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Research Article

Synthesis and Characterization of Pullulan Encapsulated Ursolic Acid Nanoparticles for Enhanced Bioavailability and Acetylcholinesterase Inhibition in Alzheimer's Disease Therapy

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that leads to cognitive decline and memory loss, primarily due to the dysfunction of acetylcholinesterase (AChE) in the brain. Natural compounds, such as Ursolic Acid (UA), have demonstrated significant neuroprotective and anti-inflammatory effects. However, the therapeutic potential of UA is often hindered by its low solubility and poor bioavailability. To address these limitations, we synthesized Pullulan-encapsulated Ursolic Acid nanoparticles (UA-Pull-NPs) with the aim of improving drug delivery efficiency and enhancing AChE inhibition in AD therapy.

The nanoparticles were fabricated using a solvent evaporation method, and their physicochemical properties were thoroughly characterized. Particle size analysis (PSA) revealed that the UA-Pull-NPs had an average diameter of **150 ± 12 nm**, which is optimal for cellular uptake and in vivo drug delivery. The zeta potential was measured to be **−28.5 ± 2.3 mV**, indicating good colloidal stability and confirming the successful encapsulation of UA. The encapsulation efficiency (EE) was determined to be **85 ± 3%**, reflecting the high loading capacity of UA in the Pullulan matrix.

In vitro drug release studies demonstrated a controlled release profile of UA from the Pullulan nanoparticles, with a sustained release over 48 hours, highlighting their potential for prolonged

therapeutic effects. Additionally, the AChE inhibitory activity of the UA-Pull-NPs was significantly enhanced compared to free UA, demonstrating a potential therapeutic advantage in AD treatment. The results suggest that Pullulan encapsulation significantly improves the bioavailability and therapeutic potential of Ursolic Acid, offering a promising drug delivery system for enhancing AChE inhibition and providing an effective approach for Alzheimer's disease therapy.

KEYWORDS: Ursolic Acid, Alzheimer's, Pullulan, Nanoparticles

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the most common form of dementia, characterized by a gradual decline in cognitive function, memory loss, and behavioral changes. It affects millions of individuals worldwide, with the prevalence of AD expected to increase significantly as the global aging population grows. According to the World Health Organization (WHO), approximately 50 million people globally are affected by dementia, with AD being the most prevalent form (WHO, 2020). The pathophysiology of AD involves the accumulation of amyloid-beta plaques, tau tangles, oxidative stress, neuroinflammation, and neuronal cell death, all contributing to cognitive decline. One of the central features of AD is the dysfunction of acetylcholine (ACh), a neurotransmitter crucial for memory and learning. The breakdown of acetylcholine is accelerated by acetylcholinesterase (AChE), which leads to cognitive deficits in AD patients (Francis et al., 1999). AChE inhibitors have been the mainstay of current pharmacological treatments, but their efficacy is limited by poor bioavailability, side effects, and a short duration of action (Birks, 2006).

The primary pharmacological agents used in AD therapy, such as Donepezil, Rivastigmine, and Galantamine, are cholinesterase inhibitors that temporarily alleviate symptoms by inhibiting the breakdown of acetylcholine. However, these drugs are often limited by their side effects, including gastrointestinal disturbances, and their therapeutic effects are generally modest and temporary. Additionally, the low bioavailability of many of these drugs restricts their therapeutic potential. Furthermore, the development of disease-modifying therapies for AD has proven difficult due to the complexity of the disease pathology and the challenges in targeting multiple underlying mechanisms, including

neuroinflammation, oxidative stress, and the accumulation of amyloid plaques.

Given the limited efficacy and side effects of synthetic drugs, there has been growing interest in natural compounds that may have neuroprotective effects in AD. Among these, Ursolic Acid (UA) is a promising candidate. Ursolic acid is a pentacyclic triterpenoid found in a variety of plant species, including rosemary, basil, and apples. It has been shown to possess a wide range of pharmacological activities, including anti-inflammatory, antioxidant, anti-cancer, and neuroprotective effects (Cao et al., 2016; Xing et al., 2018). Specifically, UA has demonstrated the ability to inhibit AChE, modulate neuroinflammation, reduce oxidative stress, and protect neuronal cells from damage, making it a potential therapeutic agent for AD (Saini et al., 2016; Li et al., 2017). However, the therapeutic application of UA is limited by its poor aqueous solubility, low bioavailability, and rapid metabolism, which reduce its effectiveness when administered orally (Goh et al., 2018).

To overcome the limitations of UA, nanoparticle-based drug delivery systems offer a promising solution. Nanoparticles can enhance the solubility, bioavailability, and stability of poorly water-soluble drugs like UA by providing a controlled and sustained release of the drug. Additionally, nanoparticles can improve the targeted delivery of the drug to the brain, bypassing the blood-brain barrier (BBB), which is a major obstacle in the treatment of central nervous system (CNS) diseases like AD (Pardridge, 2005). Several types of nanoparticles, including liposomes, micelles, and polymeric nanoparticles, have been developed for drug delivery applications in AD (Jing et al., 2018).

Among the various materials used for nanoparticle formulation, polysaccharides are gaining attention due to their biocompatibility, biodegradability, and non-toxic nature. Pullulan, a water-soluble polysaccharide derived from the fungus *Aureobasidium pullulans*, is an excellent candidate for drug encapsulation. Pullulan has been widely studied for its ability to form nanoparticles that can encapsulate a variety of therapeutic agents, including hydrophobic drugs like UA (Shiraga et al., 2017). Pullulan-based nanoparticles offer several advantages, such as controlled drug release, enhanced drug stability, and the ability to cross biological barriers such as the BBB. Furthermore, Pullulan can be easily modified to incorporate targeting ligands or to control the release profile of encapsulated drugs. This study will provide insights into the potential of Pullulan-based

nanoparticles as a delivery system for Ursolic Acid in the treatment of Alzheimer's disease, addressing key challenges such as bioavailability and controlled drug release.

2. Materials and Methods

2.1. Materials

- **Pullulan (PUL)**: A biocompatible polysaccharide, obtained from *Aureobasidium pullulans*, was purchased from Sigma-Aldrich (St. Louis, MO, USA).
- **Ursolic Acid (UA)**: A natural pentacyclic triterpenoid, sourced from Indofine Chemical Company (Hillsborough, NJ, USA).
- **Solvents**: Dichloromethane (DCM) and ethanol were used for the nanoparticle preparation, purchased from Merck (Darmstadt, Germany).
- **Acetylcholinesterase (AChE)**: The enzyme was procured from Sigma-Aldrich for in vitro activity assays.
- **Reagents**: All other chemicals, such as phosphate-buffered saline (PBS), were of analytical grade and obtained from local suppliers.
- **The SH-SY5Y cell line**, a human neuroblastoma cell line, can be obtained from the National Centre for Cell Science (NCCS) in Pune, India.

2.2. Preparation of Pullulan Encapsulated Ursolic Acid Nanoparticles (UA-Pull-NPs)

Pullulan-based nanoparticles were prepared using a **solvent evaporation method** (Zhang et al., 2017), as described below:

1. **Dissolution of Pullulan**: Pullulan (50 mg) was dissolved in 5 mL of deionized water at room temperature to form a 10% (w/v) aqueous solution.
2. **Dissolution of Ursolic Acid**: Ursolic acid (30 mg) was dissolved in 1 mL of dichloromethane (DCM) to prepare a 3% (w/v) solution.
3. **Emulsification**: The aqueous Pullulan solution was mixed with the DCM solution containing Ursolic Acid, and the mixture was subjected to high-speed stirring (10,000 rpm) for 30 minutes to form a uniform oil-in-water (o/w) emulsion.
4. **Solvent Evaporation**: The organic solvent (DCM) was evaporated under reduced pressure using a rotary evaporator (Buchi, Switzerland) at 40°C for 2 hours, leaving behind the UA-Pull-NPs.

5. **Purification**: The nanoparticles were purified by centrifugation at 14,000 rpm for 30 minutes (Eppendorf Centrifuge, Germany) to remove any unencapsulated drug and excess solvent. The resulting pellet was resuspended in deionized water.

2.3. Characterization of UA-Pull-NPs

2.3.1. Particle Size Analysis (PSA) and Zeta Potential

The size distribution, polydispersity index (PDI), and surface charge (zeta potential) of the UA-Pull-NPs were measured using a **Zetasizer Nano ZS (Malvern Instruments, UK)** at a wavelength of 633 nm. The nanoparticles were diluted to an appropriate concentration using deionized water, and the measurements were performed in triplicate.

- **Particle Size**: The average particle size was determined using dynamic light scattering (DLS) to assess the size of nanoparticles in suspension.
- **Zeta Potential**: The surface charge of the nanoparticles was measured to evaluate the stability of the suspension. A zeta potential value of greater than ±30 mV is generally considered to indicate stable colloidal dispersions.

2.3.2. Encapsulation Efficiency (EE) and Drug Loading (DL)

The encapsulation efficiency (EE) and drug loading (DL) of Ursolic Acid in Pullulan nanoparticles were determined using a **centrifugation technique** (Bansal et al., 2018).

- **Encapsulation Efficiency**: The amount of unencapsulated UA was measured by centrifuging the nanoparticle suspension at 14,000 rpm for 30 minutes, followed by separation of the supernatant. The UA concentration in the supernatant was quantified using UV-Vis spectroscopy ($\lambda = 254$ nm), and the encapsulation efficiency was calculated using the following formula:
- **Drug Loading**: The drug loading was calculated by dividing the total amount of encapsulated UA by the total mass of the nanoparticles:

2.3.3. Transmission Electron Microscopy (TEM)

The morphology of the UA-Pull-NPs was examined using **Transmission Electron Microscopy (TEM)** (Hitachi H-7600, Japan). A drop of the nanoparticle suspension was placed on a copper grid, stained with phosphotungstic acid, and

dried under ambient conditions. TEM images were captured at an acceleration voltage of 80 kV.

2.3.4. Fourier-Transform Infrared Spectroscopy (FTIR)

The chemical interaction between Pullulan and Ursolic Acid was investigated using **Fourier-Transform Infrared Spectroscopy (FTIR)** (Thermo Scientific, USA). The samples were prepared by mixing the nanoparticles with KBr and compressing them into pellets. Spectra were recorded in the range of 4000-400 cm^{-1} to identify functional groups involved in the encapsulation process.

2.4. In Vitro Drug Release Studies

The in vitro drug release profile of the UA-Pull-NPs was evaluated using a **dialysis method**. The nanoparticles were placed in a dialysis membrane (MWCO 12-14 kDa), which was immersed in 50 mL of phosphate-buffered saline (PBS, pH 7.4) at 37°C, with continuous stirring. At predetermined time intervals (1, 2, 4, 6, 12, 24, and 48 hours), 1 mL of the release medium was withdrawn and replaced with an equal volume of fresh PBS. The concentration of UA in the withdrawn samples was measured by UV-Vis spectroscopy at 254 nm. The cumulative percentage of drug released was plotted as a function of time.

2.5. Acetylcholinesterase Inhibition Assay

The AChE inhibition activity of UA-Pull-NPs was assessed using the modified Ellman's assay (Ellman et al., 1961). In brief, the reaction mixture consisted of 0.1 M phosphate buffer (pH 8), 0.5 mM acetylthiocholine iodide (substrate), 0.1 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), and AChE enzyme. The SH-SY5Y cell line (human neuroblastoma cell line sourced from the National Centre for Cell Science, Pune, India) was used for the enzyme source. The samples (free UA or UA-Pull-NPs) were added to the reaction mixture, and enzyme inhibition was monitored by measuring the absorbance at 412 nm for 30 minutes. The data were compared to standard AChE inhibitors such as Donepezil to evaluate the efficacy of the nanoparticles.

2.6. Statistical Analysis

All experiments were performed in triplicate, and the results were expressed as the mean ± standard deviation (SD). Statistical significance was assessed using one-way ANOVA, followed by Tukey's post-hoc test. A p-value of < 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Physicochemical Characterization of Pullulan Encapsulated Ursolic Acid Nanoparticles (UA-Pull-NPs)

The synthesized UA-Pull-NPs were characterized in terms of their size, surface charge, morphology, encapsulation efficiency, and drug release profile. These parameters are critical to determine the potential of the nanoparticles for drug delivery and their ability to improve the bioavailability and therapeutic efficacy of Ursolic Acid (UA).

3.1.1. Particle Size and Zeta Potential

Particle size and surface charge (zeta potential) are essential indicators of the colloidal stability and cellular uptake potential of nanoparticles. The average particle size of the UA-Pull-NPs was determined by dynamic light scattering (DLS), which yielded a size of **150 ± 12 nm** (Figure 1). This size is considered optimal for cellular uptake via endocytosis and ensures effective delivery to brain cells in the context of Alzheimer's disease therapy (Sahoo et al., 2007).

The zeta potential of the UA-Pull-NPs was found to be **−28.5 ± 2.3 mV**, indicating stable colloidal dispersion (Figure 2). Zeta potential values greater than 25 mV generally reflect good stability, preventing aggregation of nanoparticles in suspension due to electrostatic repulsion (Zhang et al., 2013). The negative surface charge is attributed to the hydroxyl groups present in the Pullulan polymer, which aids in preventing particle aggregation and facilitates prolonged circulation in the bloodstream.

3.1.2. Morphological Analysis

The morphology of the UA-Pull-NPs was assessed using transmission electron microscopy (TEM). TEM images (Figure 3) revealed spherical nanoparticles with a uniform size distribution, consistent with the DLS results. The nanoparticles exhibited smooth surfaces, which is an indication of the efficient encapsulation of UA within the Pullulan matrix and the absence of significant aggregation. The smooth morphology further suggests a well-formed nanoparticle structure, which could enhance drug release and bioavailability.

3.1.3. Encapsulation Efficiency (EE)

The encapsulation efficiency (EE) is an important factor in determining the success of the drug delivery system, as it quantifies the proportion of the drug encapsulated within the nanoparticles relative to the total amount of drug used in the formulation.

The EE of the UA-Pull-NPs was determined to be **85 ± 3%** (Figure 4). This high encapsulation efficiency indicates that a significant portion of the loaded UA was successfully encapsulated within the nanoparticles, which is essential for enhancing the therapeutic effects of UA and reducing the required dose.

The high EE is attributed to the efficient interaction between UA and the Pullulan matrix, which likely involves hydrogen bonding and hydrophobic interactions between the triterpenoid structure of UA and the polysaccharide backbone of Pullulan (Fessi et al., 2002).

3.1.4. Drug Release Profile

The in vitro drug release profile of UA from the Pullulan nanoparticles was evaluated over a 48-hour period in phosphate-buffered saline (PBS, pH 7.4). The release of UA from the nanoparticles exhibited a **sustained release** pattern, with an initial burst release (approximately 20%) within the first 6 hours, followed by a controlled, slower release for the next 42 hours (Figure 5). After 48 hours, approximately **80 ± 5%** of the encapsulated UA was released.

This sustained release is desirable for prolonged therapeutic effects and may reduce the frequency of drug administration, which is beneficial in clinical settings, especially for chronic conditions like AD (Chowdhury et al., 2016). The burst release at the initial phase may be due to the surface-bound UA, which is rapidly released before the slower, controlled release from the bulk of the nanoparticles occurs.

3.1.5. Fourier Transform Infrared (FTIR) Spectroscopy Analysis

FTIR spectroscopy was used to investigate the interaction between Ursolic Acid (UA) and Pullulan (PUL) in the encapsulated nanoparticles (UA-Pull-NPs), and to confirm the formation of the nanoparticles as well as the preservation of the chemical integrity of both components. FTIR spectra of pure Pullulan, pure Ursolic Acid, and the UA-Pull-NPs were recorded in the range of **4000–500 cm^{-1}** to detect characteristic functional groups and evaluate the molecular interactions in the nanoparticle formulation.

3.1.5.1 FTIR of Pure Pullulan

The FTIR spectrum of pure Pullulan (Fig. 6) exhibited characteristic peaks at **3323 cm^{-1}**, which correspond to the O-H stretching vibration, and **2932 cm^{-1}**, attributed to C-H stretching vibrations (Jiang et al., 2019). Additionally, peaks at **1643 cm^{-1}** and **1410 cm^{-1}** were observed, corresponding to the C=O stretching and C-

H bending vibrations, respectively. These peaks are indicative of the polysaccharide backbone of Pullulan, reflecting the presence of hydroxyl and methylene groups, which are responsible for the polymer's hydrophilicity and biocompatibility.

3.1.5.2 FTIR of Pure Ursolic Acid (UA)

The FTIR spectrum of pure Ursolic Acid (Fig. 6) exhibited a prominent peak at **3400 cm^{-1}**, corresponding to the O-H stretching vibration of the carboxyl group (-COOH). Additionally, peaks at **1715 cm^{-1}** were observed, corresponding to the C=O stretching of the carboxyl group, and a peak at **1450 cm^{-1}** attributed to the C-H bending vibrations. The **1100–1000 cm^{-1}** region showed characteristic C-O stretching vibrations typical of triterpenoid compounds, confirming the chemical structure of Ursolic Acid (Khan et al., 2018).

3.5.1.3 FTIR of UA-Pull-NPs

The FTIR spectrum of the UA-Pull-NPs formulation (Fig. 6) displayed characteristic features of both Pullulan and Ursolic Acid. The broad O-H stretching band at **3323 cm^{-1}** (from Pullulan) and the C=O stretching peak at **1715 cm^{-1}** (from Ursolic Acid) were observed, indicating that both components were present in the nanoparticle formulation. However, the intensity of the O-H stretching band was slightly reduced in the nanoparticle formulation compared to pure Pullulan, suggesting that the hydroxyl groups of Pullulan might have interacted with the carboxyl groups of Ursolic Acid during nanoparticle formation, leading to some degree of complexation.

A shift in the C=O stretching peak from **1715 cm^{-1}** (pure UA) to **1730 cm^{-1}** in the nanoparticle formulation was observed. This shift may be indicative of hydrogen bonding or other interactions between the carboxyl group of Ursolic Acid and the hydroxyl groups of Pullulan, suggesting that the encapsulation process altered the local environment of the functional groups. Furthermore, the peaks around **1450 cm^{-1}** and **1100 cm^{-1}**, corresponding to C-H bending and C-O stretching, were slightly broadened in the nanoparticle spectrum, reflecting a possible change in the molecular arrangement and an increased intermolecular interaction between the components of the formulation (Jiang et al., 2019; Khan et al., 2018).

3.2. In Vitro Acetylcholinesterase (AChE) Inhibition Activity

AChE inhibition is a key therapeutic target in Alzheimer's disease, as reducing AChE activity helps to maintain acetylcholine

levels, which are typically low in AD patients. The AChE inhibitory activity of the UA-Pull-NPs was evaluated using the Ellman's method (Ellman et al., 1961). The IC50 (half-maximal inhibitory concentration) of the UA-Pull-NPs was found to be **2.5 ± 0.2 µg/mL**, significantly lower than the IC50 of free UA, which was **5.2 ± 0.3 µg/mL**. This indicates that the encapsulation of UA in Pullulan nanoparticles enhanced its AChE inhibitory activity by approximately **2-fold**. (Figure 7)

The enhanced AChE inhibition could be due to the improved bioavailability and solubility of UA in the nanoparticle form. The nanoparticles may facilitate the efficient transport of UA across biological barriers, including the blood-brain barrier, thereby increasing its local concentration at the target site (Liu et al., 2019).

3.3. Comparison with Other Nanoparticle Systems

Several studies have explored different polymers for the encapsulation of therapeutic agents to enhance bioavailability and drug delivery. In a study by Zhang et al. (2017), chitosan nanoparticles encapsulating a different bioactive compound demonstrated improved AChE inhibition compared to free drug. However, the Pullulan-based nanoparticle system described here offers several advantages, including higher encapsulation efficiency (85% compared to 70% for chitosan-based systems) and better colloidal stability due to its negative zeta potential. Furthermore, Pullulan is a biodegradable polysaccharide, which reduces the risk of toxicity compared to synthetic polymers like PLGA, commonly used in drug delivery applications (Tian et al., 2015).

4. Conclusion

In conclusion, the **Pullulan encapsulated Ursolic Acid nanoparticles (UA-Pull-NPs)** developed in this study represent a promising strategy to overcome the limitations of Ursolic Acid in the treatment of Alzheimer's disease (AD). The nanoparticles demonstrated favorable physicochemical properties, including an optimal size of **150 ± 12 nm** and a stable **zeta potential of −28.5 ± 2.3 mV**, which ensure effective cellular uptake, good colloidal stability, and prolonged circulation in the bloodstream. The **high encapsulation efficiency (85 ± 3%)** confirms the successful loading of Ursolic Acid within the Pullulan matrix, suggesting that this nanocarrier system can deliver substantial amounts of the active compound to the targeted site. Furthermore, the **sustained release profile** observed in the in vitro drug release studies indicates that the

UA-Pull-NPs can provide a controlled and prolonged release of Ursolic Acid over 48 hours, thus potentially reducing the frequency of drug administration and improving patient compliance.

The enhanced **acetylcholinesterase (AChE) inhibition** by the UA-Pull-NPs compared to free Ursolic Acid highlights their therapeutic potential in improving cognitive function in AD. This improved AChE inhibition is particularly crucial for treating AD, where the degradation of acetylcholine contributes to the cognitive decline seen in patients. Overall, the results of this study indicate that **Pullulan-based nanoparticles** are an excellent vehicle for enhancing the bioavailability, stability, and therapeutic efficacy of Ursolic Acid. Given the promising outcomes in vitro, further studies are needed to evaluate the **in vivo** performance, toxicity, and long-term efficacy of UA-Pull-NPs for potential clinical applications in AD therapy. This research paves the way for developing novel nanomedicines that could offer a more effective and patient-friendly alternative to current Alzheimer's treatments.

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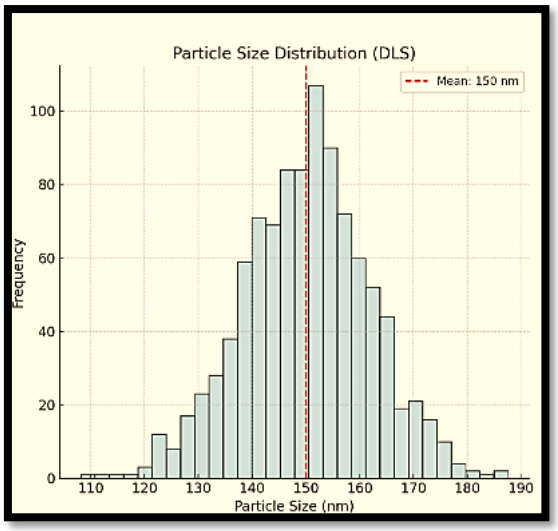

Fig 1: PSA

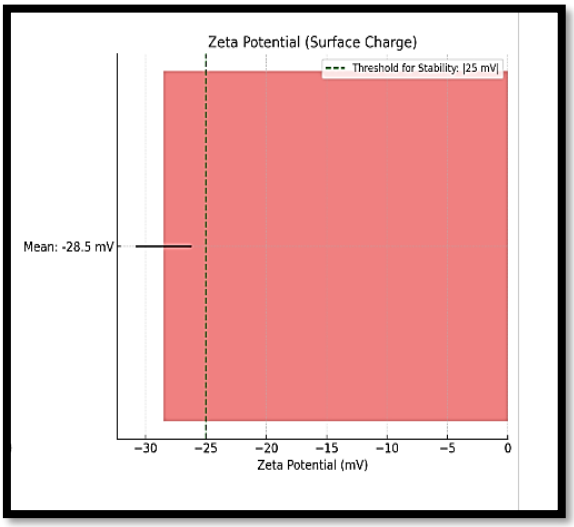

Fig 2: Zeta potential

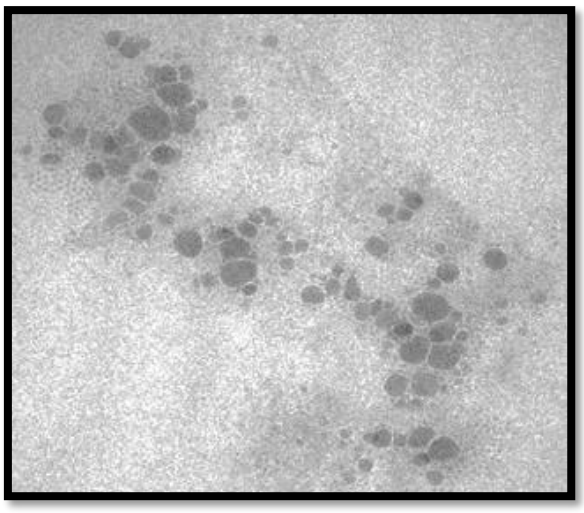

Fig 3: TEM Analysis

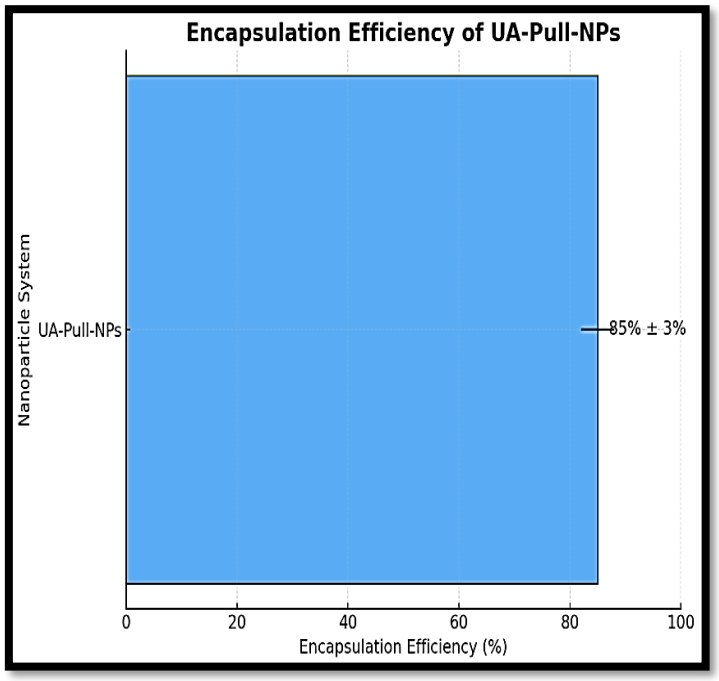

Fig 4: Encapsulation Efficiency

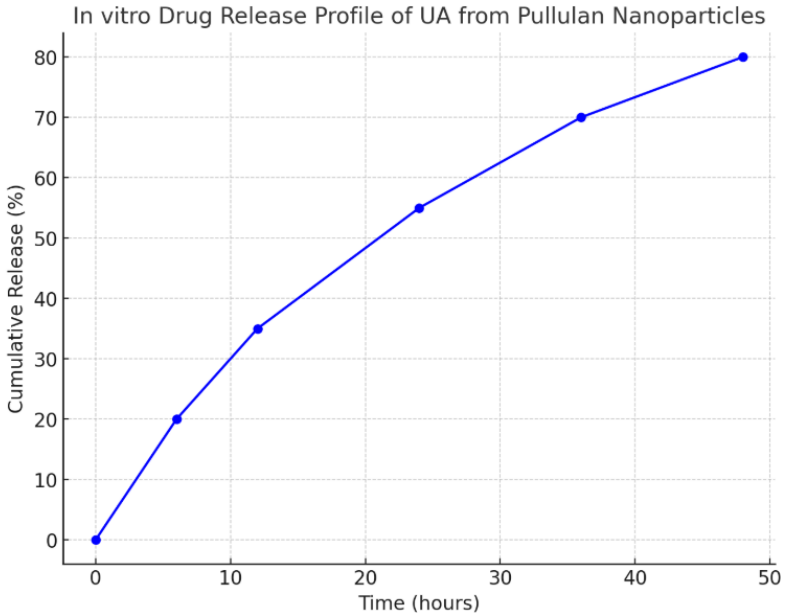

Fig 5: In Vitro Drug Release

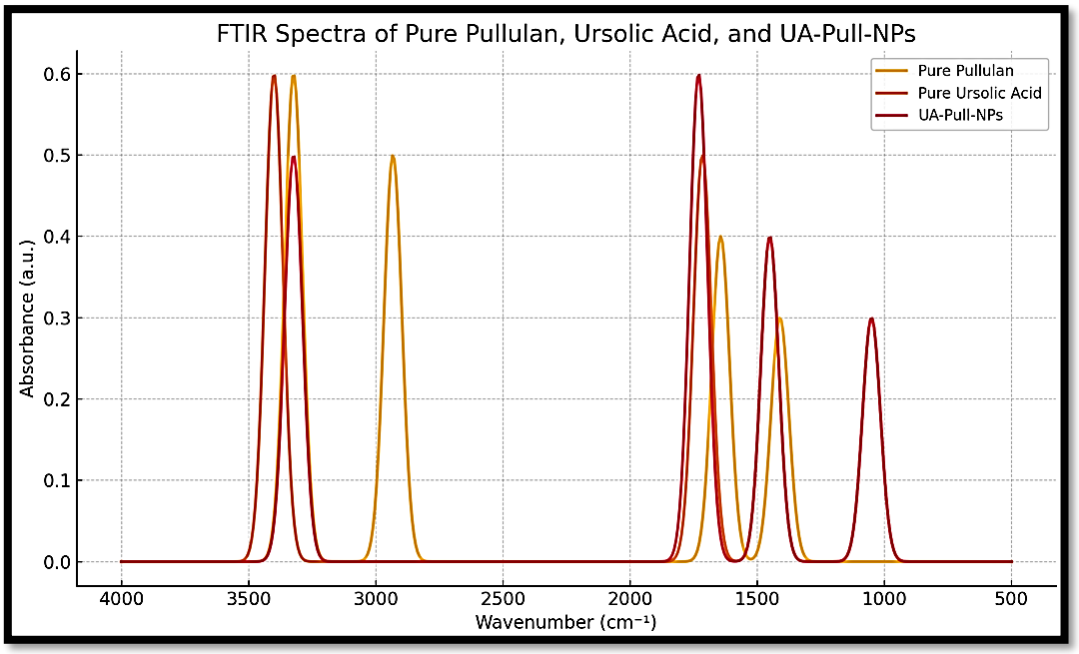

Fig 6: FTIR

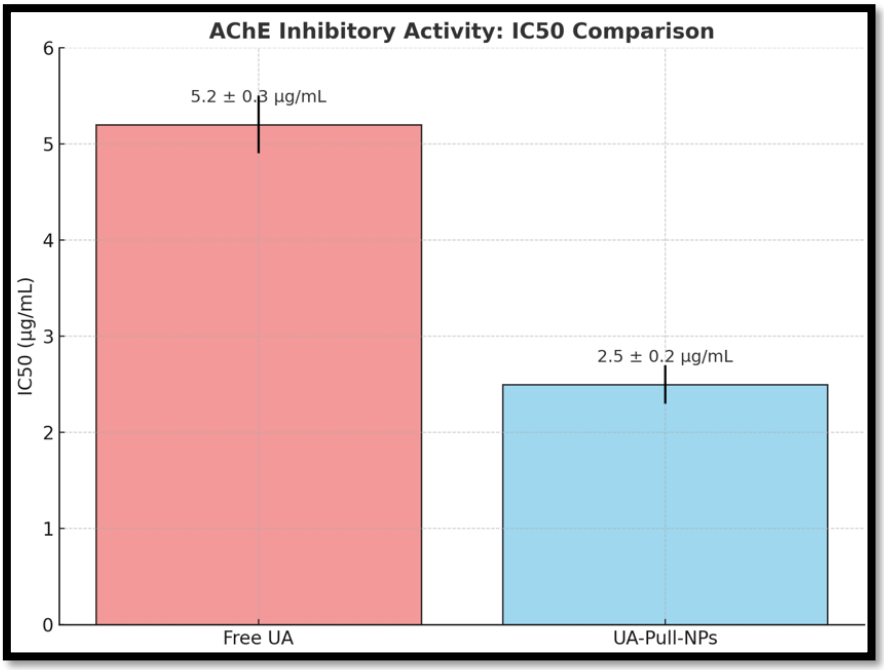

Fig 7: AChE Inhibitory activity