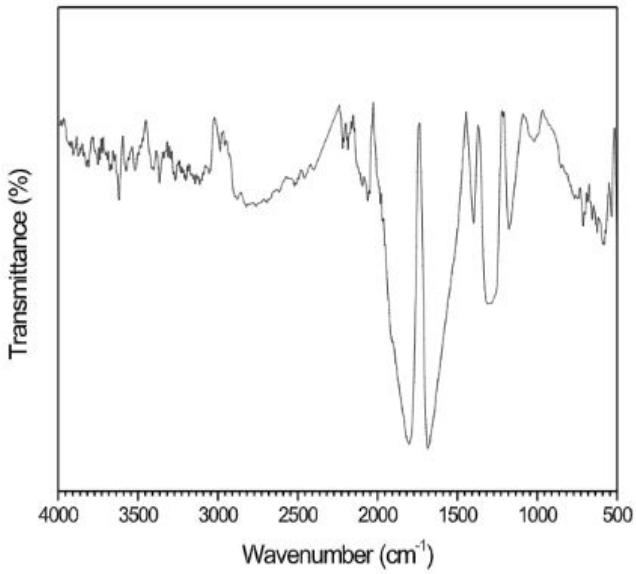


Fig.7 FT-IR absorbance plot of GO-Fe3O4 nanoparticles

The peak values were compared with Standard IR chart and different functional groups were identified (Table 1). Therefore, the absorbance peak values were compared with the standard IR chart and surface functional groups present in the graphene oxide nanoparticles were identified, which confirms the presence of GO-Fe3O4 nanoparticles.

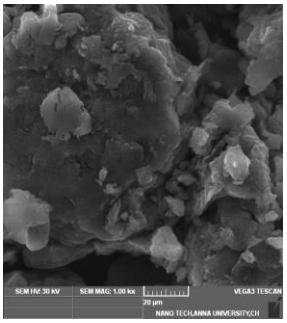
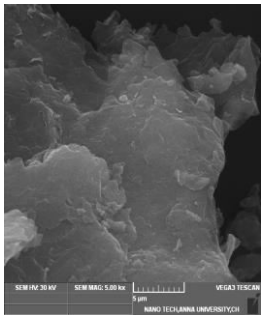
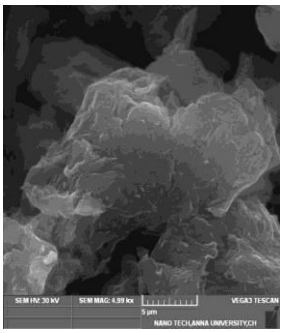
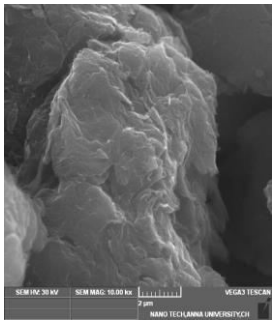
Table 1: FT-IR Functional groups present in GO-FE3O4 nanoparticles

Absorbance peak (cm^{-1})	Functional group	Compound class	Appearance
602.14	C-I stretching	Halo compound	Strong
801.20	C-Br stretching	Halo compound	Strong
1320.86	O-H stretching	Carboxylic acid	Medium
1400.30	O-H bending	Alcohol	Medium
1600.22	N-H bending	Amine	Medium

1800.24	C=O stretching	Conjugated acid halide	Strong
2200.12	CHC stretching	Alkane	Weak
3400.10	N-H stretching	Aliphatic primary amine	Medium

Scanning Electron Microscopy (SEM) of Graphene oxide nanoparticles

The GO-Fe3O4 nanoparticles were characterized by Scanning Electron Microscope (SEM) and three-dimensional images of the nanoparticles were obtained at the nanoscale. The nanoparticles were visualized at a magnification of 1.00kx,5.00kx,4.99kx,10kx,9.99kx and 10.0kx at an amplification of 30kv in a micrometer scale to nanometer scale which were shown in the Fig.8. Therefore, the three-dimensional images obtained by SEM analysis confirms the obtained particles are magnetic graphene oxide nanoparticles.

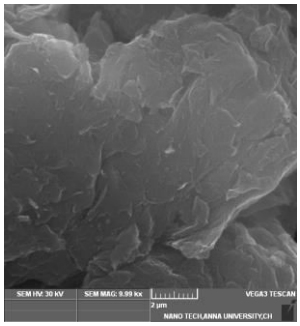
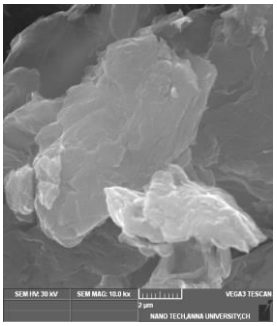



Fig.8 SEM Results

Fabrication of Magnetic Nanoemulsion

The following Fig.9 shows the fabricated magnetic nanoemulsion.

Fig.9 Figure shows the Magnetic nanoemulsion

Isolation of Biofilm forming bacteria

The sample was serially diluted as shown in Fig.10 and spread plating was performed as shown in Fig.11.To obtain pure colonies of bacteria, streaking of the culture and also slant culture was prepared which was shown in the Fig.12 and fig.13 respectively.

Fig.10 Serial Dilution of sample

Fig.11 Spread plating

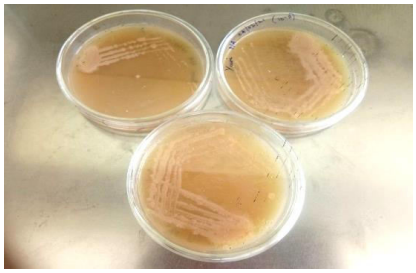


Fig.12 Streaking of isolated colonies

Fig.13 Slant culture

Fig.14 Formation of biofilm

Further, the existing culture was again freshly cultured in order to obtain the biofilm formation as shown in the figure 14.

Crystal Violet Assay

Crystal violet assay was performed in three ways such as:

1. Using Essential oil
2. Using Magnetic Nanoparticles
3. Using Magnetic Nanoemulsion

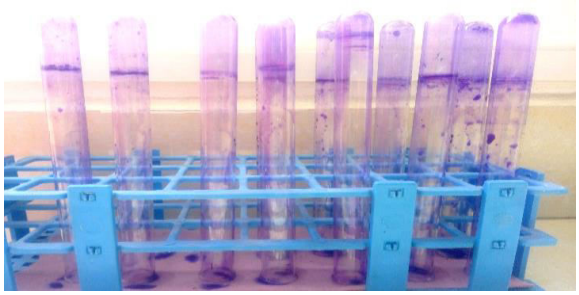


Fig.15 Crystal violet assay using Essential oil

Fig.16 Crystal violet assay using Magnetic Nanoparticles

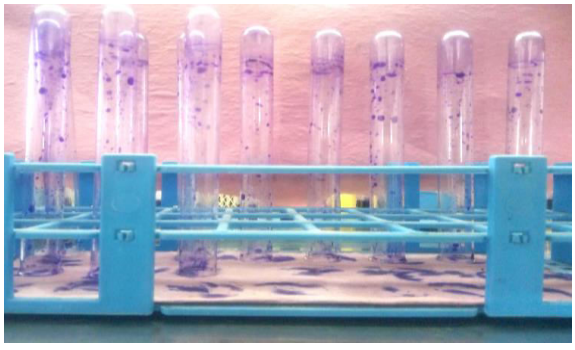


Fig.17 Crystal violet assay using Magnetic Nanoemulsion

Figures 15, 16 and 17 represents the results of Crystal violet assay performed using essential oil, magnetic nanoparticles and magnetic nanoemulsion respectively. On comparing the above three ways of crystal violet assay, the assay performed using the magnetic

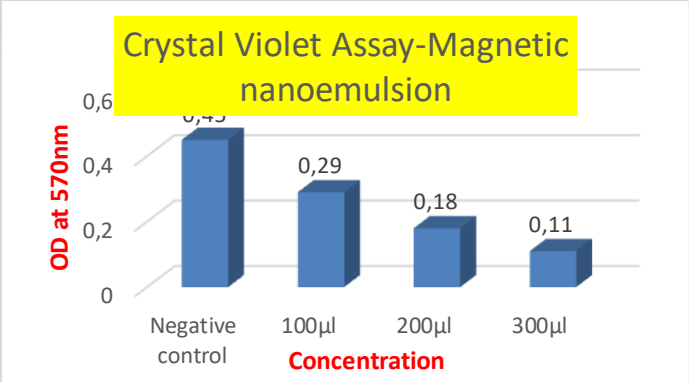


Table 2: UV readings of crystal violet assay at 570nm

Concentration of Sample	Using essential oil	Using Magnetic nano particles	Using Magnetic nano emulsion
Negative control	0.46	0.465	0.45
100µl	0.79	0.60	0.29
200µl	0.72	0.46	0.18
300µl	0.49	0.30	0.11

On comparing the above absorbance values, the biofilm inhibition was high in the use of magnetic nanoemulsion. A graph was plotted between the concentration of the test sample used in the x-axis and the absorbance values at 570nm in y-axis. With the help of the graph plotted, the biofilm inhibition can be quantified by this assay. The graphical representation of the assay was shown in the following figures.

Fig.18 Graphical Representation of Crystal Violet Assay using Essential oil

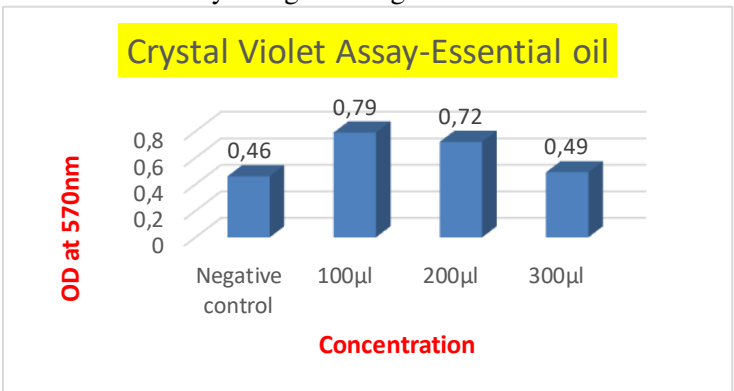
Fig.19 Graphical Representation of Crystal Violet Assay using Magnetic nanoparticles

Fig.20 Graphical Representation of Crystal Violet Assay using Magnetic nanoemulsion

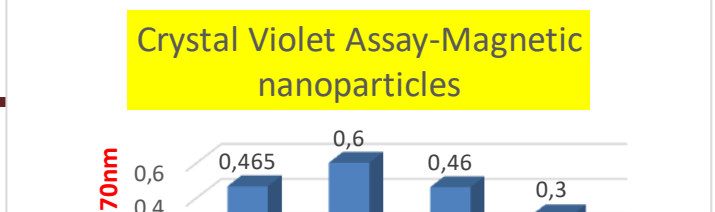
On comparing the above plotted graphical representations of the crystal violet assay, the biofilm inhibition was high while using the magnetic nanoemulsion at a concentration of 300µl.At a concentration of 300µl,the biofilm concentration was quantified as 0.11.

4.DISCUSSION

The study was performed to analyse the inhibition of the biofilm by using the magnetic nanoemulsion. As



pathogens. Thus, the synthesized magnetic nanoemulsion possess anti-biofilm activity which was proved by Crystal violet assay. This study was a combination of nanotechnology and microbiology in



effective methodology to treat the biofilm infections in the water processing environments.

5. CONCLUSIONS

Large group of traditional medicinal plants have been mostly used for different medicinal properties, which have a greater potential to cure various diseases. Furthermore, various extracts from different medicinal plant parts such as leaves, flowers, essential oils, root, and barks were also found to possess the anti-biofilm activity. This review can contribute to the development of a new approach to prevent and to treat biofilm infections. This project has been focused on fabricating an effective magnetic nanoemulsion.

ACKNOWLEDGEMENT

I would like to thank Dr.P.Dhasarathan and Mr.D.Shanmugha Sundhar for helping me to find this interesting topic.

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