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BIOLOGICAL EVALUATION OF AZELAIC-CETRIMIDE LOADED MONTMORILLONITE WITH EXPANDED GRAPHITE NANOEMULSION

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ABSTRACT

These work on improving the antibacterial and antifungal qualities of Montmorillonite (MMT) clay by intercalating cetrimide and azelaic acid. The dispersibility and stability of the prompting nanoemulsion are more enhanced by the addition of expanded graphite (EG). Techniques for characterizing completed intercalation include FT-IR, Raman spectroscopy, TEM, DLS, SEM, and XRD confirmation. Appropriate techniques have been used to ensure the effectiveness of the formulation and maintain the properties of the nanoemulsion. Emphasizing the possible medical and environmental uses, biological tests indicated notable antibacterial action against bacteria. Azelaic-Cetrimide-MMT in concert with EG offers a fresh method for creating sophisticated antibiotics. The basis for the next studies on nanoemulsion-based antimicrobial systems with improved stability and performance is established by this work.

Keywords: Montmorillonite, Azelaic Acid, Expanded Graphite, Cetrimide, Nanoemulsions, Antibacterial, Antifungal

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INTRODUCTION

Modern improvements in analytical applications have focused on utilizing nanotechnology to develop nanoemulsions, which enhance bioavailability, ensure cosmetic biocompatibility, and increase antimicrobial effectiveness. Better chances to address a number of problems have been made possible by the field of nanotechnology. In order to mitigate these dangers, it is therefore very desirable to synthesize novel materials. Microorganisms can easily attach to surfaces[1]. Natural flake graphite, a crystalline carbon allotrope, exhibits exceptional electrical and thermal conductivity due to its layered graphene structure.[2-5] Expanded graphite was a versatile carbon-containing material with pores and a large specific surface area[6-9], widely used in industries for sealing, absorption, flame resistance, and packing due to its lightweight, flexibility, and absorption properties.[10-14] EG is characterized by its unique properties and superior thermal and chemical stability.[15-17] With its non-polar surface, EG is excellent for absorbing non-polar and macromolecular compounds, particularly various types of oil.[18-19]It is typically prepared through a process involving the sequential intercalation and drying of natural flake graphite to obtain expandable graphite. It plays a role in several fields as an inorganic material owing to its excellent properties.[20-21] Rapid heating of high-temperature graphite intercalant compounds to separate the carbon layers, expanding them up to 300 times the original graphite crystal layers[22], making it useful in medical materials[23] and various other fields.[24-25]Driven by its antibacterial[26] and anti-inflammatory[27] characteristics, azelaic acid [$HO_2C(CH_2)_7COOH$] is a saturated, straight-chain dicarboxylic acid showing great biological activity. Its therapeutic value is improved by its capacity to permeate and absorb the skin. Apart from that, medical formulations of Azelaic (Yu, Wu, and Chen, 2015)acid in different dosages match the degree of acne and demand for the highest efficacy, compliance, and cosmetic acceptability.[28-29] Though non-hazardous and fit for medical usage, azelaic acid is used in dermatological therapies outside of its biological relevance.[30-32]Quaternary ammonium compounds (QACs) are cationic surfactants utilized for the preparation of organoclays, lending the clay surface a hydrophobic character coupled with antibacteriostatic capabilities, thereby providing a broad spectrum of activity[33]. Particularly against E. coli and Staphylococcus, pure forms of quaternary ammonium salts have effective killing power and

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contribute antiseptic qualities with short-term antibacterial action.[34-36] Because of their harmful effects, controlling germs in many sectors is vital; but, growing bacterial resistance to antibiotics has led to the quest for new biocidal agents, including less prone to resistance macromolecules and organoclays.[37-39] Whereas cetrimide improves permeability by interacting with skin components[40–43], the nitrogen-containing alkyl long chain influences the antibacterial effectiveness of QA salt.[44] Because of their structural structure of silicate sheets on a nano-scale, with a changeable basal distance-clay minerals are also known as layered. Because of their special properties, including chemical inertness[45], they were frequently utilized in human and health formulations as active ingredients. Two-dimensional materials like clay and graphite organize themselves, which generates worldwide interest in their monolayer exfoliation. These materials create highly anisotropic colloidal particles with a thickness of less than a nanometre and a strong aspect ratio that resemble plates. High sorption and ion exchange powers of clay are well-known. [46–48]Rich in natural content, nontoxic, high selectivity nature, MMT has a crystalline form among one of the most often occurring clay minerals[48-50] and advantage in medicines, aesthetic[51], as a result, a significant class of antibacterial medicines that have demonstrated efficacy in both preventing and treating illnesses resistant to many drugs are clay minerals treated with transition metal ions and cationic surfactants. MMT is widely recognized for its strong adsorption capacity, high exchange, and swelling capacities, making it a versatile porous sorbent material.[52-57] MMT can adsorb bacteria and has been used as the original material to prepare intercalation compounds with quaternary ammonium surfactants.[60] Mont-GO has been maximum adsorbent capacity.[61]Nanoemulsions are kinetically stable systems obtained by shearing a mixture of two immiscible liquids with a surfactant.[62] Recent trends in nanotechnology emphasize the development of nanoemulsions for enhanced drug delivery, improved bioavailability, and cosmetic applications.[63-65] Therefore, this study focuses on the biological application of Azelaic acid-QACs loaded MMT with EG nanoemulsion, investigating its biocompatibility and antimicrobial efficacy.[66-67] Emulsification is achieved using a variety of methods, including high-energy emulsification methods. Nanoemulsions are the recommended process that is self-formed. Which achieves the required equilibrium of the system, and its divergence process is also small.[68] The nanoemulsion exhibits acceptable biocompatibility, making its suitable candidate for further development in dermatological applications.

EXPERIMENTAL

Materials and Methods

Gremech Microniser, located in Navsari, Gujarat, India, supplied the 50-mesh natural flake graphite. Linseed oil was acquired from Young Chemist Pvt. Ltd., Ahmedabad; cetrimide was acquired from Sigma-Aldrich chemicals; azelaic acid ($HO_2C(CH_2)_7COOH$) was acquired from Oxford Lab Pine Chemical LLP; and montmorillonite (Banga Holding, Gujarat). The following were utilised as supplied: deionised water, 30% H_2O_2 hydrogen peroxide, potassium permanganate ($KMnO_4$), acetic acid (HAc), ethyl alcohol (C_2H_2OH), and perchloric acid ($HClO_4$).

Expanded Graphite Preparation

Peroxidation of natural flake graphite was the first step. Using magnetic stirring, a 30% H_2O_2 solution was thoroughly combined with a certain quantity of flake graphite for a brief time. The mixture was then left in a biochemical incubator at 25°C for 6 hours to produce the preoxidized graphite. Following this time, the graphite was cleaned with purified water and allowed to dry at 60°C for four hours. A significant oxidation and intercalation process made up the second stage. An $HClO_4$ solution was blended with a specified quantity of $KMnO_4$, uniformly stirred for two minutes. After adding the preoxidized graphite, the mixture was agitated for five minutes. For half an hour, the system was subjected to heat 40°C in a water bath. After that, HAc was added, and the reaction proceeded under the same circumstances for a further half hour. To produce expandable graphite, the resultant mixture was cleaned with distilled water, then dried for 24 hours at 70°C [20] (Fig.-1b). The last phase involved heating a quartz beaker for five minutes at 600°C in a high-temperature furnace. The quartz beaker was then promptly filled with the dry expanding graphite. To create expanded graphite (EG), the muffle furnace door was closed and the graphite was left to expand for 30 seconds (Fig.-1c).

Nanoemulsion Preparation

Making a 1:1 Ratio Nanoemulsion

50 milliliters of deionized water were mixed with a 1:1 mixture of expanded graphite (EG) and montmorillonite (MMT), and the mixture was sonicated for 30 minutes. To create an alcoholic solution, 1 g of cetrimide and 1 g of azelaic acid were separately dissolved in 10 mL of water. The EG/MMT mixture was then mixed with the alcoholic solution. The nanoemulsion was then prepared by probe sonication.

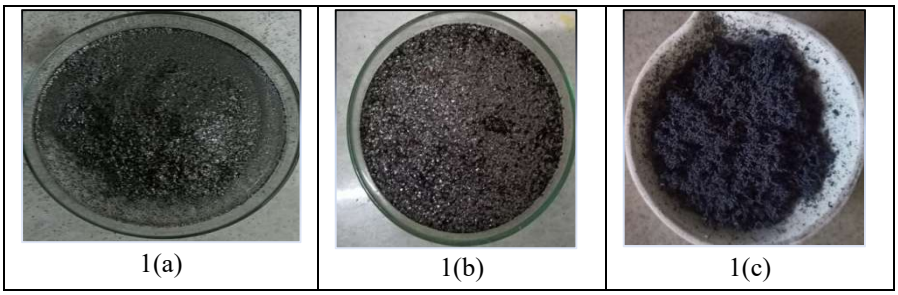


Fig.-1:(a) Natural Flake Graphite, 1 (b) Expandable Graphite, 1 (c) Expanded Graphite.

Making a 1:0.5 Ratio Nanoemulsion

50 ml of deionized water was mixed with a 1:0.5 mixture of expanded graphite (EG) and montmorillonite (MMT), and the mixture was sonicated for 30 minutes. To create an alcoholic solution, 1 g of cetrimide and 1 g of azelaic acid were separately solubilized in 10 mL of water.The EG/MMT mixture was then mixed with the alcoholic solution. The nanoemulsion was then prepared by probe sonication.

Characterization Method

Functional groups were identified by FT-IR analysis of the Azelaic-Cetrimide with EG/MMT nanoemulsion in the 4000-400 cm^{-1} range, and the compounds' presence was confirmed by Raman spectroscopy. Dynamic light scattering is used to track the nanoemulsion particle size. To verify the materials' purity and crystallinity, X-ray diffraction assessment was carried out using a Rigaku D/max 40 kV diffraction spectrometer. To examine the structural morphology of the nanoemulsions, TEM pictures and SEM - EDS mapping were captured using a Hitachi PU.

Antimicrobial Activity

The antibacterial activity of synthesized nanoemulsion samples of azelaic-cetrimide with EG/MMT nanoemulsion was investigated. According to McFarland standards, all test bacterial strains were adjusted to 0.5 after being cultivated in the nutrient broth for the entire night at 37°C. Under sterile circumstances, 100 µL of gram-positive and gram-negative strains were spread out on each nutrient agar plate. The synthesized Azelaic-Cetrimide with EG/MMT was injected into each well of a 10 mm diameter. After the plates were incubated for 24 hours at 37°C, the diameter of the inhibition zone was measured.[69]

RESULTS AND DISCUSSION

FTIR Analysis

The carbon structure's vibrational frequencies were used to characterize it using Fourier Transform Infrared spectroscopy. The C-O-C stretch vibration in EG is represented by distinctive peaks at 2360 cm^{-1} in the FTIR spectra, which is displayed in Fig.-3. Strong absorption band is also shown by the 891 cm^{-1} and 906 cm^{-1}, which are frequently ascribed to the vertical bending of C-H in alkenes and conjugated ring structure. The bending vibration of the Al-OH group in MMT is shown by the peak at 914 cm^{-1}. The bond stretch vibrations of Si-O bonds are signified by bands at 1008 cm^{-1}, 1031 cm^{-1}, which are compatible with MMT.[70] The vibration linked to the carbonyl group is responsible for the peak at 1105 cm^{-1}, 1946 cm^{-1}. This could be the result of surface functionalization with the carbonyl group. Peaks at 1188 cm^{-1} and 1292 cm^{-1}, 1946 cm^{-1}, and 1776 cm^{-1} are referred to the -C=O in carboxylic acid and the $–CH_3$ bending and C-N stretching conjugated system of the emulsion, and combination bands in Expanded Graphite are responsible for the peaks at 1892 cm^{-1}, 1992 cm^{-1}, and 2017 cm^{-1}. Alkyl chains in quaternary ammonium and azelaic acid are represented by peaks at 2565 cm^{-1}, 2754 cm^{-1}, 2852 cm^{-1}, and

2952 cm-[1], which show alkynes C-H stretching vibrations and symmetric and asymmetric –CH2 stretching.[71] The existence of free hydroxyl groups in both samples is suggested by the high-frequency peak range between 3691 cm-[1] and 3695 cm-[1]. Furthermore, no new peaks were found, suggesting that expanded graphite and MMT were physically compounded with cetrimide and azelaic acid without any chemical processes taking place. As a result, expanded graphite and montmorillonite show excellent chemical compatibility with cetrimide and azelaic acid.

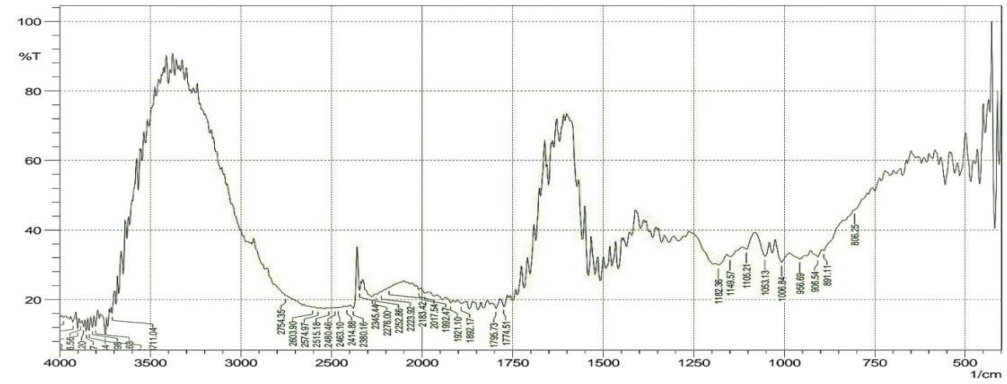


Fig.-1(a): Expanded Graphite

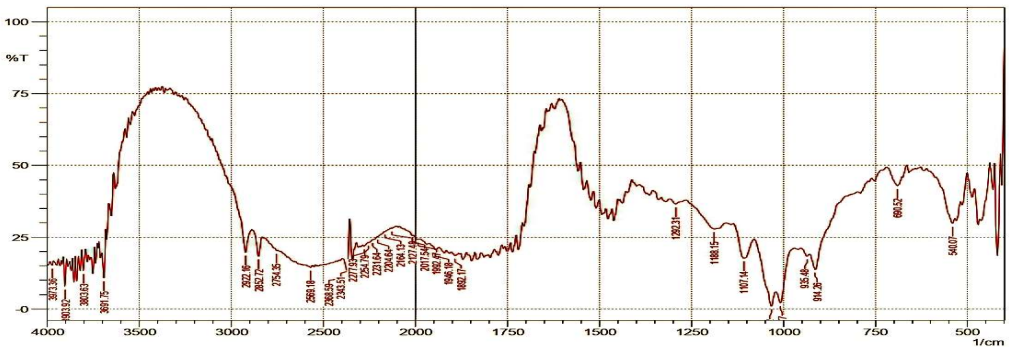


Fig.-2(b): 1:1 Ratio of Nanoemulsion Sample

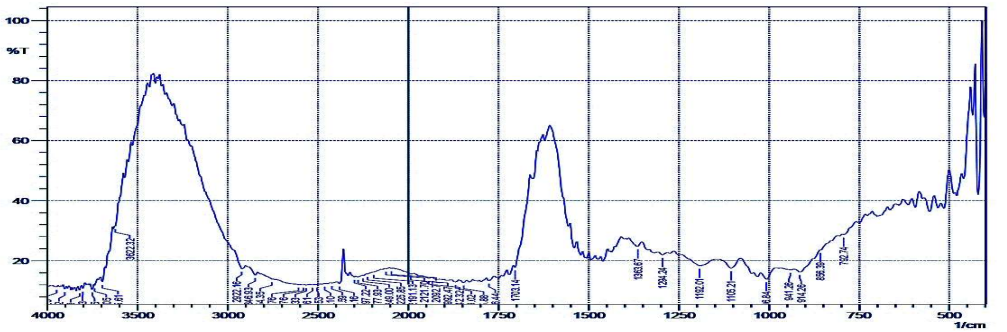


Fig.-2(c): 1:0.5 Ratio of Nanoemulsion Sample

Raman Shift

One of the most effective and instructive methods for examining the disorder of carbon material is Raman spectroscopy. The Raman spectra (inviva reflex, 532 nm) for EG nanoemulsion samples of 1:1 and 1:0.5 were shown in Fig.-3(a). The graphite layers' increased interlayer spacing results in the EG's G peak at

1583 cm-[1], D peak at 1364 cm-[1], and 2D peak at 2723 cm-[1]. In the case of EG, the defect caused by oxidation and intercalation in the graphite structure also results in a shoulder in the G peak on the left side. The production of sp2 carbon atoms at higher rating temperatures and layer structures is indicated by an increase in ID/IG and I2D/G of EG 0.13 to 0.50.[72-73] The 1:1 and 1:0.5 nanoemulsion spectra show a G peak at 1583 cm- and a D peak at 1364 cm- and 1365 cm-, as shown in Fig.-3(b) and (c). For the 1:1 and 1:0.5 ID/IG nanoemulsion samples, the corresponding ratios are 0.13, 0.52, and 0.13. By the presence of a long alkyl chain, which is a feature of the QA molecule, the spectra of Aza show C-C stretching bands at 1059 cm- and C-H bands at 2926 cm-[1] and 2928 cm-[1].[74]

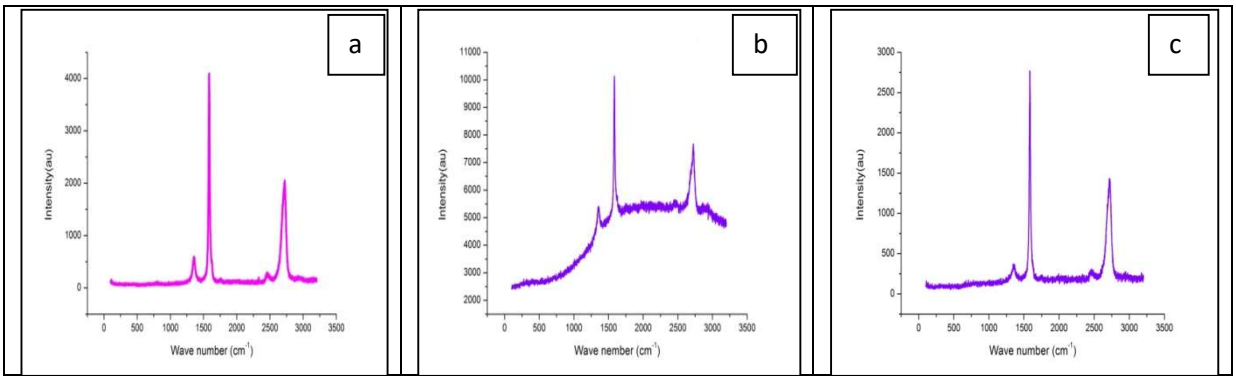


Fig.-3:(a) EG, (b) 1:1 Nanoemulsion, (c) 1:0.5 Nanoemulsion

XRD Analysis

Figure-4 displays the nanoemulsion sample's XRD pattern. The (100), (002), and (004) crystal planes were represented by the three distinct distinctive diffraction peaks of 1:1 ratio sample 1, which are situated at 2θ equal to 21.88° (d=4.06Å), 26.73° (3.33 Å), and 55.02° (1.66 Å) accordingly. Sample 2's 1:0.5 ratio 2θ value is 21.59° (100), 26.65° (002), and 29.58(211) °, while the distances between the lattice planes (d) are 7.28 Å, 3.341 Å, and 3.017 Å. The good crystallinity of the EG was suggested by the significant reflection at 2θ equal to 26.73, (002) plane.[21] The successful intercalation of EG with the crystal structure of azelaic cetrimide was confirmed by the XRD measurements.

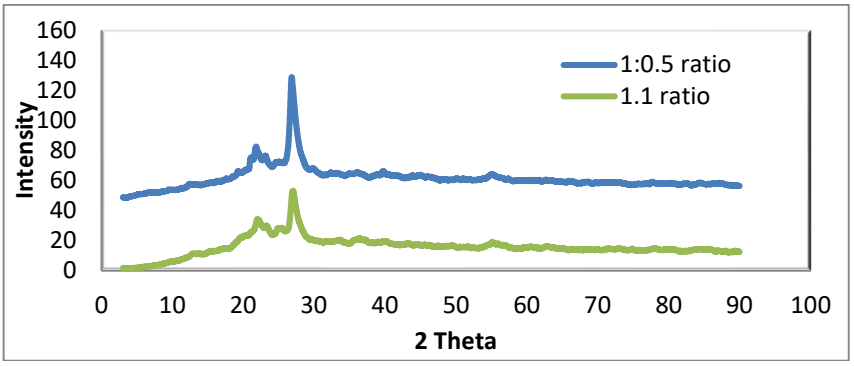


Fig.-4: XRD Graph

Dynamic Light Scattering (DLS)

A particle size analyzer (Lite sizer 500) was used to ascertain the nanoemulsions' particle dispersion. The temperature was 250, and the scattering cell was surrounded by a refractive index (n=1.33) that matched the filtered (0.1µm) bath. Dynamic light scattering was applied to examine the Aza-cetrimide particle size distribution with EG/MMT. A common method for determining the size distribution of suspended tiny particles. When applied to nanoemulsions, DLS provides crucial information about the size and arrangement of droplets in the emulsion. The sample showed a wide size distribution, with the majority of the particles distributed within a small range, as shown by the data shown in Fig.-5a& b. The average diameter and size of the particles were roughly 0.71 nm, 1116.5 nm, and 2074 nm, respectively.[74]

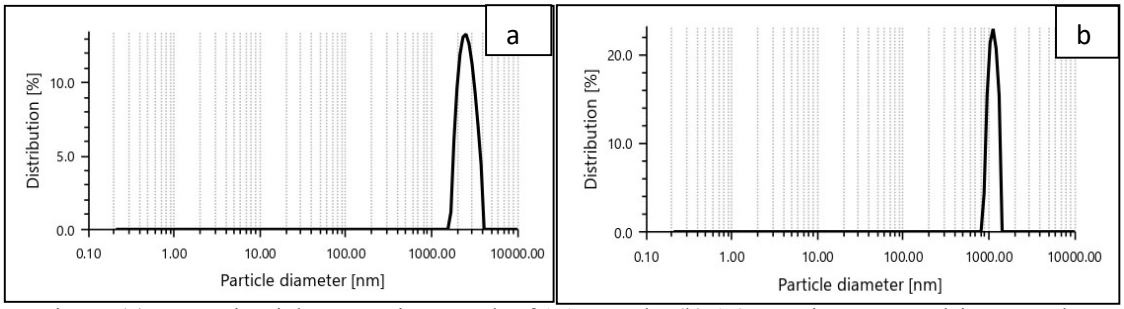
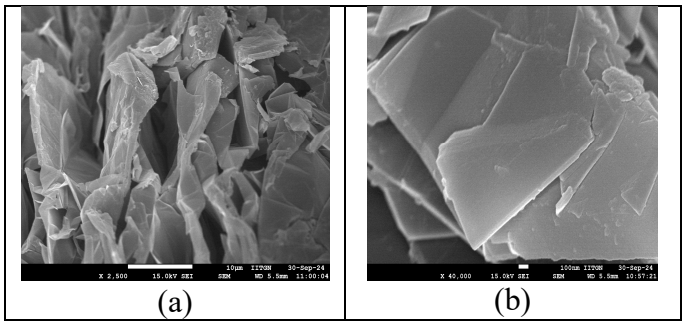


Fig.-5: (a) Dynamic Light Scattering Result of 1:1 Sample, (b) 1:0.5 Ratio Nanoemulsion Sample

FE-SEM Morphology

The surface morphology of the Azelaic-Cetrimide deposited in expanded graphite was assessed using FE-SEM morphology. The nanoemulsion particles had a distinct spherical shape and were uniformly distributed throughout the gel foundation. The gel layer barrier was formed by the embedded pores in the alginate gel. It was evident from Fig.-6(a) and (b) that the layer spacing was increased and that the EG multilayer structure contained several pores [9,21]. SEM-EDS mapping (EDS-Oxford INCA ENERGY 250 EDS) of EG was shown in Fig.-6(c).



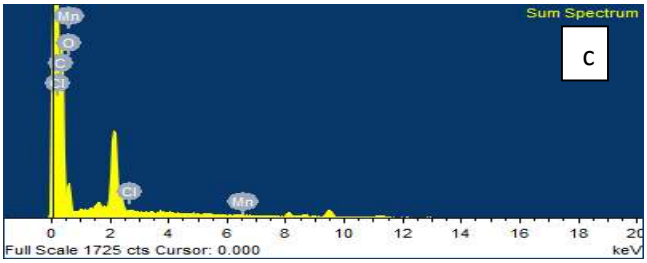
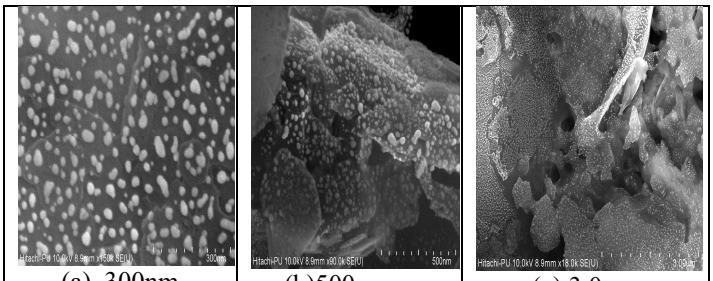
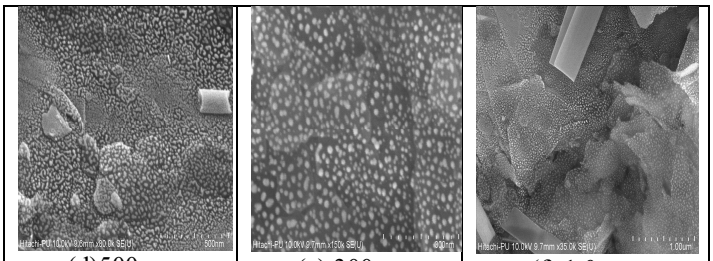


Fig.-6: (a &b) FE SEM Images and (c) EDS Mapping of Expanded Graphite





(d)500nm (e) 300nm (f) 1.0 µm

Fig.-7: (a)-7(c), sample-1:0.5; 7(d)-7(f)

Azelaic-cetrimide pores with MMT are seen inside Fig.-7(a) to (e), which are the result of solid-liquid products changing during thermal exfoliation. It was discovered that the surface of the nanoemulsion and the spaces between the enlarged graphite layers were loaded unevenly in SEM pictures.

HR TEM Analysis

The size and shape morphology were studied via high-resolution transmission electron microscopy,using the JEM 2100 plus model,Fig.-8(a-b).

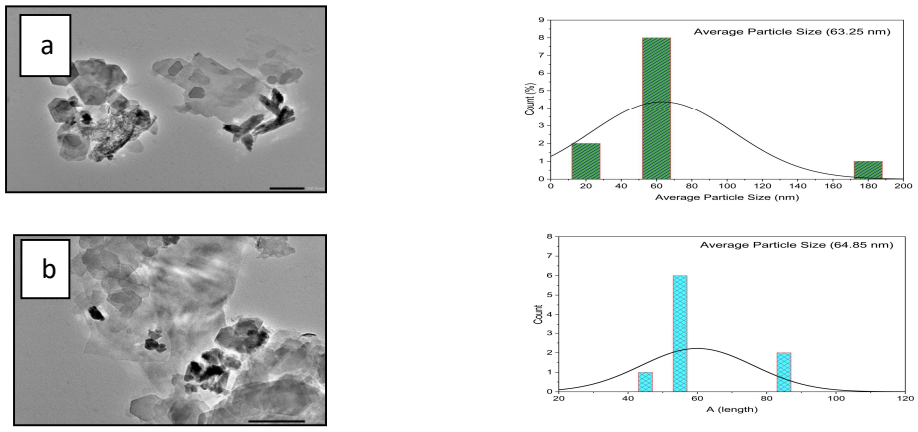


Fig.-8: (a)TEM Analysis with Size Distribution Curve of 1:1 Nanoemulsion and (b) 1:0.5 Nanoemulsion

Antimicrobial Activity

A novel nanoemulsion compound's antifungal and antibacterial properties were investigated. Figure-9&10illustrate the nanoemulsions encouraging inhibitory effects against a variety of bacterial and fungal species. Aza-cetrimide-MMT loaded on EG had exceptional antibacterial and antifungal action against both Gram-positive and negative bacteria, according to results of amicrobial activity investigation. Following a 24-hour incubation period, the zones of inhibition for samples 1:1 and 1:0.5 against Pseudomonas aeruginosa and Staphylococcus aureus are 33.74 mm and 24.70 mm, respectively, and 34.20 mm and 25.42 mm. Sample 1:0.5 showed outstanding antibacterial effectiveness against Pseudomonas aeruginosa bacteria based on the aforementioned analysis (Table-2).

Table-2: Antimicrobial Activity of Aza-cetrimide –MMT Loaded on EG Nanoemulsion

Bacteria & Fungi	Zone of inhibition(mm)	
	Sample 1:1	Sample 1:0.5
P. aeruginosa	33.74	34.20
S. aureus	24.70	25.42
Candida albicans	17.28	15.80

CONCLUSION

A novel nanoemulsion compound's antifungal and antibacterial properties were investigated. The substance demonstrated encouraging inhibitory effects on a range of bacterial and fungal species. These results point to the compound's possible application in the management of various microbial diseases. To sum up, the biological assessment of montmorillonite loaded with azelaic acid, cetrimide, and expanded

graphite shows encouraging outcomes that point to the possibility of employing clay-based nanoemulsions to increase the therapeutic efficacy of these substances. Significant inhibition against target bacterial strains was found in the antimicrobial testing, indicating that this formulation may be a useful therapeutic option for inflammatory diseases and skin infections.

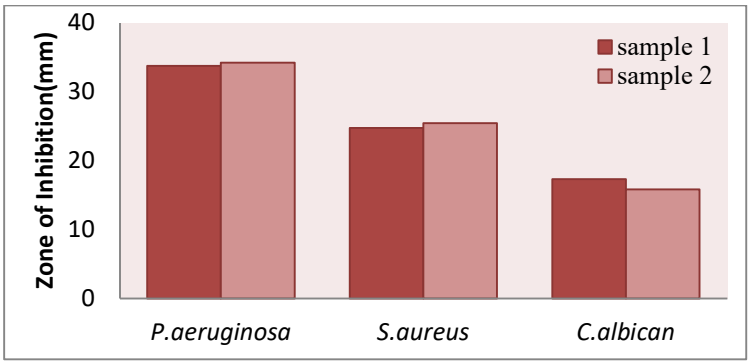
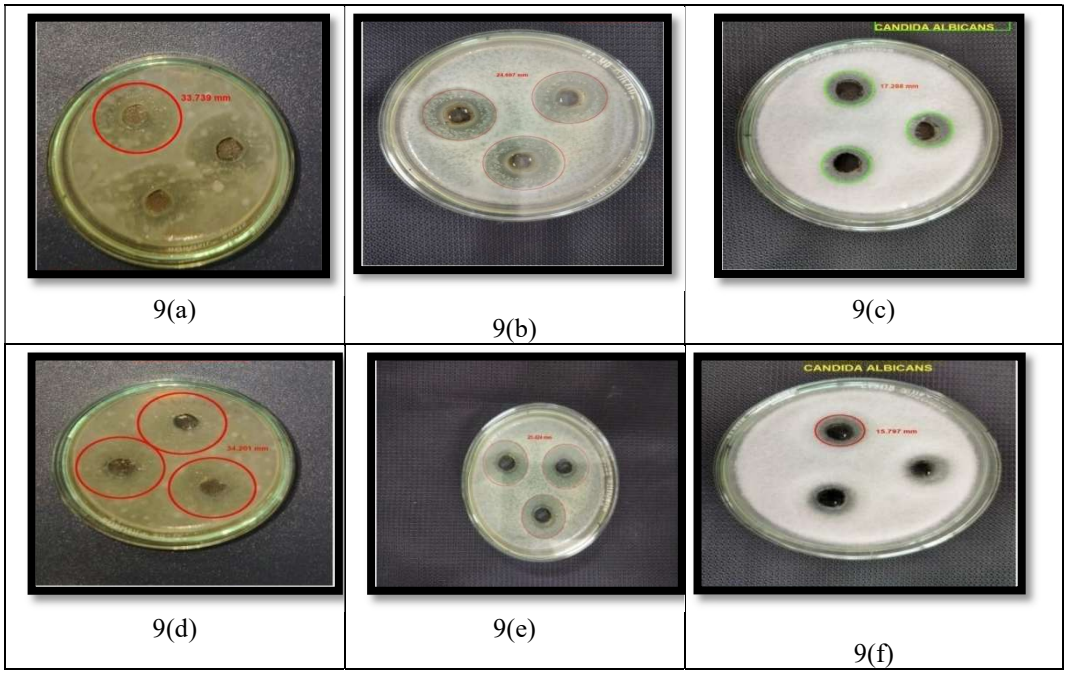


Fig.-9: Antimicrobial Zone of Inhibition of Aza-cetrimide –MMT Loaded on EG Nanoemulsion



9(a) 9(b) 9(c)

9(d) 9(e) 9(f)

Fig.-10: Antimicrobial Activity of Sampe-1:1 (a) *P. aeruginosa* (b)*S. aureus* (c)*Candida albicans, sample*- 1:0.5 nanoemulsion(d) *P. aeruginosa* (e) *S. aureus* (f) *Candida albicans*.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

The present work is related to *SDG-3: Good Health and Well-being*. All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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