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Alginate-based nanoencapsulation of drugs using poly-β-hydroxybutyrate extracted from soil *Bacillus* spp.- Azithromycin and Cephalexin

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ABSTRACT: Conventional drug delivery methods like tablets, capsules, syrups, ointments are lacking in producing sustained drug release due to their low bioavailability and erratic plasma drug levels. Polymeric nanoparticles as nanocarriers have the potential of better drug delivery systems because of their better biocompatibility, sustained and controlled-release properties, and subcellular size. The aim of the research work is to undergo alginate-based nanoencapsulation of drugs azithromycin and cephalexin with PHB extracted from *Bacillus* species obtained by our unique preparation technique, along with comparative studies including antimicrobial and anti-inflammatory studies with those nanoencapsulated drugs.

KEYWORDS : drug delivery method, alginate-based nanoencapsulation, PHB, *Bacillus* spp., anti-inflammatory and antimicrobial studies.

I. INTRODUCTION

Traditional drug delivery methods (such as tablets, capsules, syrups, ointments, etc.) are poor in producing sustained release due to their low bioavailability and erratic plasma drug levels[1]. Polymeric nanoparticles as nanocarriers have undergone extensive research in the pharmaceutical and medical fields due to their potential as drug delivery systems because of their better biocompatibility with tissue and cells, controlled and sustained-release properties, and subcellular size to penetrate[2]. Unlike the conventional drug delivery system, nanocarriers prolong the payload's time in the blood and stop it from breaking down too quickly in the biological environment. They increase bioavailability as well [3]. However, nanocarrier surfaces may be easily modified to avoid phagocytosis if extended blood presence is necessary[4]. The surface of NCs is also altered to improve medication delivery and target-site targeting. In order to modify the surface of NCs, either tagging ligands[5] or hydrophilic polymers [6] are used on their outside. The process of creating drug-loaded nanoparticles with diameters between 1 and 1000 nm is known as nanoencapsulation. Solid submicron-sized medication carriers known as nanoparticles can be either biodegradable or not[7,8]. Nanospheres and nanocapsules are both collectively referred to as nanoparticles. Nanospheres have a structure like a matrix. Drugs may be contained inside the particle or absorbed at the surface of the sphere. Drugs encapsulated in vesicular systems known as nanocapsules are contained within a cavity made up of an inner liquid core and a polymeric membrane. In this instance, the active ingredients are typically dissolved in the inner core; however, they might potentially be adsorbed to the surface of the capsule[9]. Nanotechnology can assist in lowering medicine doses in addition to managing targeting and release[10]. Polymer-coated nanoparticles have the potential to operate as carriers of medications and other bioactive substances, sheltering them from the harsh microenvironment and allowing for prolonged, regulated release of the drug or bioactive ingredient. Some polymers disintegrate readily in the body due to hydrolytic and enzymatic breakage of sensitive bonds contained in the polymer, lowering unwanted side effects and accompanying toxicity[11].

1.2 NANOENCAPSULATION

Nanoencapsulation is the process of encapsulating particles of a medicine or substance of interest with various materials to create nano-scale particles that may have therapeutic or biological advantages. The enclosed substance is also known as the internal phase, the core material,

the filler, or the fill. The encapsulating material is referred to as the exterior phase, shell, covering, or membrane. The core material for distinct nanoparticles can vary widely in size, shape, and composition; the enclosed particles can likewise vary in size and form[12]. There are several methods to create polymeric nanoparticles in which alginate-based nanoencapsulation plays an outstanding role. Due to its biocompatibility, biodegradability, and non-toxicity, alginate has been thoroughly investigated in recent decades for use in tissue engineering, wound healing, drug and gene delivery, and non-toxic applications[13].In the scope of this work, azithromycin and cephalexin will serve as the pharmacological agents to be encapsulated within the biocompatible natural polymer, polyhydroxybutyrate (PHB).

1.3 AZITHROMYCIN - DRUG CHARACTERISTICS

An antibiotic with a broad spectrum and a high degree of tissue penetration is azithromycin. It was first given FDA approval in 1991. In addition to being used in place of other macrolides for specific sexually transmitted diseases, it is primarily used to treat respiratory, gastrointestinal, and genitourinary infections. Azithromycin, sold under the brand names Zithromax (in oral form) and Azasite (as an eye drop), is an antibiotic medication used for the treatment of a number of bacterial infections including intestinal illnesses, pneumonia, traveller's diarrhoea, strep throat, and middle ear infections. It could be used for malaria together with other drugs. It can be ingested or administered intravenously. There has been no evidence of danger from using it while pregnant [14]. Although its safety during nursing has not been established, it is probably safe. The antibacterial action of azithromycin is shallow yet somewhat wide. Some Gram-positive bacteria, some Gram-negative bacteria, and many unusual microorganisms are all inhibited by it. In the population in 2015, a gonorrhoea strain with strong azithromycin resistance was discovered. However, it is not frequently used as monotherapy due to the low barrier to resistance development [15]. Azithromycin resistance in *Streptococcus pneumoniae* is increasing as a result of widespread usage[16].

1.4 CEPHALEXIN - DRUG CHARACTERISTICS

It is an antibiotic called cephalosporin of the first generation. Dihydrothiazine and beta-lactam are both components of cephalosporins. Cephalexin is used to treat bacterial infections, such as upper respiratory infections, by inhibiting the formation of bacterial cell walls, which causes disintegration and ultimately cell death, infected ear, skin maladies, bladder infections, and bacterial and bacterial bone infections. It works similarly to other agents within this class, including intravenous cefazolin, but can be taken by mouth [17].

1.5 PHB AS BIOPOLYMER

Polyhydroxyalkanoate (PHA) is a group of linear polyester biopolymers produced by bacteria during the fermentation of sugars and lipids that are accumulated in the cell as a carbon and energy storage body under circumstances of nutrient deficiency and in the presence of excess carbon sources[18]. The most prominent member of the family of biodegradable thermoplastic polyhydroxyalkanoates, Poly 3-hydroxybutyrate (PHB), has the properties of high melting temperature, high crystallinity, and low permeability to O2, H2O, and CO2. PHB is an excellent substitute for synthetic polymers, and it has mechanical qualities similar to polypropylene[19]. PHB has gained commercial interest since it exhibits thermoplasticity and compost biodegradability. With stronger water vapor barrier qualities than polypropylene and greater oxygen barrier values than both polyethylene terephthalate and polypropylene, PHB offers a lot of potential in food packaging applications[20]. PHB is biocompatible and won't cause an inflammatory reaction when implanted in people. PHB can be used as a carrier for slow drug release because PHB's internal deterioration is a gradual process[21]. PHB can be processed more easily and with less brittleness by blending it with other polymers or plasticizers and lowering the processing temperature[20,22]. PHB can be synthesized either artificially or naturally occurring biological substances. In reaction to excessive carbon sources or a lack of vital nutrients, *Bacillus* and some other microorganisms produce PHB[23]. Extraction of the biopolymer PHB from bacterial strains like *Bacillus* is thought to be a low-cost procedure[24].

The ultimate aim of the research work is to undergo nanoencapsulation of drugs like azithromycin and cephalexin with PHB extracted

from *Bacillus* species obtained by our unique preparation technique, along with comparative studies including antimicrobial and anti-inflammatory studies with those nanoencapsulated drugs.

II. MATERIALS AND METHODS

2.1 ISOLATION OF MICROORGANISMS FROM THE SOIL:

The soil sample with a depth of 1-3 cm was obtained from Sowripalayam (latitude: 11.0115913, longitude: 77.0089332) in Coimbatore, Tamil Nadu, India, with a sterile spatula. About 20 g of soil sample was obtained. The sample was stored at 4 °C in sterile polypropylene bags. 1 g of the soil sample is dissolved in 10 ml of distilled water and was shaken for 2 minutes. From the solution, 1 ml is transferred in 9 ml of sterile distilled water. To get dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} for the isolation of organisms, the serial dilution is done. A nutrient agar medium was plated with 0.1 ml from each dilution. Plates were incubated for 48 hours at 37 °C . Isolated *Bacillus* colonies were obtained and cultured in nutrient broths[25].

2.2 PRELIMINARY SCREENING OF PHB PRODUCING BACTERIAL STRAINS USING SUDAN BLACK TECHNIQUE:

A quantity of 0.1 g of Sudan black powder was introduced into 75 ml of 95% ethanol. Subsequently, the solution was adjusted to a total volume of 100 ml by incorporating 25 ml of distilled water. This solution underwent a filtration process twice. The resultant mixture was then transferred onto petri plates containing *Bacillus* culture, following the streaking procedure. After an incubation period of 20 minutes, the plates were observed[25].

2.3 EXTRACTION BY SODIUM HYPOCHLORITE - CHLOROFORM PHASE METHOD:

To separate PHB as pellet, a volume of 10 ml of *Bacillus* culture was subjected to centrifugation at 5000 rpm for a duration of 15 mins. The resulting supernatant was carefully discarded. To the pellet, 2 ml each of acetone and ethanol were added for the purpose of removing impurities, which were subsequently discarded. 2 ml of a 4% Sodium hypochlorite solution was introduced, and the mixture was allowed to incubate at 40°C for 30 minutes. After the incubation period, the entire mixture received an additional 1 ml of acetone, 1 ml of ethanol, and 2 ml of chloroform.2 ml of a 4% Sodium hypochlorite solution were introduced, and the mixture was allowed to incubate at 40°C for 30 minutes. After the incubation period, the entire mixture received an additional 1 ml of acetone, 1 ml of ethanol, and 2 ml of chloroform[25].

2.4 NANOENCAPSULATION OF DRUGS BY SODIUM ALGINATE BASED CONTROLLED GELIFICATION BY CA 2+ IONS:

0.2 g of polyvinyl alcohol (the primary binder) was dissolved in 20 ml of distilled water. This mixture was allowed to dissolve completely, forming a clear solution, by using a magnetic stirrer at a temperature range of 80°C to 95°C for a duration of 45 minutes. Subsequently, PHB was introduced into the mixture and allowed to dissolve. Furthermore, 0.2 g of sodium alginate (the secondary binder) was incorporated into the mixture.Each drug sample, weighing 500 mg, was individually added to the solution, resulting in the formation of viscous fluids. 7 g of calcium chloride was dissolved in 50 ml of water, and this solution was poured onto two petri plates. Subsequently, the viscous fluids were individually added dropwise to the petri plates using a syringe of the appropriate size[26].

2.5 DRUG CHARACTERISATION BY FTIR ANALYSIS:

FTIR analysis is used for characterization since it can identify certain molecular vibrations in a molecule's chemical bonds. On the basis, Comparison of functional groupings for PHB, drugs (Azithromycin, Cephalexin) and PHB combined with drugs were done.

2.6 ANTI INFLAMMATORY STUDIES USING PROTEIN DENATURATION INHIBITION ASSAY:

A volume of 0.2 ml of 1% bovine serum albumin, along with 4.78 ml of phosphate buffer, was added to 0.02 ml of both nanoencapsulated drug samples. This mixture was then incubated in a water bath at a temperature of 37°C for a duration of 15 minutes. Subsequently, the same mixture was heated to 70°C for only 5 minutes. Finally, the mixture was allowed to cool. Using a UV spectrometer or Colorimeter, the turbidity was

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measured at 660nm. Water is considered as a test solution[27].

2.7 ANTIMICROBIAL ACTIVITY USING WELL DIFFUSION METHOD:

To carry out an antimicrobial procedure employing several bacterial species, including *Escherichia coli* and *Staphylococcus aureus*, the bacteria were all isolated. The antibacterial assay is determined by media prepared using peptone, beef extract, yeast extract, sodium chloride, and nutrient agar throughout the investigation. The media was autoclaved for 15 minutes at 121°C after being pH-adjusted. The media was transferred onto two petri plates and were allowed to solidify[28].

2.7.1 WELL DIFFUSION METHOD:

The drug solution was made up by dissolving 10µl, 20µl, 30µl, 40µl, and 50µl of encapsulated drugs of azithromycin and cephalexin in 1000µl of distilled water in separate Eppendorf tubes. Cotton swab inoculation was used to introduce the test strains of *Staphylococcus aureus* and *E. coli* into agar plates. Five equally spaced wells were created on the agar spread using a sterile cork borer. The samples are inserted into the wells accordingly using a sterile micropipette. The plates were incubated at 37°C for 48 hours[28].

III. RESULTS AND DISCUSSION

3.1 ISOLATION OF MICROORGANISMS FROM SOIL :

From the soil sample, microorganisms were separated, and isolated colonies were produced through serial dilution. Bacterial colonies having various morphological traits were chosen, and each colony was given a number. These colonies were kept for further research by being streaked on nutrient agar plates.

Strain No.	Colony Morphology
C1	Large , irregular colonies
C2	Small, dried colonies
C3	Large, dried colonies
C4	Large colonies
C5	Small, irregular colonies
C6	Large colonies

Six different bacterial colonies from the crowded plate technique were selected as strains C1, C2, C3, C4, C5, and C6.

3.2 PRELIMINARY SCREENING OF PHB PRODUCING BACTERIAL STRAINS USING BLACK SUDAN TECHNIQUE:

Six colonies were displayed in Sudan black staining. They are C1, C2, C3, C4, C5, and C6. These isolates tested positive for the presence of Sudan-black absorption patterns and lipophilic PHB granules.

Strain No.	Observation
C1	Minimum production of PHB
C2	Minimum production of PHB
C3	Moderate production of PHB
C4	Minimum production of PHB
C5	Maximum production of PHB
C6	Minimum production of PHB

C1, C2, C4, and C6 showed the minimum absorption pattern, which represents the minimal production of PHB. Similarly, C3 showed a moderate absorption pattern. Whereas C5 showed the maximum absorption pattern, representing the maximum production of PHB.

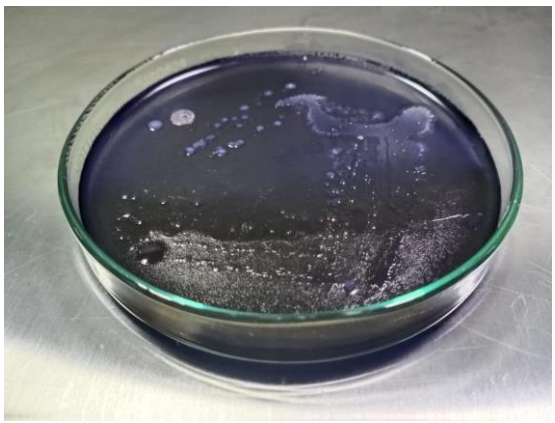

Fig 3.1 Sudan Black Technique (C5)

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3.3 EXTRACTION BY SODIUM HYPOCHLORITE - CHLOROFORM PHASE METHOD:

Three phases are formed in the centrifuge tube. The upper phase contains sodium hypochlorite solution, the middle phase of chloroform containing undisturbed cells, and the bottom phase contains chloroform with PHB. The bottom phase containing chloroform with PHB was transferred using a micropipette into a petri plate and allowed to evaporate for 20 minutes at 70 °C in a water bath. PHB powder is gathered in an Eppendorf tube from the petri plate by scratching.

Fig 3.2 Extracted Poly-β-hydroxybutyrate

3.4 NANOENCAPSULATION OF DRUGS BY SODIUM ALGINATE BASED CONTROLLED GELIFICATION BY CA 2+ IONS:

Nanocapsules were formed on the surface of the $CaCl_2$ solution. The nanocapsules were stored in Eppendorf tubes for further studies.

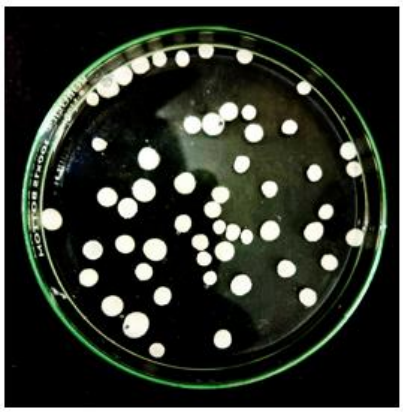

Fig 3.3 Nanoencapsulated azithromycin

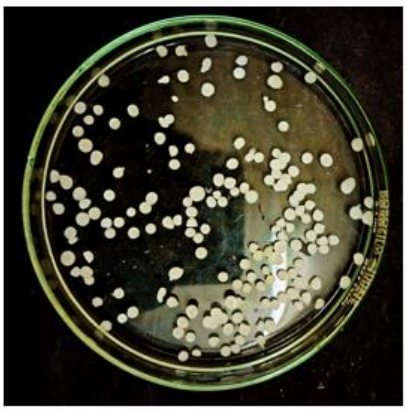

Fig 3.4 Nanoencapsulated cephalexin

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3.5 CHARACTERISATION BY FTIR (FOURIER TRANSFORM INFRARED) SPECTROSCOPY ANALYSIS :

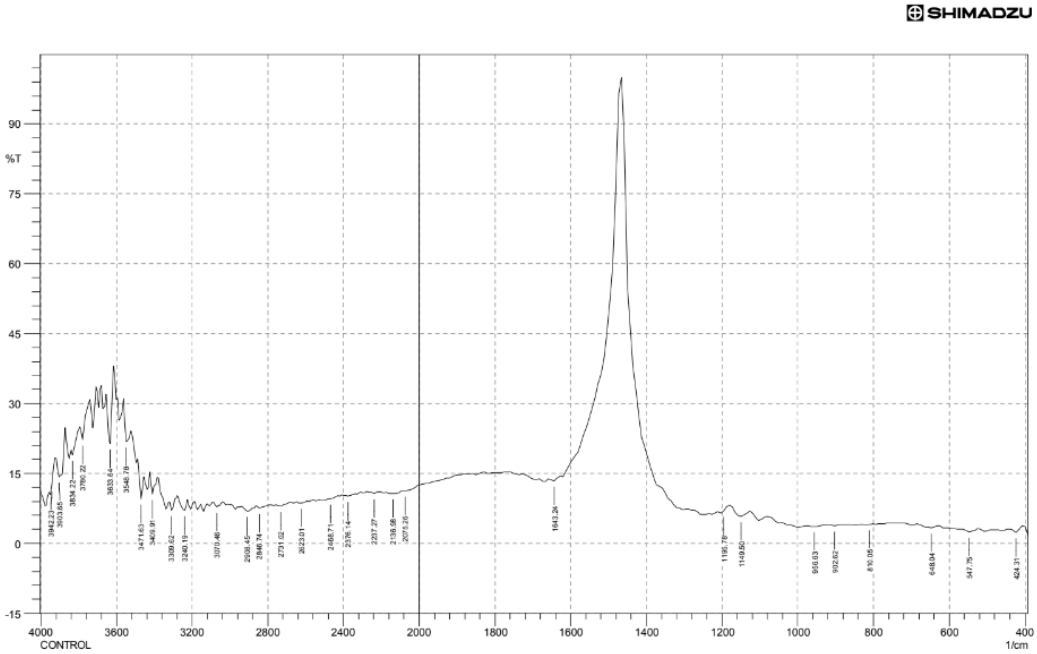

Fig 3.5 : FTIR Analysis of PHB (Control)

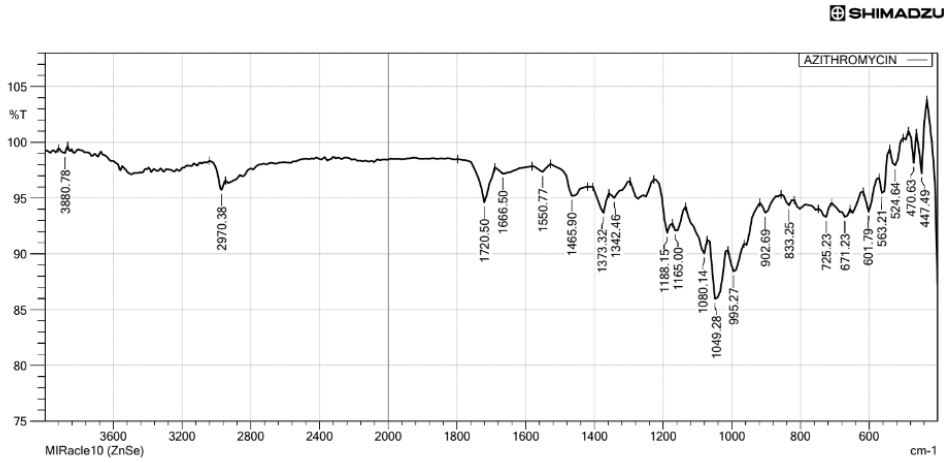

Fig 3.6 : FTIR analysis of azithromycin standard

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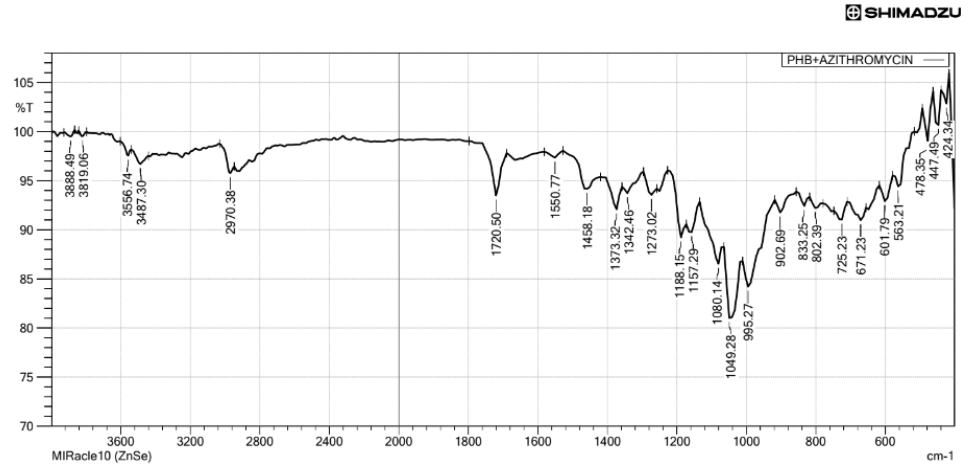

Fig 3.7 : FTIR analysis of PHB + azithromycin

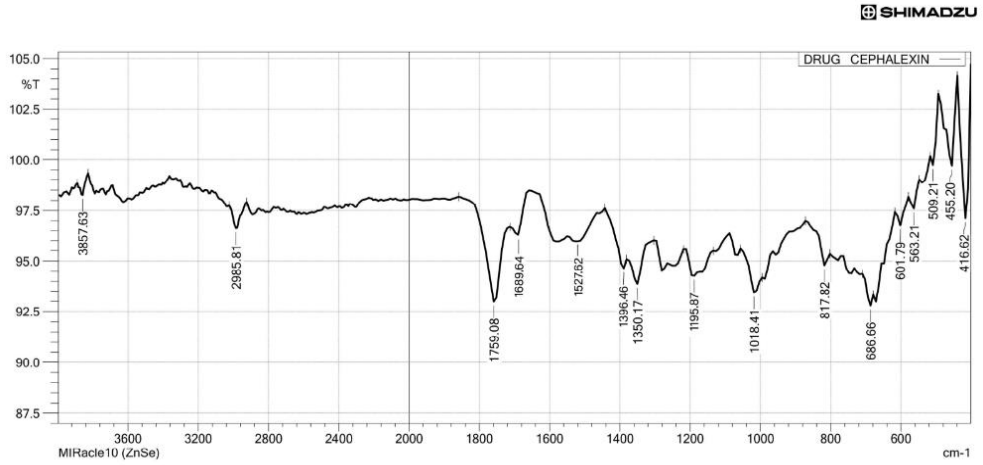

Fig 3.8 FTIR analysis of cephalexin standard

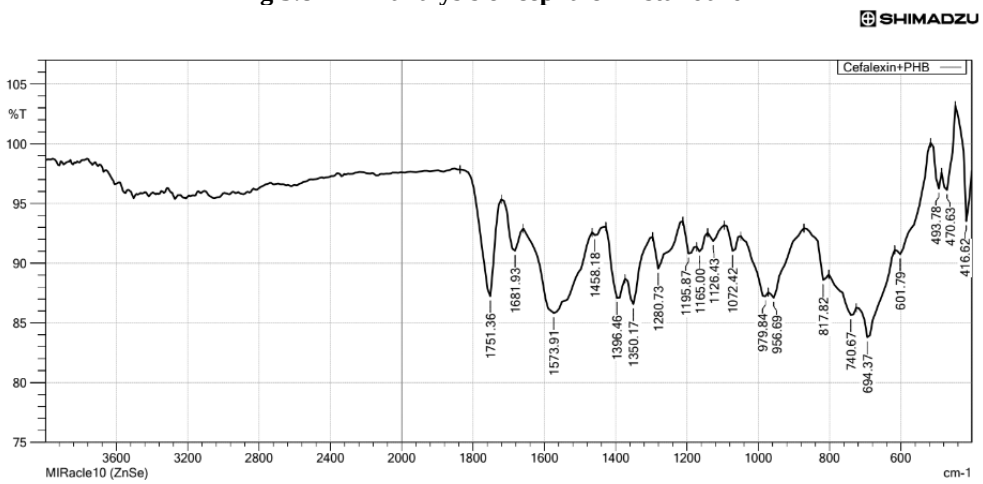

Fig 3.9 FTIR analysis of cephalexin + PHB

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Table : Comparison of FTIR results

S. NO.	PHB (cm^{-1})	AZITHROMYCIN (cm^{-1})	AZITHROMYCIN + PHB (cm^{-1})	CEPHALEXIN (cm^{-1})	CEPHALEXIN + PHB (cm^{-1})	FUNCTIONAL GROUPS AND COMPOUNDS (in stretching)	APPEARRENCE
1.	3471.63	-	3487.30	-	-	O-H stretching (alcohol)	**Strong**
2.	1643.24	1666 .50	-	1689.64	1681.93	C=N(imine/oxime)	**Medium**
3.	1195.78	1188.15	1188.15	1195.87	1195.87	C-O stretching (tertiary alcohol)	**Medium**
4.	-	1550.77	1550.77	1527.62	1573.91	N-O stretching (nitro compound)	**Strong**
5.	-	1373.32	1373.32	1396.46	1396.46	O-H bending (alcohol)	**Medium**
6.	810.05	833.25	833.25	817.82	817.82	C=C bending (alkene)	**Medium**
7.	648.04	671.23	671.23	586.36	601.79	C-Br stretching (Halo compound)	**Strong**
8.	547.75	563.21	563.21	586.36	-	C-Cl stretching (Halo compound)	**Strong**

(**Source** : IR spectrum standards - Sigma Alrich : IR Spectrum Table (sigmaaldrich.com))

DESCRIPTION:

The peak 1 value of 3471.63 cm^{-1} stretching in Fig 3.5 reveals the presence of the O-H functional group, which is similar to the peak value of 3487.30 cm^{-1} in Fig 3.7 and indicates the presence of PHB in encapsulated azithromycin.

The peak 3 value of 1188.15 cm^{-1} stretching in Fig 3.6 shows the presence of C-O stretching (tertiary alcohol), which is similar to the peak value of 1188.15 cm^{-1} in Fig 3.7 and indicates the presence of the azithromycin drug in the encapsulated product.

The peak 3 value of 1195.78 cm^{-1} stretching in Fig 3.5 shows the presence of C-O stretching (tertiary alcohol), which is similar to the peak value of 1195.87 cm^{-1} in Fig 3.9 and indicates the presence of PHB in encapsulated Cephalexin.

The peak 6 value of 817.82 cm^{-1} stretching in Fig 3.8 shows the presence of C=C bending, which is similar to the peak value of 817.82 cm^{-1} in Fig 3.9 and indicates the presence of Cephalexin drug in encapsulated products.

3.6 ANTI INFLAMMATORY STUDIES :

The drugs were able to inhibit protein denaturation in a concentration-dependent manner. The inhibitory effect of the drug at different concentrations (10–50 µg/ml) on protein denaturation is shown in the graphs.

CONCENTRATION(AZITHROMYCIN) in µg/ml	ABSORBANCE at 660 nm
10	0.02

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20	0.05
30	0.09
40	0.14
50	0.10

DESCRIPTION:

The table provides a comprehensive dataset of concentration and corresponding absorbance values at 660 nm for Azithromycin, a commonly used antibiotic, in the context of anti-inflammatory studies. Azithromycin has gained attention for its potential anti-inflammatory properties and this dataset is instrumental in elucidating its behaviour under specific experimental conditions.

CONCENTRATION(CEPHALEXIN) in µg/ml	ABSORBANCE at 660 nm
10	0.03
20	0.07
30	0.16
40	0.14
50	0.11

DESCRIPTION:

The table presents concentration and absorbance values obtained during anti-inflammatory studies involving cefalexin, a commonly used antibiotic and anti-inflammatory agent. This dataset was generated to assess the relationship between the concentration of cephalexin solutions and the corresponding absorbance readings at 660 nm.

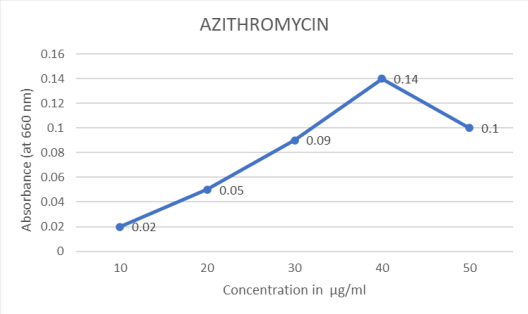

Fig 3.10 Anti-Inflammatory result of Azithromycin

The graph of Azithromycin drug regarding anti-inflammatory studies is plotted in accordance with concentration (at x-axis) and absorbance (at y-axis) and shows highest absorbance value of 0.14 when the concentration is at 40 µg/ml.

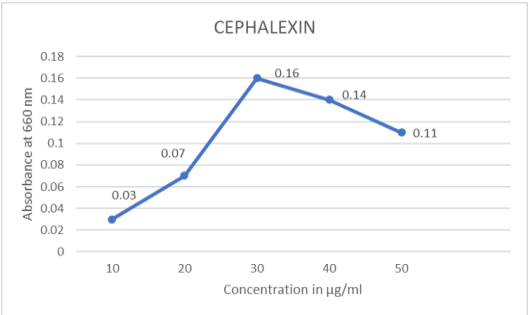

Fig 3.11 Anti-inflammatory result of Cephalexin

The graph of Cephalexin drug regarding anti-inflammatory studies is plotted in accordance with concentration (at x-axis) and absorbance (at y-axis) and shows highest absorbance value of 0.16 when the concentration is at 30 µg/ml.

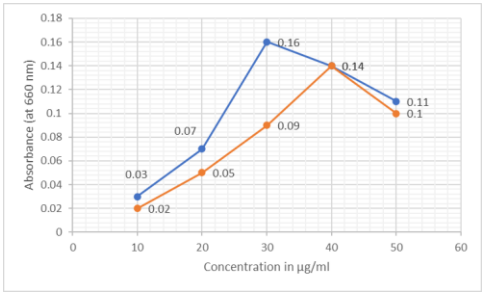

Fig 3.12 Comparison of Anti-Inflammatory Studies of Azithromycin And Cephalexin

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3.7 ANTIMICROBIAL ACTIVITY USING WELL DIFFUSION METHOD:

Two distinct strains were used to perform the antibacterial activity. The developed PHB with medicines Azithromycin and Cephalexin has antibacterial action against the pathogens *Escherichia coli* and *Staphylococcus aureus* and was represented by the inhibition zone in the plate.

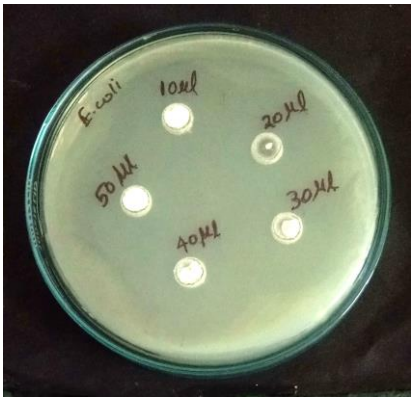

Fig 3.13 Azithromycin against *E.Coli*

Fig 3.14 Azithromycin against *Staphylococcus Aureus*

The test organisms *E.coli* and *S.aureus* are highly sensitive to Azithromycin. The drug should be tested in a lower concentration in future.

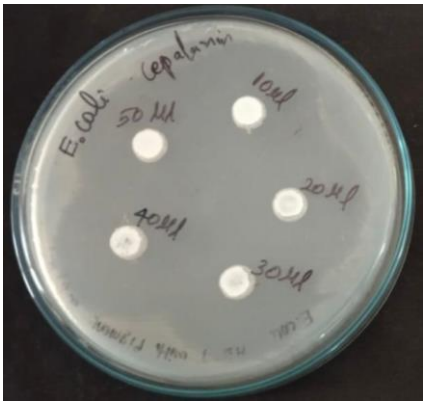

Fig 3.15 Cephalexin against *E.Coli*

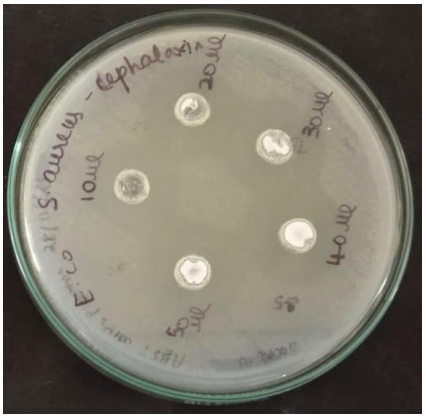

Fig 3.16 Cephalexin against *Staphylococcus Aureus*

The test organisms *E.coli* and *S.aureus* are sensitive to cephalexin. Resistance of nano encapsulated Cephalexin is comparatively lower than the nano encapsulated Azithromycin.

IV. CONCLUSION

In this study, we successfully employed the biopolymer Poly-β-hydroxybutyrate (PHB) to encapsulate the pharmaceuticals azithromycin and cephalexin, utilising the alginate-based nanoencapsulation technique. The source of our poly-β-hydroxybutyrate (PHB) was *Bacillus* species isolated from soil samples. Notably, the extraction procedure for PHB from *Bacillus* species is cost-effective and uncomplicated.

Due to PHB's biodegradable and biocompatible characteristics, it was chosen as the biopolymer for nanoencapsulation. This guarantees that the drugs included within PHB can be gradually digested and excreted from the body without harm. PHB is a viable and affordable material option for nanoencapsulation applications due to its low cost.

We used the alginate-based ionic gelification process for nanoencapsulation, which showed high encapsulation effectiveness and simplicity of use. Fourier-transform infrared spectroscopy (FTIR) was used to characterise the encapsulated pharmaceuticals, confirming the existence of PHB and the associated medications within the encased particles.

Furthermore, we evaluated the encapsulated medicine's anti-inflammatory and antibacterial properties. Notably, it was discovered that the anti-inflammatory effects of both cephalexin and azithromycin in capsule form were found to be comparable with those of their free counterparts. It is important to note that compared to encapsulated cephalexin, encapsulated azithromycin and cephalexin showed superior resistance to microbial growth in tests with *Staphylococcus aureus* and *Escherichia coli.*

Collectively, our results indicate that the use of PHB for the nanoencapsulation of azithromycin and cephalexin is a viable strategy for the safety of these medications and the streamlined nanoencapsulation process. The goal of upcoming research should be to improve the nanoencapsulation procedure and test the encapsulated drugs *in vivo* stability and effectiveness over the course of time. This research may lead to improvements in therapeutic results and drug delivery strategies.

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