NANOPARTICLE

Research Article

Evaluating the Antimicrobial Effects of *Vernonia Amygdalina* through Encapsulation with Starch Nanoparticles

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ABSTRACT

Starch, a naturally abundant polymer, holds significant potential in polymeric drug delivery systems due to its biocompatible and biodegradable nature. However, native starch extracted directly from plants faces limitations such as low thermal stability and hydrophilicity. To address these challenges, starch nanoparticles have emerged as a promising drug delivery system that offer advantages like cost-effectiveness, high drug loading capacity, and controlled release of drugs. By incorporating phytochemicals from the plant Vernonia amygdalina, these nanoparticles are expected to exhibit improved stability and enhanced antimicrobial effectiveness. The collection and authentication of Vernonia amygdalina, preparation of phytochemical extracts, standardization of nanoparticle production processes along with the characterization, through Scanning Electron Microscopy (SEM), Dynamic Light Scattering (DLS), Fourier-Transform Infrared Spectroscopy (FTIR), and X-ray Diffraction (XRD), of the obtained nanoparticles are carried out in this study. Furthermore, the antimicrobial activity of the polymer nanoparticles against gram-positive and gram-negative bacteria is assessed using the Agar well diffusion method. This study aims to provide insights into the development of phytochemical-encapsulated starch nanoparticles and investigate their potential as antimicrobial formulations.

Keywords: Vernonia amygdalina, Starch Nanoparticles, Phytochemicals, Phytoextracts, Nanoprecipitation, Probe Sonication, Antimicrobial Activity, Nano Encapsulation, DLS, SEM, FTIR

INTRODUCTION

Vernonia Amygdalina, which can be seen in the region of African and Asian continents, is a small shrub with an average height of 2m and is commonly called "Bitter Leaf". *V.amygdalina* is very rich in minerals, especially phosphorus, calcium, potassium, magnesium, zinc,iron and some vitamins like vitamin A, C and E [1]. It belongs to the family of Asteraceae and does not produce

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seeds but its cultivation is usually done by stem planting and mostly grows in tropical areas [2]. The phytochemical studies of the extract of the plant *Vernonia amygdalina* had resulted in the isolation of flavonoids, saponins, alkaloids, tannins, phenolics, terpenes, steroidal glycosides, triterpenoids, and several types of sesquiterpene lactones [3-5] The aqueous and ethanol extracts of *V.amygdalina* leaves had shown antimicrobial effects against S*taphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* [2,6].

Phytochemicals, defined as biologically active compounds present in various plant parts such as leaves, barks, seeds, and roots, are essential sources of medicinal agents [7]. These compounds are classified as secondary metabolites and can produce pharmacological or toxicological effects in both humans and animals [8]. Categories of these secondary metabolites encompass phenolics, alkaloids, saponins, flavonoids, terpenoids, tannins, steroids, glycosides, and volatile oils, which find extensive applications in pharmaceuticals, cosmetics, and food additives. The preference for bioactive substances derived from medicinal plants is often attributed to their perceived safety and environmental sustainability in contrast to synthetic alternatives. The traditional knowledge associated with these plants has significantly facilitated the identification of novel bioactive compounds, which is a vital foundation for contemporary drug development [9].

Alkaloids are low-molecular-weight nitrogen-containing compounds recognized for their strong biological activities, which likely serve defensive roles in plants [10]. Terpenoids, or isoprenoids, are synthesized from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) and are classified into categories such as monoterpenes, sesquiterpenes, and triterpenes [10]. Flavonoids, the largest group of bioactive compounds, are significant phytonutrients and include subclasses such as flavanols, flavones, and isoflavones [11]. They are characterized by two benzene rings linked through a heterocyclic pyran ring, and variations in their side groups contribute to their diverse biological activities [12]. Phenolic compounds are lipid-soluble due to the hydroxyl group present in their chemical structure so they can react with the cellular membranes of microbes that leads to loss of cell membrane integrity. These compounds have mechanisms that show antimicrobial activity like disturbing microbial membranes, weakening cellular metabolism, control biofilm formation, inhibiting bacterial capsule production, attenuating bacterial virulence by controlling quorum-sensing, and reducing microbial toxin production [13].

Bacterial infections continue to be a major health challenge as they are leading causes of long term health diseases and high mortality rates worldwide. While antibiotics have been the go-to treatment due to their effectiveness and low cost. The extensive use of these drugs has resulted in development of bacterial resistance to multiple antibiotics [14]. It is crucial to develop new antimicrobial agents to address this issue and nanoparticles have emerged as a promising solution in this field. Nanoparticles to exhibit antibiotic activity must interact with bacterial cells. This interaction occurs through a combination of electrostatic, van der Waals, receptor-ligand, and hydrophobic forces [15-18]. NPs penetrate the bacterial membrane via phagocytosis and endocytosis and accumulate within the cell disrupting the membrane's structure and impacting cellular metabolism [19]. Nanoparticle carriers can direct antibiotics to the site of infection reducing the risk of systemic side effects [19]. Nanoparticles enhance antibiotic activity by disrupting bacterial membranes through mechanisms like pore formation and increased permeability [20] these lead to cell leakage and death. They also alter membrane structure and lipid composition impairing essential functions [18]. Additionally, nanoparticles generate reactive oxygen species that cause oxidative damage further wearing the bacteria [19]. By penetrating biofilms nanoparticles make bacteria more vulnerable to antibiotics and hinder the development of resistance and ultimately improving the treatment efficacy.

Starch is one of the most abundant and widely available natural polymers and can be sourced from a variety of fruits and vegetables including papaya, banana, corn, potato, rice, and sago [21,22]. Starch is valuable because of its abundance, renewability, biodegradability and non-toxic properties making it useful for many applications [23].

The chemical makeup of starch is primarily composed of two polysaccharide fractions: amylose and amylopectin and are crucial in defining the structure and properties of native starch [21,22,24]. Amylose is a linear polymer that is linked by α-D-(1-4) glycosidic linkages that makes 20–30% of total starch and amylopectin is a branched chain polymer with α-D-(1-4) glycosidic linkages in linear chain and α-D-(1-6) branched linkage [25]. Native starch is characterized for having a semi-crystalline nature with varying crystallinity that depends on the amount of amylopectin and amylose. The degree of crystallinity of the starch is influenced by the ratio of these two polymers where amylopectin predominantly contributes to the crystalline structure. The varying levels of

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amylopectin and amylose in different starch types results in unique melting behaviors which is related to their crystalline structure [21,22,24].

Starch granules are insoluble in water at room temperature because of their stable and well-organized semi-crystalline structure [26,27]. Upon exposure to thermal treatments like cooking their ordered structure becomes disorganized due to granule swelling, leaching of amylose and disarray of amylopectin [26]. Cooling, drying or storage of gelatinised starch leads to recrystallisation or retrogradation [28]. Retrogradation happens when amylose molecules shrink due to decreased kinetic energy and brownian motion which leads to formation of new hydrogen bonds between the hemiacetal oxygen and the adjacent hydroxyl groups in d-glucopyranosyl residues which leads to stronger bonding and precipitation of amylose in aqueous media [29,30].

Starch nanoparticles offer several advantages over native starch mainly in various applications such as drug delivery, food industry, and other material sciences. Due to the reduction of starch granules to nano size the nanoparticles have a higher surface area-to-volume ratio this increases their reactivity and interactions with other substances and also they have better solubility, which improves the bioavailability of nutrients or drugs [31]. Additionally, starch nanoparticles can be engineered for controlled release of encapsulated substances. They exhibit greater stability against environmental factors [32-34]. Starch nanoparticles are typically derived from renewable resources contributing to more sustainable product development. Starch nanoparticles are generally biocompatible and non-toxic, making them suitable for biomedical applications [35]. Drug adsorption efficiency increases with the surface area of the adsorbent and decreases as particle size grows. Due to their high surface area and ability to be functionalized, nanoparticles are ideal for targeted drug delivery [35,36]. Inside nanoparticle carriers the encapsulated drugs are shielded from harmful chemical reactions this helps to preserve their effectiveness and ensure targeted delivery to the site of action.

Our study focuses on the development of the starch nanoparticles loaded with the phytoextracts of *Vernonia amygdalina* and aims to evaluate the antimicrobial efficacy of these encapsulated nanoparticles, exploring their potential for the natural, sustainable alternative for the antibiotics. This approach of synthesis and evaluation seeks to contribute to the growing field of plant-based nanotechnology in antimicrobial research.

MATERIALS AND METHODS

Materials

The *Vernonia amygdalina* leaves were collected from the Medicinal Lives Nursery in Hosur, Bengaluru, Karnataka, and authenticated by the University of Agricultural Sciences, Bangalore.Waxy maize soluble starch powder of laboratory grade was procured. Other chemicals and reagents, including sodium hydroxide (NaOH), ethanol, nutrient agar, ciprofloxacin antibiotic, and bacterial strains (E. coli and Staphylococcus aureus), were supplied by PES University, Bangalore.

Extraction of Phytochemicals from Vernonia amygdalina

To prepare the phytochemical extract from Vernonia amygdalina, fresh leaves were collected and sun dried. The dried leaves were then ground into fine powder. Following the procedure outlined in Ekam and Ebong (2007), 25 grams of powder was dissolved in 100 ml of chilled ethanol and left to infuse overnight. The extract was subsequently filtered and the resulting filtrate was placed in a petri dish and allowed to dry for 48 hours ensuring complete evaporation of the ethanol [38]. The dried extract obtained is then utilized for synthesis of phytochemicals encapsulated with starch nanoparticles and was carefully collected.

Synthesis of Starch-Encapsulated Nanoparticles

Starch nanoparticles were encapsulated with the phytochemical extract of *Vernonia amygdalina* using a modified Nanoprecipitation and ultrasonication method, as previously explained by [38]. A 1.5% starch suspension was prepared by dissolving 2.25 grams of pure starch in 150 ml of 0.1 M NaOH, which was then heated to 80°C and stirred for 30 minutes. The phytochemical extract of 1.5 % was prepared by dissolving 0.9 grams of the concentrated extract in 60 ml of ethanol. 10 ml of the extract was added dropwise to 100 ml of the hot starch slurry at a 1:10 ratio. The mixture was stirred for an additional 30 minutes, with ethanol removed during heating. In order to increase the encapsulation efficiency, the resulting solution was subjected to probe sonication at 20 kHz for 40 minutes, with sonication cycles of 5 minutes on and 1 minute off, to prevent excessive heating.

Purification of Phytoextract-encapsulated Starch Nanoparticles

To purify the synthesised scratch nanoparticles, a series of differential centrifugation steps were carried out [15,39]. Initially

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the nanoparticle suspension was centrifuged at 4000 rpm to separate the mixture into a precipitate and a supernatant. Subsequent centrifugation steps were performed at 6000, 8000 and 9000 rpm to further isolate the nanoparticles. After each centrifugation the pellet was washed several times with distilled water and ethanol to remove any remaining plant extracts. The purified nanoparticles pellet was then subjected to a final centrifugation. To ensure complete purification 5 ml 70% diluted ethanol was added to assist in the drying process and the sample was vacuum dried for approximately 24 hours yielding powder of encapsulated starch nanoparticles.

Characterization of Nanoparticles

Dynamic Light Scattering (DLS)

The size distribution and stability of synthesized phytochemicals encapsulated starch nanoparticles were determined using Dynamic Light Scattering (DLS). Dynamic light scattering is a technique used to determine the size and stability of particles in suspension by analyzing variations in scattered light intensity, when the laser light interacts with moving nanoparticles and the resulting scattering patterns provide insights into their hydrodynamic radius and particle size distribution within a specific medium [40]. The size distribution and zeta potential of the synthesized nanoparticles were measured and analyzed using HORIBA Scientific SZ-100. The dried sample was diluted in ultrapure water and measurements were conducted at 25.3°C, with a scattering angle of 90°. Zeta potential analysis was performed under the same temperature conditions with a conductivity of 0.438 mS/cm and an applied voltage of 3.3 V.

Scanning Electron Microscopy (SEM & EDX)

Structural features of the phytoextract-encapsulated starch nanoparticles were examined using Scanning electron microscopy, where the samples were sputtered with gold and micrographs of the samples were recorded with the following settings: The electron beam was operated at an accelerating voltage (EHT) of 5.00 kV with a working distance (WD) of 7.63 mm to acquire images at different magnifications of 500X, 2KX and a working distance of 7.69 mm for 500X. The signal used for imaging was set to SE1 (secondary electrons) for enhanced surface resolution. Energy-dispersive X-ray (EDX) analysis was performed on the phytoextract-encapsulated starch nanoparticle sample using a Zeiss Smart EDX system. The analysis was conducted with an accelerating voltage of 20 kV, a magnification of 500x, a take-off angle of 34.2°, and a resolution energy of 128.8 eV.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra of starch nanoparticles encapsulated with phytoextracts of *Vernonia amygdalina* is performed. The absorbance was recorded from 399.28 cm^{-1} to 4000.50 cm^{-1}.

Antimicrobial Activity Evaluation

Agar Well Diffusion Assay

The antibacterial activity of synthesized nanoparticles was evaluated using agar well diffusion method. Two bacterial strains were used: *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative). A loopful of bacteria from pure cultures of *Staphylococcus aureus* and *Escherichia coli* was transferred into freshly prepared and sterilized nutrient broth in two separate 5 mL eppendorf tubes. The tubes were shaken to ensure even dispersion of the bacteria. The resulting inoculum was then poured onto a 15 cm diameter petri dish. Afterwards, a freshly prepared and sterilized nutrient agar was poured into the dish and gently mixed to ensure uniform distribution of the inoculum. Once the agar had solidified five wells were created using the back of a sterile 1 ml microtip.

For the assay, the following treatments were added to the wells:

1. Negative control: 50 µL of Distilled water was added to the first well.
2. Test sample with water: 50 µL solution containing 0.2 g of phytochemical encapsulated starch nanoparticles in 2 mL water was added to the second well.
3. Negative control: 50 µL DMSO was added to the third well.
4. Test sample with DMSO: A solution containing 0.1 g of the sample in 1 mL DMSO was added to the fourth well.
5. Positive control: Standard antibiotic ciprofloxacin (1×10^{-6} M) was added to the fifth well.

The plates were incubated at 37°C for 24 hours. After incubation, the zones of inhibition around each well were measured to assess the antibacterial activity of the test samples.

Control Samples

Ciprofloxacin, a standard antibiotic, served as the positive control, prepared by diluting a 10 mg/ml stock solution to a

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concentration of 10 µg/ml. Two negative controls were used: sterile water and a 10% (0.1 gm/1 ml) dimethyl sulfoxide (DMSO) solution, to evaluate antimicrobial efficiency of the Starch nanoparticles encapsulated with phytoextracts of *Vernonia amygdalina* dissolved in water and DMSO, respectively, against *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) bacteria.

Measurement of Antimicrobial Activity

After 24 hours of incubation, the petri plates were analyzed to determine the inhibition zones surrounding the samples and controls. The measurement of these zones was conducted using ImageJ software, with the petri plate diameter considered as the reference scale. Based on this scale, the inhibition zone diameters were precisely calculated to assess the antimicrobial activity of the samples.

RESULTS AND DISCUSSION

Physicochemical Properties of Starch- Encapsulated Nanoparticles

Particle Size and Zeta Potential

The Dynamic Light Scattering (DLS) analysis of the synthesized nanoparticles revealed an average hydrodynamic diameter of 289.6 nm with a mode of 264.4 nm. Indicating a slight skew toward larger particle sizes. The Polydispersity Index (PDI) was 0.714 suggests a moderately broad size distribution. The PDI scale ranges from 0 to 1 where the values closer to 1 indicate heterogeneity while the values below 0.5 suggest homogeneity [41]. Despite the relatively broad distribution they obtained size distribution (Figure 1) shows a single dominant peak that confirms the nanoparticles predominantly fall within the 289.6 nm size range consistent with the intended formulation.

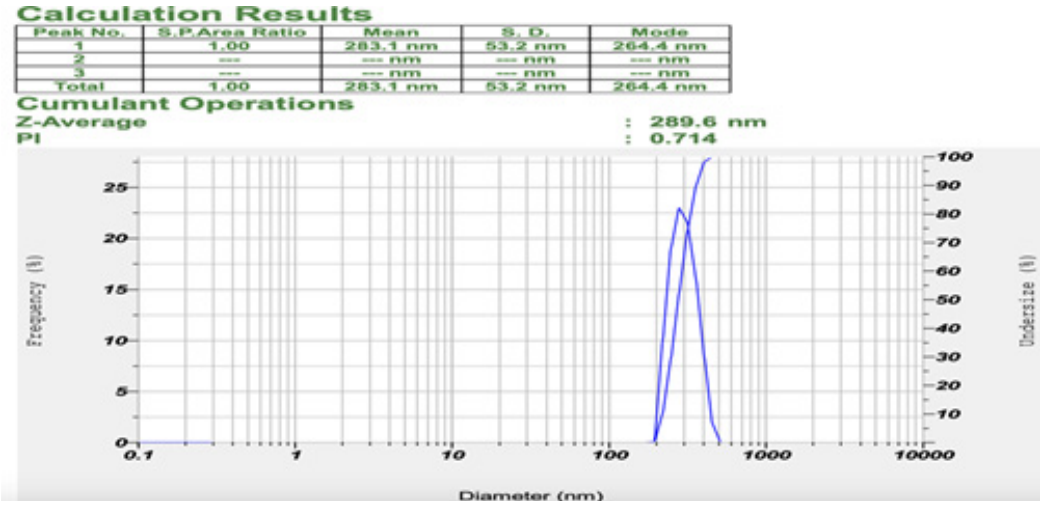

Figure 1. DLS analysis of phytochemicals encapsulated starch nanoparticles.

The zeta potential of samples was measured to analyze the surface charge and stability. The results revealed the Zeta potential of the phytochemical-encapsulated starch nanoparticles was -19.9 mV. The electrophoretic mobility was calculated to be 0.000155cm^3/V·s (Figure 2).

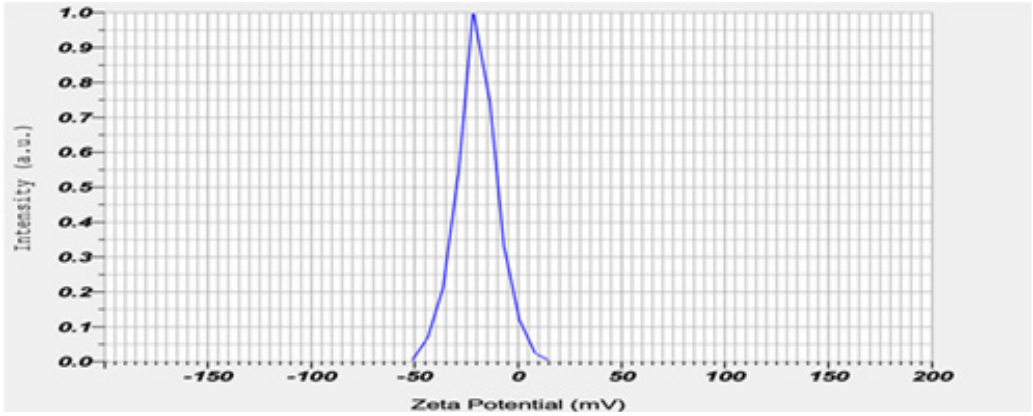

Figure 2. Results of Zeta potential measurement of phytochemical-encapsulated starch nanoparticles showing a value of -19.9 mV.

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Morphological and Elemental Characterization – SEM images and EDX

Scanning electron microscopy (SEM) is a widely used technique for high-resolution imaging of surfaces, essential for characterizing nanoscale materials by providing information on size, size distribution, and nanoparticle (NP) dispersion within various matrices [42]. By integrating an X-ray spectrometer with SEM, researchers can perform element mapping and point analysis [43], allowing for qualitative and quantitative assessments through energy-dispersive spectroscopy (EDX). The mean particle size calculated through ImageJ software was 324.6 nm and the EDX analysis of the Starch encapsulated nanoparticles showed the presence of elements; Sodium (1 % weight), Silicon (0.5% weight), Chlorine (0.4% weight) and Potassium (0.3% weight), in addition to Carbon (55% weight) and Oxygen (42% weight), which are typically found in starch. This indicates the phytochemical constituents of *Vernonia amygdalina* being present in the matrix of Starch nanoparticles (Figures 3 & 4).

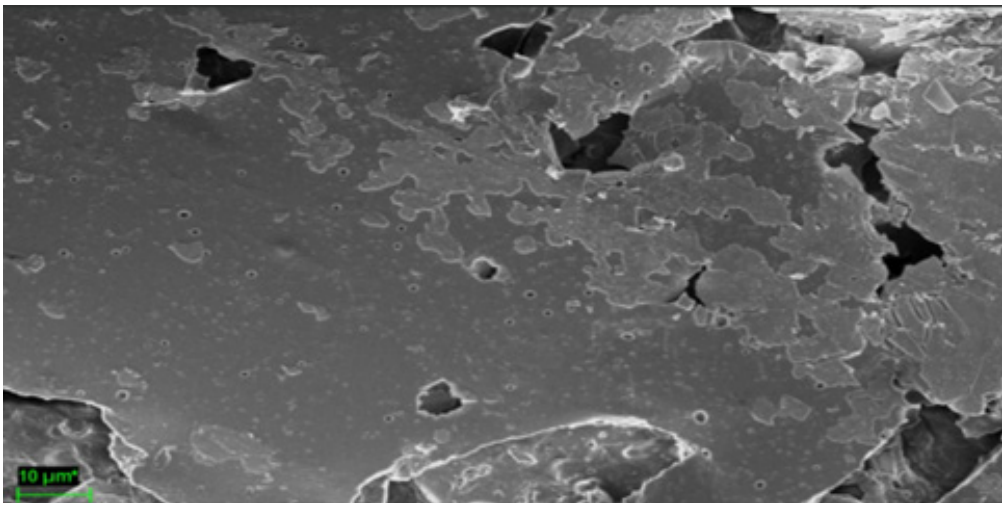

Figure 3. SEM images of Phytochemical encapsulated starch nanoparticles at 2.00KX magnification.

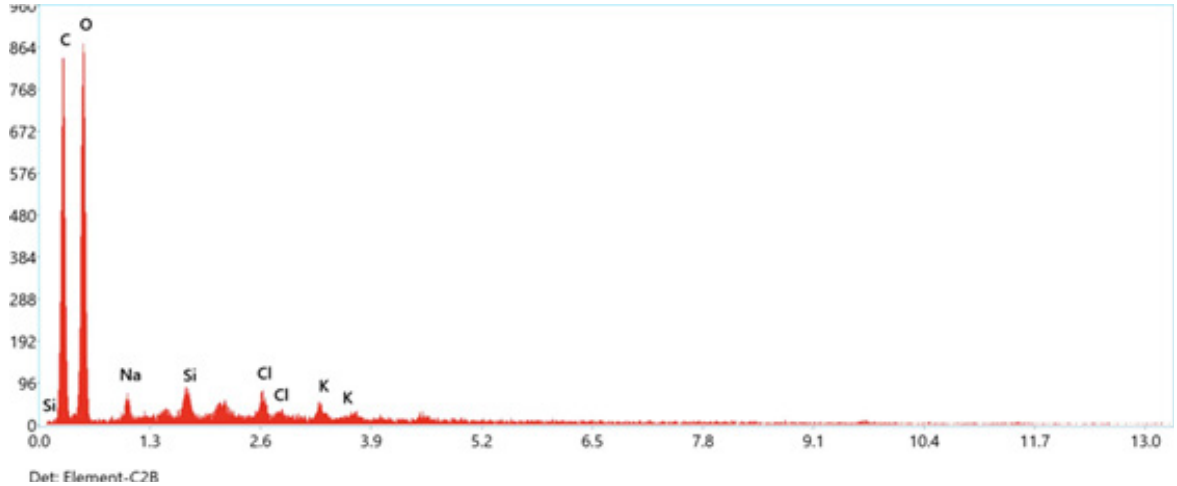

Figure 4. EDX analysis of Phytochemical encapsulated starch nanoparticles at 2.00KX magnification.

Functional Groups – FTIR spectra

The FTIR spectrum of the starch nanoparticles encapsulated with phytoextracts of *Vernonia amygdalina* is shown in Figure 5. The FTIR results of the sample give several key insights. The broad peak at the 3429 cm^{-1} corresponds to the hydroxyl(OH) and amino group(NH) stretching vibrations, indicating the presence respective groups. Peaks at 2924 cm^{-1} and 2854 cm^{-1} constitute to alkyl functional group i.e, C-H stretching vibrations. Additional peaks at the 1641 cm^{-1} and 1454 cm^{-1} are associated with O-H bending and aromatic ring skeleton

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vibrations, respectively. Peaks between 1150 cm^{-1} and 950 cm^{-1}, especially at 1026 cm^{-1}, indicates the presence of C-O-C bonds, which can be seen in saponin structures [44]. These results align with the earlier research findings of Monjezi et al., 2019 and Ch'ng, Yung Sing, et al., 2017 [45,46], which indicates the successful encapsulation of starch nanoparticles with phytoextracts containing alkaloids, flavonoids, and saponins.

The prominent O–H and N–H stretching vibrations indicate strong hydrogen bonding which are crucial for stabilizing the encapsulated bioactive compounds. The identified aromatic and ester groups indicate the presence of alkaloids, flavonoids, and saponins, which are beneficial for antimicrobial activity. The findings are consistent with the conventional FTIR spectrum reported by Ch'ng, Yung Sing, et al., 2017 [45,46] for *Vernonia amygdalina* extracts and Monjezi et al., 2019 for starch nanoparticles.

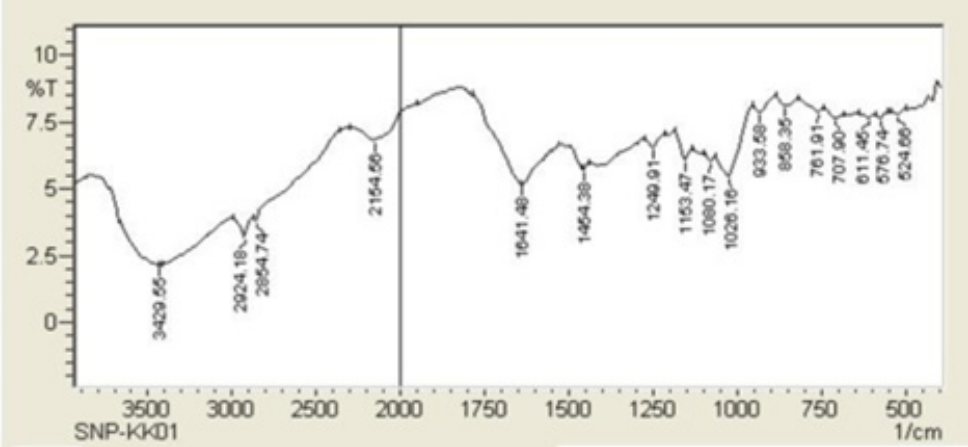

Figure 5. FTIR spectra of Phytochemical-encapsulated starch nanoparticles.

Antimicrobial Activity

The antimicrobial activity of starch-encapsulated nanoparticles was compared in two different solvents: water and 10% DMSO, with Ciprofloxacin (0.1 µg/ml) as the standard antibiotic. Starch-encapsulated nanoparticles dissolved in 10% DMSO showed a ZOI of 11.29 mm and 18.31 mm for *E.coli* and *S. aureus* respectively, where gram positive bacteria showed a greater zone of inhibition. Compared to Ciprofloxacin, which had a ZOI of around 22.6 mm and 25.6 mm for *E.coli* and *S. aureus* respectively (Figures 6 & 7) (Tables 1 & 2).

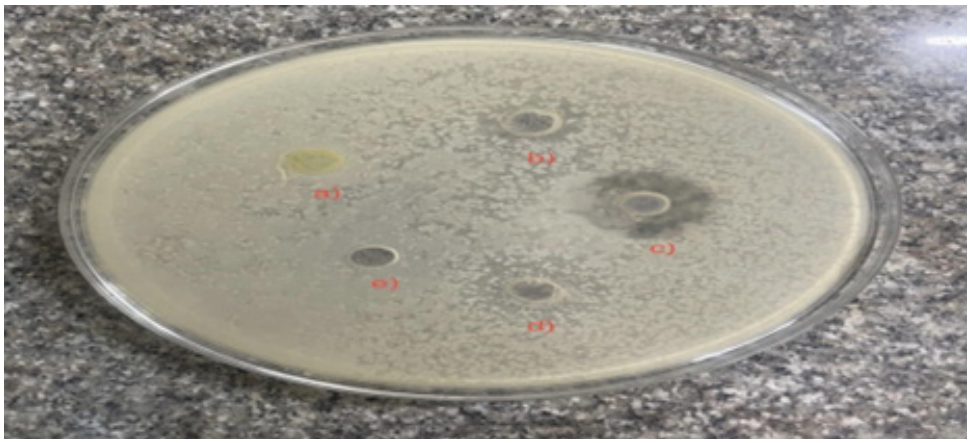

Figure 6. Agar well diffusion assay against S.Aureus where **a)** Phytoextract-encapsulated starch nanoparticles dissolved in water, **b)** Phytoextract-encapsulated starch nanoparticles dissolved in 10% DMSO, **c)** Antibiotic standard- Ciprofloxacin, **d)** Negative control- water and **e)** Negative control- 10% DMSO Solution.

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Table 1. Antimicrobial Effect Against Gram-Positive Bacteria Staphylococcus aureus

	Sample (Conc - 0.2/20 mg/ml)	Average Zone of inhibition: ZOI(mm)	ZOI of Std Antibiotic Ciprofloxacin (Conc - 0.1µg/ml) (mm)
a)	Starch encapsulated nanoparticles dissolved in water	-	1.6 mm
b)	Starch encapsulated nanoparticles dissolved in 10% DMSO sol	18.31mm	

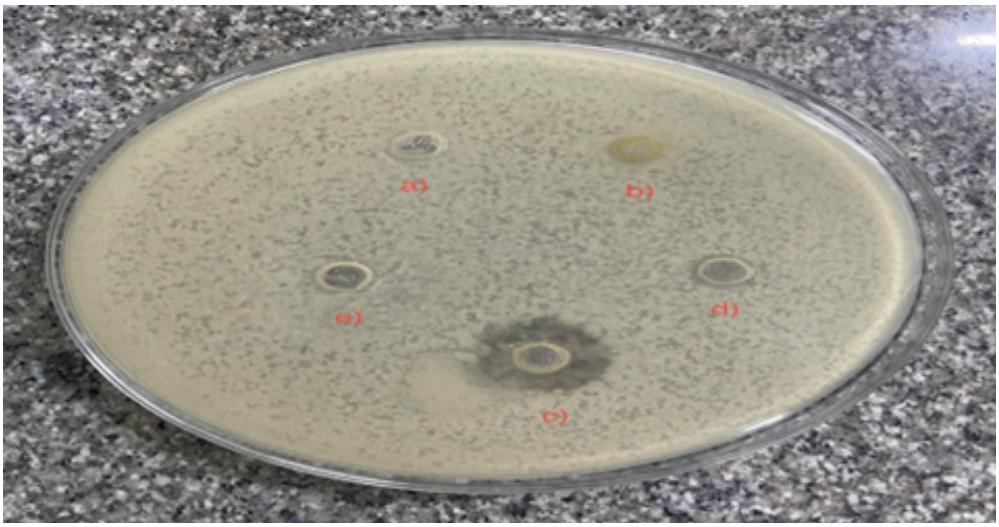

Figure 7. Agar well diffusion assay against *E.coli* where a) Negative control- Water b) Phytoextract-encapsulated starch nanoparticles dissolved in water, c) Antibiotic standard- Ciprofloxacin, d) Phytoextract-encapsulated starch nanoparticles dissolved in 10% DMSO and e) Negative control- 10% DMSO Solution.

Table 2. Antimicrobial Effect Against Gram-Negative Bacteria Escherichia coli

	Sample (Conc - 0.2/20 mg/ml)	Average Zone of inhibition : ZOI(mm)	ZOI of Std Antibiotic Ciprofloxacin (Conc - 0.1µg/ml) (mm)
a)	Starch encapsulated nanoparticles dissolved in water	-	1.62 m
b)	Starch encapsulated nanoparticles dissolved in 10 % DMSO sol	11.29 mm	

Future Directions

Future research on this topic could focus on enhancing the antimicrobial efficacy of starch-encapsulated nanoparticles by optimizing the loading of *Vernonia amygdalina* phytochemicals to improve their bioavailability. Enhancing the encapsulation efficiency to increase the retention and controlled release of active compounds could enable more effective and targeted delivery systems. Developing *Vernonia amygdalina* based antimicrobial formulations could be a natural and more cost-effective alternative to synthetic drugs. Improving the stability of these nanoparticles under different environmental conditions could extend their shelf life and practical usability. These advancements hold significant potential for expanding the role of plant-based nanotechnology in pharmaceuticals and healthcare by offering sustainable and accessible therapeutic solutions which address the issue of microbial resistance and support the development of innovative and eco-friendly treatments.

CONCLUSION

Our research highlights the synthesis and characterization of starch-encapsulated nanoparticles loaded with *Vernonia amygdalina* phytochemicals which demonstrate their

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potential as effective antimicrobial agents. The nanoparticles exhibited antimicrobial activity against both *Staphylococcus aureus* and *Escherichia coli* whose inhibition zones were comparable to the standard antibiotic ciprofloxacin when dissolved in Dimethyl Sulfoxide (DMSO). These findings further showcase the potential of using starch-based nanocarriers as a biodegradable, cost-effective and eco-friendly alternative for antimicrobial applications. The use of phytochemical extracts also enhances the bioactive potential by offering a natural solution to address the growing issue of antibiotic resistance.

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CONFLICTS OF INTEREST

The authors declare there is no conflict of interest.

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