6.1 Introduction

Biogenic routes of producing metallic nanoparticles (MNPs) by reducing metal ions with secondary metabolites of plant essential oils is a one-step process that is environmentally benign and biologically safer [285]. This biogenic synthesis of MNPs is quick, can be performed at moderate temperatures and pressures, and is a relatively straightforward process. [15,69]. Essential oils (EOs) are complex lipids containing numerous volatile and organic bioactive compounds. They have numerous uses in the pharmaceutical, cosmetic, and food industries [286]. Because of their remarkable physicochemical properties and bio-reductant activities, MNPs have distinctive antimicrobial and catalytic efficacies [15]. Silver, zinc, nickel, titanium, and copper-based NPs are conventionally used for antibacterial and catalytic applications. In contrast, their toxicity, non-biodegradability, and position as a producer of nanowaste are the main constraints that limit their economic prospects in the nanomedicine, agriculture, and food industries [287]. Magnesium (Mg)-based nanoparticle, in contrast, is biocompatible and considered safer for biotic systems. The United States Food and Drug Administration (FDA) has recognized Mg-based NPs as a safe alternative with highly effective antibacterial properties due to the unique and stable physicochemical properties of Mg NPs [74], which include a large optical and electrical band gap, thermodynamic stability, a low dielectric constant, and a small refractive index [92]. Due to the unique properties of MgNPs, massive research has been conducted to create, characterize, and utilize nanoparticles. In regards, MgNPs are produced commercially predominantly through top-down (physical) and bottom-up (chemical, Biological) techniques, such as ultrasonication, microwave irradiation, wet impregnation, laser-vaporization pathways, sol-gel, and hydrothermal routes [1,11,15,70,94,288,289].

In contrast, the use of toxic compounds in the synthesis of Mg-based NPs increases their toxicity and pollutes the environment. On the other hand, physical methods, require a great amount of energy, making them less cost-effective [74]. Whereas, biological techniques, are referred as 'greener synthesis' and among them plant derived essential oil are more preferable because of their abundance of biomolecules and phytochemicals, ease of extraction, simple availability and management, low cost, high bio-reducing efficacy, biosafety, and rapid reaction rates, they are preferred over other techniques [289,290]. The essential oil derived NPs exhibited antifungal, antibacterial, antiparasitic, or antiviral properties [291–293]. In addition, they can be utilized in a variety of environmental and biomedical applications, including drug delivery systems, biosensors, and wound healing solutions [289]. It is anticipated that essential oil derived NPs will soon be implemented more broadly on the market as a result of the encouraging findings of a large number of studies previously cited.

6.2 Experimental Section

6.2.1 Material

Ultrapure deionized water was used throughout the process. 99.9% pure high-grade magnesium acetate salt (Mg $(CH3COO)_2$) was purchased from Sigma Aldrich, > 99% Acetone was purchased from Merck life sciences, and Sodium hydroxide pellets (NaOH) were purchased from SRL private limited. Peppermint essential oil was purchased from the G.S. Chemical testing lab & allied industries. The media component, like Luria broth and agar powder, was purchased from SRL, and Nutrient agar was purchased from HIMEDIA. The bacterial strain like E. coli (ATCC 25922 strain), S. aureus (ATCC 29213 strain) was procured from ATCC, India. The fungal strain, Candida albicans (ATCC 10231) was procured from ATCC India.

6.2.2 Synthesis of Magnogel

All the glass wares were washed with aqua regia before performing the reaction, For the synthesis of Magnogel, the 2 ml of peppermint essential oil was diluted with 8 ml of acetone. Briefly, magnesium acetate salt (0.5 M) and 5 ml diluted essential oil were mixed in, and the solution was stirred for 5 minutes at 55ºC, and then 1 mL NaOH (1M) was added to maintain a pH close to 12, and the solution was kept at 55ºC under constant stirring (800 rpm) for 2 hrs. After 2 hrs. of reaction, the solution color changed from light yellowish to white turbid solution. Afterward, the solution was kept still for 2 hrs., and collected the gel-like supernatant; further supernatant was centrifuged and collected the supernatant gel; the collected gel was further washed with deionized water and used for further characterization and application.

6.2.3 Characterization

Various analytical techniques were implemented in the characterization of fabricated biogenic Magnogel, and UV-Vis absorption spectra were recorded by using Elico SL210 spectrophotometer with scanning ranging from 200-800 nm. Size and shape estimation of Magnogel were carried out by FEI Tecnai G2 20 TWIN high-resolution transmission electron microscope (HRTEM) with an accelerating voltage of 200 kV, Fourier transform infrared (FTIR) spectroscopy was performed using Nicolet iS5, THERMO Electron Scientific Instruments LLC instrument in the range of 400-4000 cm-1 using ATR technique. X-ray photoelectron spectroscopy (XPS) was carried out using K-Alpha (Thermo Fisher Scientific).

6.2.4 Microbial Culture

ATCC procured *E. coli* (Gram - ve) and *S. aureus* (Gram + ve) bacteria that were grown in Luria Bertani Broth at 37º C with continuous shaking of 220 rpm for 14 hrs. and 24 hrs., respectively. Whereas fungal strain, *candida albicans* in the same media composition were grown at 37º C for 24 hrs. at 220 rpm shaking [294].

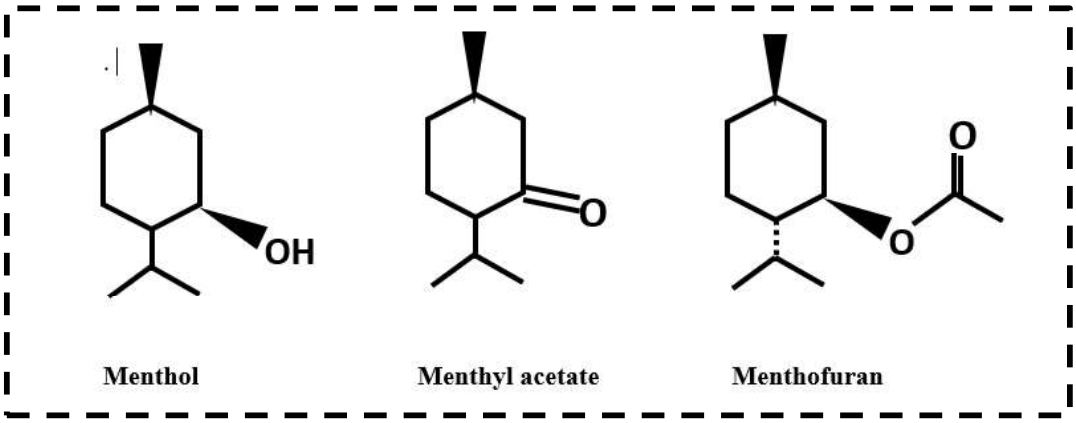
6.2.5 Antimicrobial activity

The antimicrobial activity of Magnogel was investigated on two bacterial species, Gram-positive *S. aureus* and Gram-negative *E. coli and Candida albicans* fungi. Disk diffusion method and spreading method were used to investigate its bactericidal properties with respect to conventional drugs, Gentamycin and Veromycin, and antifungal activity was evaluated using disk diffusion method. Following standard protocol, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the Magnogel were determined against the above bacterial and fungal strains. MIC is the minimum concentration of an antibacterial agent that causes no bacterial growth (i.e., no turbidity was observed) after 12 hrs. of incubation at 37 °C and of MBC is the lowest concentration of an antibacterial agent that is required to kill a bacterium over 24 hrs. at 37 °C. The growth of the *E. coli*, *S. aureus* bacteria, and *candida albicans* fungi in the presence of Magnogel (at MIC dose) was performed by measuring plate colony counting method, and the plate with no further bacteria growth was considered as MBC concentration of Magnogel.

Mechanism behind antimicrobial properties

Previous reports suggested that peppermint EO is composed of approximately 17 compounds (99.37%) out of which Menthol (53.28%), Menthyl acetate (15.1%) and Menthofuran (11.18%) are formed >80% of EO. It was assumed that peppermint essential

oils targeted bacterial and fungal cells in different mannered compared to drugs. The EOs is hydrophobic in nature, which enables their easy incorporation into the microbial cell membrane. The peppermint EOs are rich in menthol concentration, which is an phenolic monoterpene constitute hydroxyl group around the phenolic ring and exhibits its antimicrobial activity through the disruption of the cytoplasmic membrane [295,296]. Menthol, which is a phenolic component of the peppermint essential oil, helps to reduce the magnesium ions; the electron-rich moieties present in the oxygen of phenolic compound help to reduce the metal cation, whereas menthyl acetate and carboxylic acids present in EOs stabilize the NPs [286,297].



6.2.6 Oxidative stress measurement

Oxidative stress estimation on bacterial cells was carried out by using previously developed method of reduction by nitro blue tetrazolium (NBT) dye. For this measurement, the 300 µl of bacterial cells was taken in four centrifuge tubes (2ml) and treated them with MIC concentration of Magnogel, and controls. Further, 150 µl of NBT dye (10 mg ml^{-1}) was added into bacteria containing tubes and incubated this mixture at 37 ◦C for 1hrs. The reaction was stopped by adding 100 µl of 0.1 M HCl solution. The bacterial pellet was then collected after 10 minutes of centrifugation at 6,000 rpm.

The particulate was then treated with 600 µl of DMSO to extract the reduced NBT. Using a UV–vis spectrophotometer, the absorbance of the obtained solution containing formazan blue was then measured at 575 nm [294,298].

6.2.7 Chorioallantois membrane (CAM) assay for angiogenic response

The CAM (Chorioallantois membrane) assay serves as a widely adopted technique for investigating the influence of NPs on angiogenesis. In our study, we utilized the CAM assay model to explore the effects of synthesized Magnogel on vascular structures. Fertilized eggs were sourced from a poultry farm situated in Varanasi, Uttar Pradesh. Subsequently, these eggs were placed in an egg incubator and allowed to incubate for a span of four days prior to the commencement of the experiment. Upon the completion of the incubation period, a small aperture was made on the eggshell's outer surface to facilitate the visualization of the developing blood vessels. Filter paper discs fabricated from Whatman filter paper were exposed to a Magnogel containing the MIC and MBC concentration of *E. coli* for a minute at room temperature. Subsequently, these treated discs were placed onto the blood vessels within the CAM layer. Employing a Mgnus MagZoom TZM6 Trinocular Stereo Zoom Microscope, we conducted comprehensive observations at distinct time intervals to closely monitor the influence of Magnogel on the progression and maturation of the blood vessels. Untreated eggs were utilized as a reference point of comparison[299–302].

6.2.8 CAM assay for antibacterial study

CAM assay model used to investigate the antibacterial effect of Magnogel. For that purpose, a tiny hole was drilled in the shell of the egg after four days of incubation period at 37 ºC so that emerging blood vessels could be observed. Afterward, the eggs were infected with *E. coli* bacteria, whereas another set of infected eggs was treated with an

MBC concentration of Magnogel for *E. coli* bacterial strain and incubated for 12 hrs. at 37º C; the eggs were carefully poured onto the sterilize Petri dish and visualized the antibacterial effect.

6.3 Physical Evaluation of the Magnogel

6.3.1 Organoleptic Properties: Magnogel was tested for physical appearance, color, texture, phase separation, and homogeneity. These characteristics were evaluated by visual observation. Homogeneity and texture were tested by pressing a small quantity of the Magnogel between the thumb and index finger. The consistency of the formulations and the presence of coarse particles were used to evaluate the texture and homogeneity of the Magnogel. Immediate skin feel (including stiffness, grittiness, and greasiness) was also evaluated [303].

6.3.2 Spreadability Test: The spreadability of Magnogel was evaluated by measuring the spreading diameter of 400 mg of the Magnogel between two glass plates (10 cm × 20 cm) after one minute. The standard weight was applied to the upper glass surface. This experiment repeats in a triplet [304,305].

6.3.3 pH Test: One gram of Magnogel was dispersed in 25 mL of deionized water, and the pH was determined using a pH meter. Measurements were made in triplicate. The pH meter was calibrated with standard buffer solutions (pH 4, 7, and 10) before experiment [304,306].

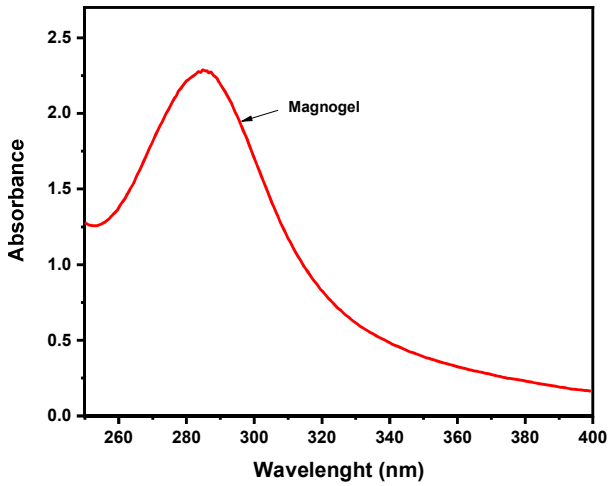
6.4 Result and discussion

The decrease in the color intensity of the essential oil from pale yellow to turbid white provided the initial visual confirmation of bioproduction mechanism that converted magnesium ions into MgNPs. Besides the visual identification, the origin of the single

specific absorption band at 285 nm confirmed the formation of small-sized Magnogel[74]. (Figure 6.1a). The FTIR analysis was used to depict the functional role of phytochemicals present in inessential oil as capping agents and in stabilizing the produced NPs. Studies were carried out for Magnogel and peppermint essential oil to interrogate possible reducing agents as demonstrated in Figure. 6.1 b, a strong band at 2946, 2878, 1713, 1454, 1380 ,902 and 761 cm^{-1} were observed for peppermint essential oil, while at 2951, 2343 ,1707, 1448, 902, and 761 cm^{-1} for biosynthesized Magnogel. The peak observed at 2951 is characteristic of the C–H group present in monoterpenes structures. Whereas, the emergence of the peak at 2343 cm^{-1} can be assigned to the triple bond C≡C presents in the Magnogel. There is a peak at 1707 cm^{-1} which is indicative of the presence of a stretch C=O bond in due to aliphatic ketone. After reducing magnesium and biomineralization of MgNPs, the peaks at 2946, 1713, and 1454 cm^{-1} shifted to 2951, 1707, and 1448 $cm^{-1,}$ respectively. The comparison of the FTIR spectrum of the Magnogel with the peppermint essential oil revealed the presence of compounds containing the bands such as C–H, C≡C, and C–O responsible for reducing and capping magnesium ions [15,286,287,290].

The particle size was evaluated using High-resolution transmission electron microscopy (TEM), and images are shown in Figure. 6.2, which displayed the formation of aggregated MgNPs with a size range of 30-40 nm in diameter. Such low dimensional nanoparticles possess a high surface-to-volume ratio anticipating high surface reaction-based usages like antimicrobial activity [286,307].

(a)



(b)

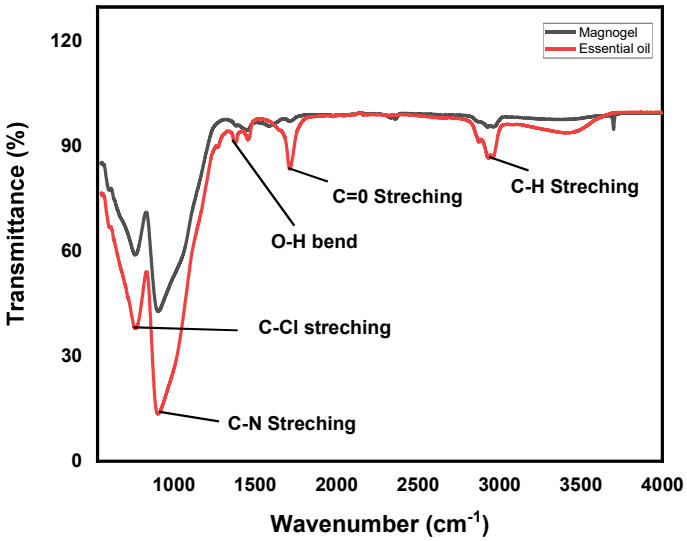


Figure 6.1 (a) UV-Vis spectra of Magnogel (b) FTIR spectra of Magnogel and peppermint essential oil.

(a) **(b)**

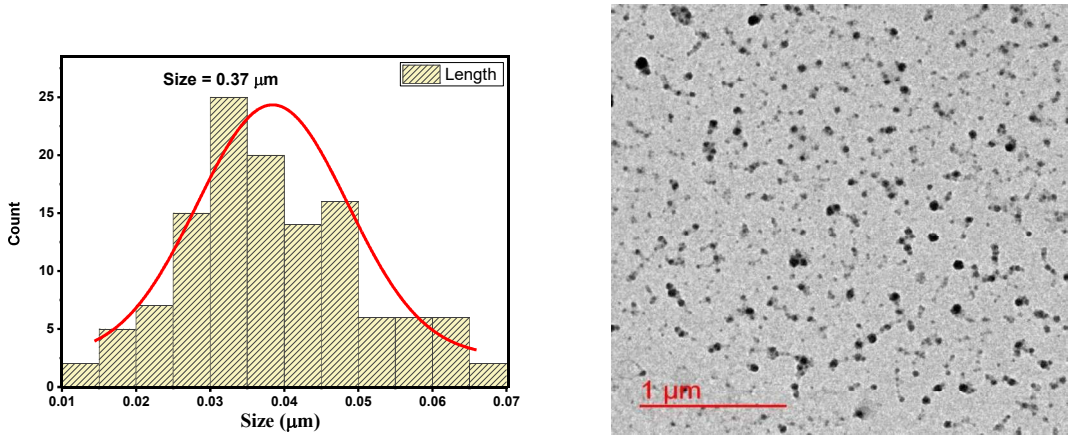


Figure 6.2 (a), Particle size distribution curve, which was calculated using TEM image (b) HR-TEM image of the Magnogel at 1µm scale bar

Mg 1s XPS analysis revealed a peak at 1303.7. This is completely consistent with the data acquired for Mg $(OH)_2$. The O 1s region shows one sharp peak at 530.5 eV corresponding to the adsorbed water [308]. (Figure 6.3 a, b) Mg $(OH)_2$ is anticipated to be the most frequent species on the surface of MgNPs (Magnogel) due to moisture in the air [74]. Organoleptic results showed that the Magnogel had an appealing white opaque appearance with a smooth texture, and they were all homogenous with no signs of phase separation. The odor of Magnogel is very pleasant and refreshing, like mint. An immediate skin contact feels a little cooling sensation, no grittiness, and a little greasiness [309]. The pH of prepared Magnogel is 6.4, while the optimal pH range for topical gel application is 4.5–6.5 [304]. A gel with a higher pH may cause skin irritation, while a gel with a lower pH causes dermatitis. Hence, highly acidic or alkaline gels alter the skin's surface pH, which has a negative effect on the epidermal barrier function and dermal, and it takes many hours to restore these characteristics. Furthermore, Magnogel shows maximum spreading (6 cm) in less time.

Good spreadability is ideal for topical application and can be guaranteed if the sample has a spreading range from 5–7 cm [303,304]. For faster healing of wound process, excellent spreading is foremost. These results make Magnogel an potential candidate for topical application of bacterial infection and wound.

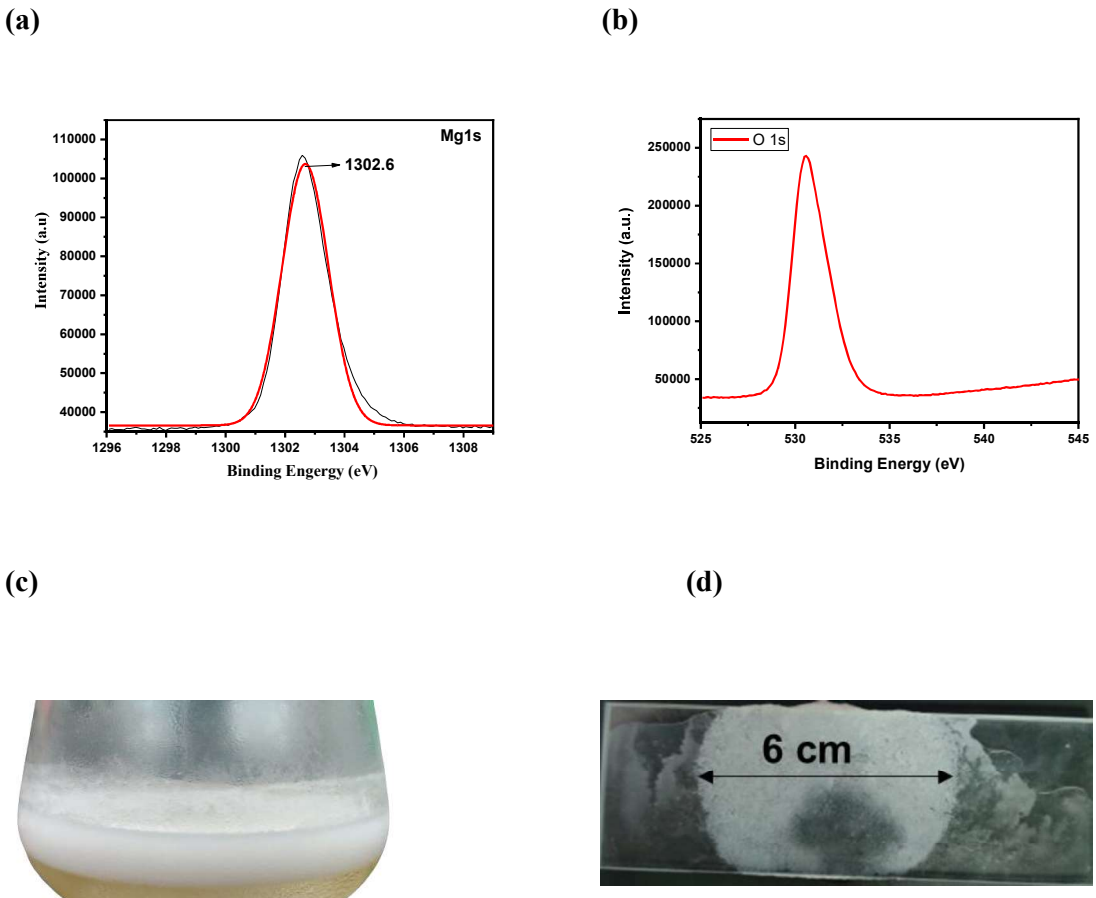


Figure 6.3 (a), XPS spectra of Mg 1s (b) XPS spectra of O 1s (c) Physical image of Magnogel as supernatant (d) Spreadability of Magnogel

6.4.1 CAM assay analysis

Chick embryo blood vessel visuals were captured at various time intervals spanning up to 6 hours post-application of treatments, utilizing a stereo microscope. The findings illustrated in Figure 6.4 disclose that Magnogel treatments did not impede the formation of blood vessels nor disrupt the integrity of vascular structures in chick embryos. To quantify aspects concerning blood vessel stability, such as size, junctions, and length (Figure 6.4) Angiotool and ImageJ Software were employed. The analysis highlighted that the size remained consistently uniform across all samples. However, there were more notable alterations in the length and junction parameters compared to the untreated reference. The antibacterial assay on the E. coli infected chick egg treated with Magnogel demonstrated no observable alteration, death, and fouling, whereas untreated chick egg died, and fouling odor was observed. (Figure 6.5)

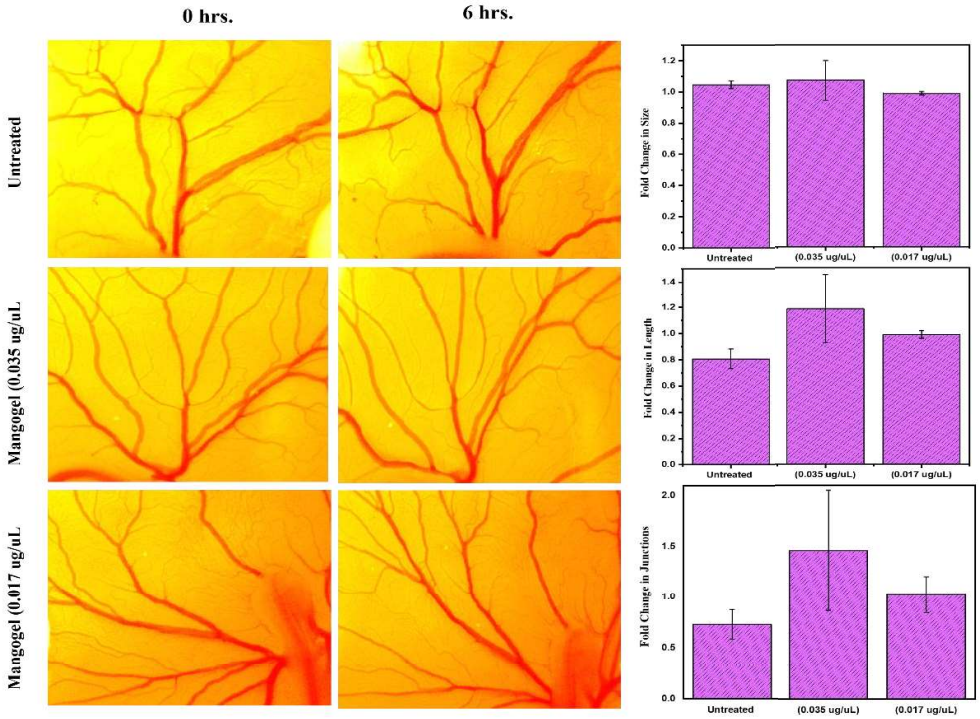


Figure 6.4. CAM assay: (a-f). Growth of blood vessels treated with Magnogel at 0.035 µg/µl at 0.017 µg/µl,and untreated at 0 hrs and 6 hrs (g-i) The images are quantified with respect to length, junction, and size using ImageJ and Angiotool software. The black arrows indicate the change in blood vessels of the treatment groups at 0 hrs and 6 hrs time points. These experiments are performed in triplicate and represented as the mean±SD. No significant differences from UT embryos are observed (*p>0.05).

Figure 6.5. E. coli infected fertilized control egg and Magnogel treated eggs.

6.4.2 Antimicrobial study analysis

The concentration of Magnogel in which there was no bacterial growth observed (i.e., no turbidity was observed) after 12 hrs. incubation at 37 °C was considered to be the minimum inhibitory concentration (MIC) Interestingly, the MIC of Magnogel were found to be different such as 0.017 $\mu g \mu l^{-1}$,0.008 $\mu g \mu l^{-1}$ and 0.035 $\mu g \mu l^{-1}$ for *E. coli, S. aureus* and Candida albicans, respectively. Whereas, the MBC concentration is the concentration of Magnogel at which the bacterial cell compromised and no any further growth observed even after further incubation at preferable condition, the MBC concentration of microbes was found to be 0.035$\mu g \mu l^{-1}$,0.017 $\mu g \mu l^{-1}$ and 0.07 $\mu g \mu l^{-1}$ for *E. coli, S. aureus* and *Candida albicans*, respectively. Furthermore, the MIC concentration of E. coli and S. aureus bacteria which spreaded in Petri plate, shown negligle growth after incubation at 37 ºC for 14 hrs. (Figure 6.6)

Antibacterial property

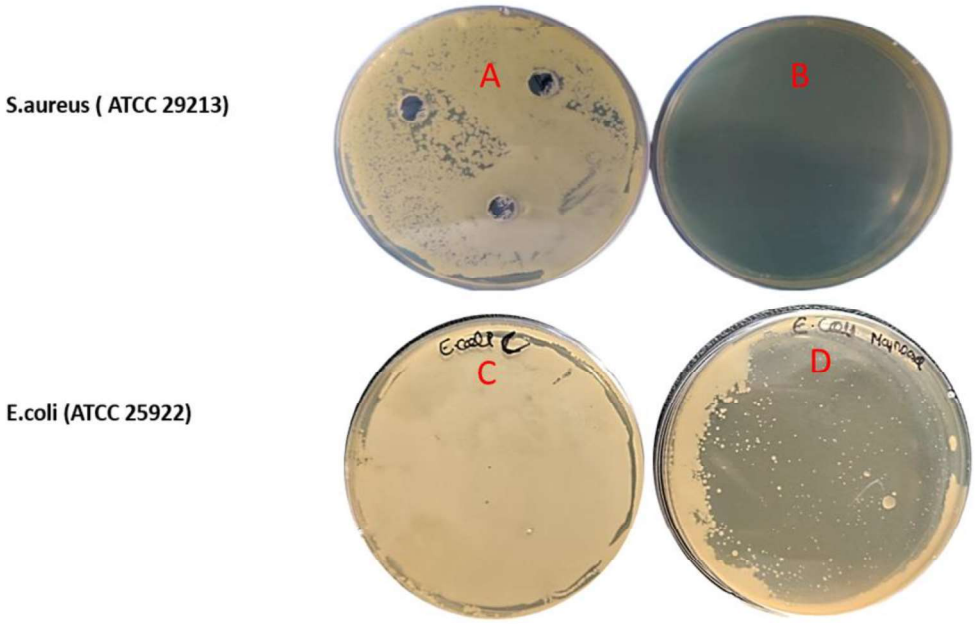


Figure 6.6 Spreadability antibacterial test of Magnogel with E. coli and S. aureus(A) Control S. aureus (B) Magnogel treated S. aureus (c) Control E.coli (D) Magnogel treated E.coli

In Figure 6.7, Magnogel shows 20 mm ZOI against *E. coli* bacteria and 15 mm ZOI observed against *S. aureus* bacteria. In contrary, conventional drugs gentamycin and veromycin demonstrate 22 mm and 17 mm ZOI for *E. coli* and *S. aureus* bacteria respectively, which is almost same for Magnogel. Additionally, essential oil taken as control which shows negligible antibacterial effect against both bacterial cells.

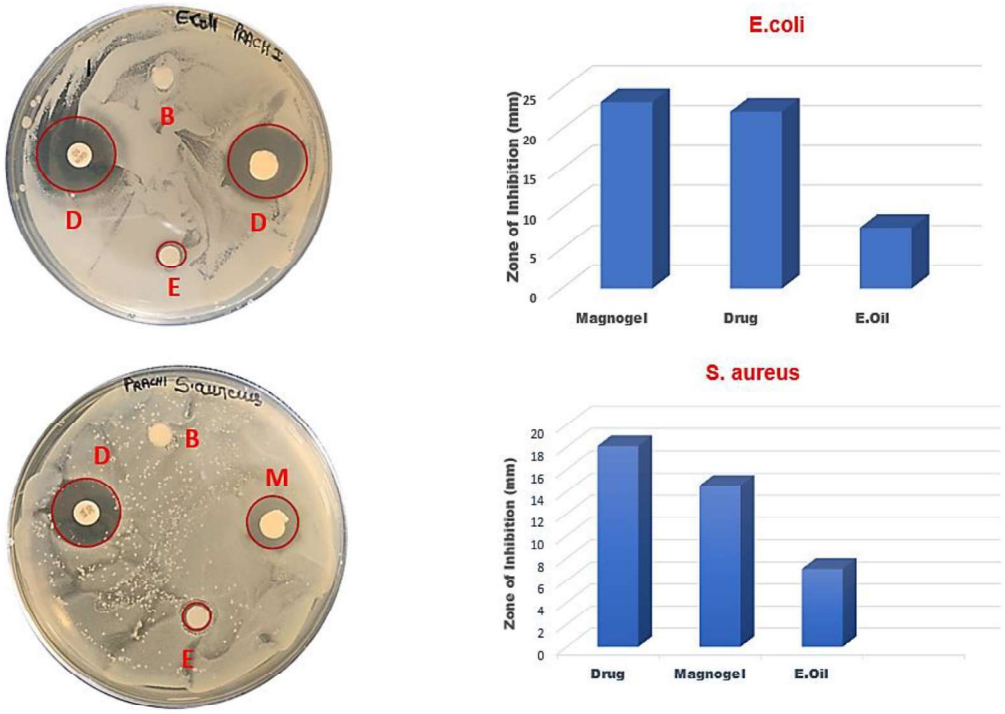


Figure 6.7 (a) Zone of inhibition seen due to the antibacterial action of Magnogel in E. coli bacteria (D ; drug , M; Magnogel ,E ; essential oil , B; bacterial cellulose) (b) Relative antibacterial action with respect to conventional drug and control seen in histogram plot (c) Zone of inhibition seen due to the antibacterial action of Magnogel in S. aureus bacteria (D ; drug , M; Magnogel ,E ; essential oil , B; bacterial cellulose) (d) Relative antibacterial action with respect to conventional drug and control seen in histogram plot.

Antifungal property

In Figure 6.8, spreading test demonstrated negligible growth of fungus into Magnogel treated plate, whereas full growth observed in control plate (Untreated)m. In Figure 6.9 Magnogel shows 25 mm ZOI against *candida albicans fungus*. In contrary, conventional drug Cotrimazole demonstrate 15 mm and essential oil demonstrate 12 mm ZOI for *candida albicans fungus.*

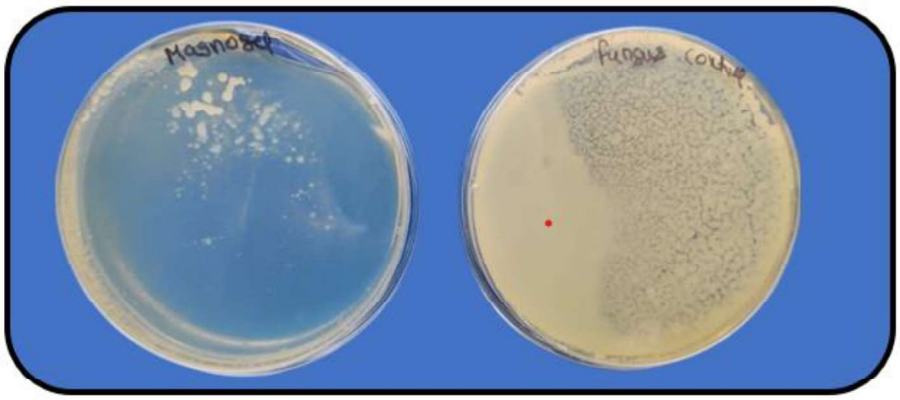


Figure 6.8: Spreadability plate test of Magnogel against candida albicans fungus

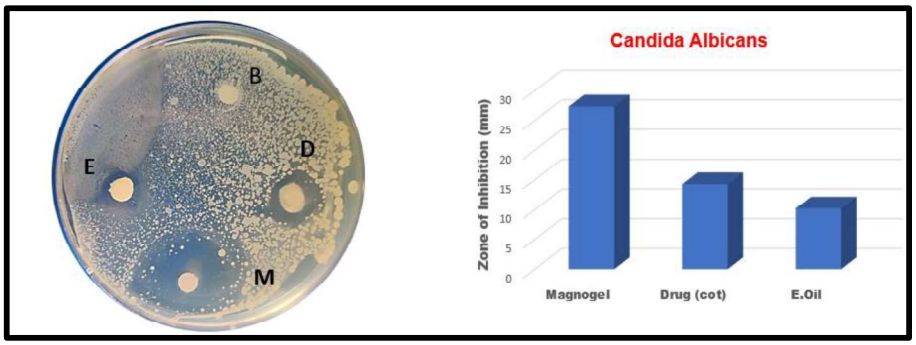


Figure 6.9: Zone of inhibition seen due to the antibacterial action of Magnogel in Candida albicans fungus (D; drug, M; Magnogel, E; essential oil, B; bacterial cellulose) (b) Relative antibacterial action with respect to conventional drug and control seen in histogram plot

According to previous reports, the main causative reason behind microbial cell death in the case of metal NPs is the production of reactive oxygen species (ROS) via a series of single electron reductions. The bacterial cells incubated with Magnogel, shows generation of reactive oxygen species. In this instance, ROS generation was measured using the nitrobluetetrazolium (NBT) reduction method. Due to the formation of formazan by ROS species, the light-yellow color of the NBT due solution transforms to blue color. Compared to the individual control sample, Magnogel-treated cells produced a significant amount of reactive oxygen species (ROS) (Figure 6.10).

(a) **(b)**

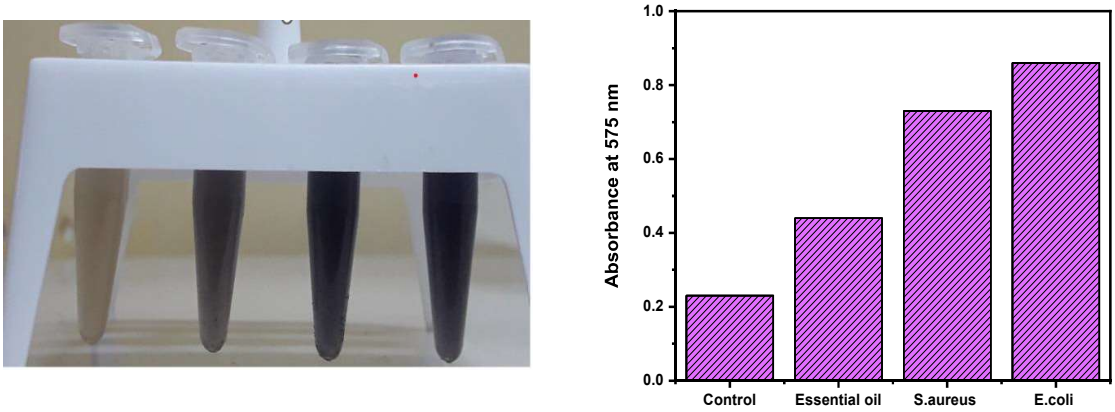


Figure 6.10 (a) NBT assay for Magnogel after incubation for 1 hour with bacterial cells (b) Histogram plot depicting the amount of ROS produced during NBT assay at MIC concentration of *E. coli* and *S. aureus*

Conclusion

For the first time, the peppermint essential oil was utilized as a capping and reducing agent for the development of biocompatible Magnogel, which is endowed with exceptional physicochemical, antibacterial, antifungal, and angiogenic properties. The well-characterized Magnogel (30-40 nm) is suitable for various biomedical applications in view of its greener synthesis route and devoid of harmful chemicals and solvents. CAM assay on chick egg shows enhanced angiogenic response and biocompatibility of Magnogel. The antibacterial and antifungal efficacy of Magnogel against pathogenic bacteria and human fungi has been investigated. Consequently, it is safe to suggest that the incorporation of peppermint essential oil into nanomaterials can serve as a natural bio-adsorbent in materials science, and similar strategies can be applied to other abundant, renewable, natural, and safer extractives. Due to their significant antifungal, antibacterial, and angiogenic activities, the study opens a new door for the fabrication of essential oil-mediated MgNPs to develop smart infectious disease-combating strategies.