MANUFACTURING OF ACACIA NILOTICA LEAVES EXTRACT INCORPORATED CHITOSAN BEAD FOR POTENTIAL BIOACTIVITIES

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Abstract

***Acacia nilotica*, a medicinal plant with a rich historical context in traditional medicine, is noteworthy for its extensive application in the field of medicinal manufacturing. It is imperative to conduct a comparative analysis of the extract post-loading onto biopolymer versus its crude form. This study aimed to scrutinize the effects of *A. nilotica* leaves extract on the physicochemical properties and various biological characteristics of chitosan beads. The incorporation of the extract into chitosan beads was achieved using the calcium chloride ionic gelation method. The resulting beads, characterized by distinct chitosan/extract ratios (1:2, 1:3, and 1:4 w/w respectively), underwent characterization through Fourier Transform Infrared Spectroscopy (FTIR), X-ray Diffraction (XRD) analysis, particle size distribution evaluation via Dynamic Light Scattering (DLS), and Transmission Electron Microscopy (TEM). The *in vitro* antimicrobial efficacy was rigorously assessed against bacterial strains, including *Escherichia coli* and *Staphylococcus aureus*, as well as the fungal pathogen *Candida albicans*, utilizing the agar diffusion assay. The investigation further evaluated the diphenylpicrylhydrazyl (DPPH) radical scavenging and acetylcholinesterase inhibition (AChE) activities under in vitro conditions. The beads demonstrated a particle size of 1299 d.nm, with a Polydispersity Index (PdI) of 0.58, in contrast to the crude extract which presented a size of 771 d.nm and a PdI of 0.31. TEM analysis indicated a predominantly spherical morphology and distinctly aggregated particles with well-defined peripheries. High-Performance Liquid Chromatography (HPLC) analysis conducted over various incubation time intervals confirmed the strong adhesion of the loaded extract to the chitosan matrix. The beads exhibited notable antimicrobial and antioxidant capacities along with significant AChE activities at higher concentrations. This investigation elucidates the prospective benefits of synergistically incorporating *A. nilotica* extract into chitosan for applications in health and food packaging.**

Keywords: **Chitosan; A. Nilotica Leaves Extract; Antimicrobial; Acetyl Cholinesterase Inhibition Activity**

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Introduction

Natural medicinal flora possesses the capacity to prolong lifespan, foster optimal health, and facilitate self-healing processes (ElDeeb *et al*. 2024). The therapeutic attributes of these flora are ascribed to bioactive compounds, referred to as phytochemicals, which exert distinct physiological influences on the human organism (Begum *et al*., 2023; Basheera John and Vimala, 2023; Refaz *et al*., 2023). Prominent bioactive c; ompounds identified within this flora encompass alkaloids, flavonoids, tannins, phenolic compounds, steroids, resins, fatty acids, and gums, each of which can elicit considerable physiological reactions (Joshi *et al*., 2009). Considering the escalating resistance of pathogens to current antibiotic therapies, there exists a sustained imperative to identify novel antimicrobial agents to confront this resistance predicament; (Haroun *et al*., 2011; Haroun *et al*., 2013a; Haroun *et al*., 2013b; Cai *et al*., 2021). The emergence of novel diseases is propelling the global utilization of natural compounds within medical applications, particularly in encapsulated formulations for the management of diverse ailments in contemporary medicine. The ongoing rise of bacterial resistance compromises the effectiveness of various antibiotic classes and compels the development of efficacious strategies to address therapeutic obstacles (Haroun *et al*., 2014; Haroun *et al*., 2015; Rizk *et al*., 2018; Aliero *et al*., 2023). Acacia nilotica, a multifaceted nitrogen-fixing tree legume classified under the Mimosaceae family, is prevalent in subtropical and tropical locales, including regions of Africa and India (Agunu *et al*., 2005). Phytochemical investigations of its aerial components have identified flavonoids and polyphenolic substances within the flowers. Acacia is particularly abundant in antioxidant phenolics, such as condensed tannins, gallic acid, catechin, and other analogous compounds (ElToumy *et al*., 2011). Empirical studies have demonstrated that the antimicrobial characteristics of A. nilotica can be assessed through extracts derived from its foliage and bark. Nevertheless, current understanding remains scant regarding the specific impacts of its bioactive compounds on drug-resistant bacteria, particularly those implicated in diarrheal diseases (Teshome *et al*., 2019). Research has indicated pronounced antimicrobial efficacy against pathogens such as Staphylococcus aureus, E. coli, and *Salmonella typhi* in comparative assessments involving various acacia species. Ethanol and petroleum ether extracts have exhibited antibacterial properties against numerous bacterial strains, including *S. aureus* and *Klebsiella pneumoniae* (Okoro *et al*., 2014; Rasul *et al*., 2024). A multitude of investigations has underscored the antimicrobial and antioxidant capabilities of *A. nilotica* extracts, illustrating their health advantages for both human and animal populations (Kalaivani and Mathew 2010; Haroun *et al*., 2010; Abdulhamid *et al.*, 2019). Moreover, extensive research concerning *A. nilotica* flower and bark extracts integrated into chitosan beads has evaluated their antimicrobial efficacy relative to alternative agents in experimental settings. Notably, *A. nilotica* extracts have manifested substantial antimicrobial impacts against various pathogens, including *S. mutans* and *H. pylori* (Odewade *et al*., 2020; *Amshi et al*., 2022; Bhat *et al*., 2023; Byakod and Padma, 2023). Recent explorations into novel drug discovery from medicinal flora adopt an integrative methodology that amalgamates molecular, botanical, and phytochemical approaches. However, there persists a deficiency of information pertaining to the antimicrobial effects of *A. nilotica* leaves extract against prevalent pathogenic bacteria associated with human infections. This research endeavor seeks to investigate the inhibitory properties of chitosan-loaded leave extract from *A. nilotica*, employing calcium chloride ionic gelation, against specified pathogenic bacteria. Concurrently, Alzheimer's disease is characterized by diminished levels of acetylcholine in the cerebral context, a neurotransmitter crucial for memory retention and cognitive functions. Acetylcholinesterase (AChE) is an enzyme responsible for breaking down acetylcholine. By inhibiting AChE, acetylcholine levels can be increased, which may improve cognitive function (Hebert *et al*., 2003; Thangavelu and Ramasamy, 2015). This mechanism is critical for the development of Alzheimer's treatments, as it helps regulate acetylcholine levels in the brain. Investigating the kinetics and efficacy of AChE inhibitors, particularly those derived from *A. nilotica*, could offer valuable insights for creating innovative Alzheimer's therapies. Incorporating *A. nilotica* leaves extract into chitosan beads through ionic gelation represents a novel addition to existing research.

Materials and Methods

The leaves of *A. nilotica (L.)* Delile (subsp. nilotica) were collected from Upper Egypt in April 2024. The identification of the plant was verified by the Department of Flora Agricultural, Museum,

Ministry of Agriculture, and Herbarium of the Department of Botany, Faculty of Science, Cairo University. Voucher specimens have been preserved in the herbarium at the National Research Center, located on El-Tahrir Street, Dokki, Cairo, Egypt.

Extraction and isolation.

The powdered form of air-dried leaves from A. nilotica was defatted with chloroform (99.5%) and then extracted with a methanol:water mixture (7:3) at room temperature. The resulting extract was filtered, evaporated under reduced pressure, and then lyophilised. The dry extract was re-dissolved in 2 l of deionised water and extracted with 99.5% ethyl acetate. When the solvent evaporated, the extract mixed with the residual aqueous phase to form a dark brown solid. This extract was saved for future analyses.

Immobilization of the extract via the ionic gelation methodology.

Chitosan beads impregnated with *A. nilotica* leave extract were synthesised using ionic gelation of the divalent cation calcium chloride ($CaCl_2$), as described by Opanasopit *et al.* Chitosan (10 wt%) was solubilized in a 1% acetic acid solution at 40°C until completely dissolved, then cooled to room temperature. Various extract concentrations (20, 30, and 40 wt%) were added to the chitosan solution while stirring. The divalent cation ($CaCl_2$) was dissolved in distilled water to make 10 wt% solution. The extract-loaded beads were generated by gradually adding the chitosan/extract combination to the divalent cation solution while magnetically stirring (1000 rpm for 90 min) at room temperature. The resulting beads were separated by centrifugation of the previously saturated mixture.

Quantitative determination of the released phenolic compounds using HPLC

A 10 µL aliquot of the extracts was analyzed utilizing a Shimadzu LC-20AT high-performance liquid chromatography (HPLC) system, which was outfitted with a quaternary pump, a solvent degasser, an auto-sampler, and a photodiode array (PDA) detector. The separation of compounds was accomplished on a Kinetex C18 column (150 × 4.6 mm, 5 µm; Agilent Technologies, Santa Clara, CA, USA) employing a gradient elution technique. Mobile Phase A was composed of water adjusted with phosphoric acid (pH 2.3), whereas Mobile Phase B consisted of acetonitrile. The gradient elution proceeded from 5% to 90% of Mobile Phase B over a duration of 45 minutes, maintained at a consistent temperature of 35°C and a flow rate of 0.8 mL/min. The components of the mobile phase were subjected to filtration and degassing through Macherey-Nagel (MV) filters with a pore size of 0.20 µm and were processed in an Elma-Elmasonic P sonication bath prior to each daily HPLC analysis. Detection occurred over a wavelength spectrum of 190-800 nm, with quantification executed at wavelengths of 280, 320, and 360 nm. (±) Catechin hydrate (purity ≥96%, sourced from Sigma) was employed as a standard for the calibration curve.In vitro biological activity (Opanasopit *et al*., 2008).

Antimicrobial examination Preparation of microbial cultures.

The nutrient medium was prepared following the manufacturer's guidelines, containing 10 g/l glucose, 6 g/l peptone, 3 g/l yeast extract, 1.5 g/l meat extract, and 28 g/l agar. It was sterilized at 120°C for 20 minutes. Pathogenic strains, including *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 8739), and *Candida albicans* (ATCC 10231), were sourced from the Laboratory of Chemistry of Natural and Microbial Products at the National Research Centre in Egypt. The cultures were maintained on nutrient agar medium and stored at 4°C in a refrigerator.

Inhibition zone technique

The bacterial strains were cultured in nutrient media at 37°C for 24 h, while the yeast was incubated on potato dextrose agar (PDA) at 27°C for 48 h. The culture media were diluted 1:1000 with sterile 0.9% saline solution, yielding a suspension of approximately 5×10^9 microorganisms per ml. The samples, including the *A. nilotica* leaves extract, were tested for antibacterial activity on nutrient agar plates using the agar well diffusion method described by Agarry *et al*. 2005. Freshly prepared nutrient agar was poured into 12 cm Petri dishes and inoculated by evenly spreading the pathogenic strain suspension across the surface. Pathogen concentrations were standardized to the 0.5 McFarland standard (which corresponds to approximately 1.5×10^8 CFU/ml). Wells were created in the agar, and 100 µl of each sample was introduced into the wells. The inoculated plates were incubated at 37°C for 24 h, and the formation of inhibition zones around the wells indicated antibacterial activity. Determination of minimum inhibition concentrations (MIC)

The MIC of the extract from the sample was defined as the minimal concentration of the sample in µg/ml that inhibits the growth of the tested pathogen. Thereby indicating the absence of discernible bacterial proliferation (Eve *et al*. 2020). Five distinct concentrations of each evaluated sample were formulated (12.5, 25, 50, and 150 mg/ml) for the purpose of assessing the MIC. Data are mean of three replicates in each group.

Cell count method

This methodology was employed to corroborate the preceding MIC findings. A precise weight (0.2 g) of the tested samples was solely introduced into 100 ml conical flasks containing 25 ml of nutrient broth media. Blank flask contained nutrient broth only without addition of any samples. The flasks were duplicated and subsequently inoculated with pathogenic cells and incubated at 37°C for 24 h. The serial dilution technique was implemented for the cells suspension of each culture, with 0.01 ml utilized to inoculate nutrient agar media in Petri dishes. Following a 24 h incubation period, the cells were enumerated, and the number of colony-forming units (CFU/ml) was computed utilizing the following equation (Vembadi *et al*., 2019). Data are mean of three replicates in each group:

CFU/ml = (number of colonies × dilution factor) / volume of plated culture (1)

In vitro examination of antioxidant activity

In vitro assessment of antioxidant activity (specifically, the diphenylpicrylhydrazyl radical DPPH scavenging activity) was conducted in accordance with the methodology outlined by (İlhami *et al*. 2005). Two very diluted concentrations, to overcome the interference of high concentration colour depth with the optical density reading range of the spectrophotometry (0.01 and 0.05 µg) of the evaluated material were placed in test tubes. One ml of absolute ethanol was added to each test tube and subsequently vortexed. Each test tube received one ml of DPPH, which was allowed to incubate in the dark for thirty minutes before the absorbance was measured against the blank using a UV-visible spectrophotometer at a wavelength of 517 nm. The blank consisted of 1 ml ethanol, while the control comprised 1 ml ethanol plus 1 ml DPPH. The DPPH scavenging activity was calculated as:

% Inhibition = read of control - read of test / read of control x100 (2)

In vitro Acetyl cholinesterase inhibition assay

The inhibitory activity of acetylcholinesterase (AChE) was measured using a colorimetric assay following the method described by (Ellman *et al*. 1961). In this assay, 150 µl of 100 mM sodium phosphate buffer (pH 8.0), 10 µl of the test solution at various concentrations (3.12, 6.25, 12.5, 25, 50, 100, and 200 µM), and 20 µl of AChE solution (5.32×10^{-3} U) were combined in a 96-well microplate and mixed thoroughly. After a 15-minute incubation at 25°C, the reaction was initiated by adding 10 µl of 0.5 mM 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB) and 10 µl of the substrate acetylthiocholine iodide (0.71 mM). The hydrolysis products, 5-thio-2-nitrobenzoate and 2-nitrobenzoic-5-mercaptothiocholine, were monitored spectrophotometrically at 412 nm at 5-min intervals for 15 min using a 96-well microplate reader (PerkinElmer) in triplicate experiments. Donepezil was used as a reference compound for comparison. The results were expressed as IC_{50} (µg/ml), and the percentage of inhibition was calculated using the formula:

Inhibition (%) = [(Ac−As) / Ac] ×100 (3)

Where Ac and As are the absorbance's of the reference and sample as measured by the UV-visible spectrophotometer, respectively. Methanol in phosphate buffer (pH 8) served as the blank sample

Statistical analysis

Data are mean percentage ±SD of three replicates in each group. Statistical analysis is carried out using One Way Analysis of variance combined with post hoc (Least Significant Variance, LSD), computer program coupled with Co-state computer program, where different letters are significant at $p \leq 0.05$.

Results and Discussion

Physicochemical characterization

The Fourier Transform Infrared Spectroscopy (FTIR) analysis was conducted on the crude extract of *A. nilotica* leaves, the chitosan beads containing the extract, and the raw chitosan (refer to Figure 1) to validate the presence of functional groups within the loaded extract and to elucidate the possible interactions between the extract and chitosan. The spectrum of the loaded *A. nilotica* extract reveals a broad band in the range of 3286-3626 cm^{-1}, which can be attributed to the -OH stretching of alcohols and phenols, as well as the -NH stretching associated with amides, thereby confirming the existence of amide linkages. In contrast, the spectrum of raw chitosan exhibits characteristic peaks at 1650, 1319, and 1025 cm^{-1}, which are assigned to N-H bending, C-N (amide II), and C-O stretching vibrations, respectively. This indicates that the asymmetric stretching of the N-H bonds (amide A) within the amino groups is more pronounced in raw chitosan when juxtaposed with the loaded sample (Rubilar *et al*. 2013; Suriyatem *et al*., 2018; Chaiwong *et al*. 2020). Such displacements may arise from interactions between the chitosan matrix and the phenolic compounds

inherent in the *A. nilotica* leaves extract (Bitencourt *et al*., 2014). Furthermore, in the spectrum of the raw extract, the characteristic peaks ranging from 1700 to 1670 cm^{-1}, which are attributed to C=O stretching vibrations (indicative of carboxylic acid groups), suggest the presence of phenolic acids. The asymmetrical stretching of N-O groups has shifted to 1458 cm^{-1}, while the characteristic peak around 3410 cm^{-1} corresponds to the -OH stretching vibration, indicating the presence of alcoholic -OH groups and hydrogen-bonded phenols. Overall, the spectrum of the loaded extract demonstrates numerous characteristic absorption peaks of the crude extract being displaced, which likely indicates the attachment of the extract onto the chitosan beads. This data corroborates findings from prior studies (Siripatrawan *et al*., 2010; Wu *et al*., 2013).

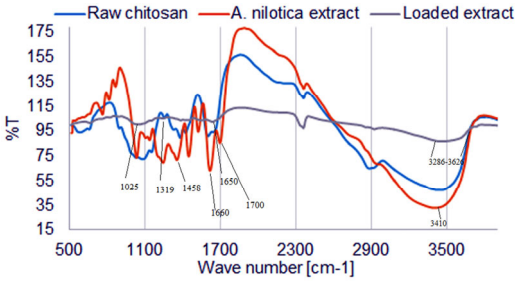

Figure 1. FTIR spectra of the loaded A. nilotica extract in compared with the raw chitosan and the crude extract

Figure 2 illustrates the X-ray Diffraction (XRD) patterns of the loaded *A. nilotica* leaves extract in comparison with raw chitosan. The distinctive peak of chitosan at 2θ 19.88° signifies the amorphous nature of chitosan. Upon the incorporation of the extract into chitosan, the aforementioned strong diffraction peak was no longer observable. Specifically, at 2θ 19.88°, the bead containing the loaded *A. nilotica* leaves extract exhibited a broad peak. This observation suggests that hydrogen bonding between chitosan and polyphenols restricts the mobility of molecular chains and impedes the crystallization process. Literature indicates that when tea polyphenols are combined with chitosan or starch, varying concentrations of tea polyphenols do not yield distinct diffraction peaks (Wu *et al*. 2009; Hussain *et al*., 2022). These findings imply that an interaction between the chitosan polymer matrix and the phenolic compounds present in the extract has been established.

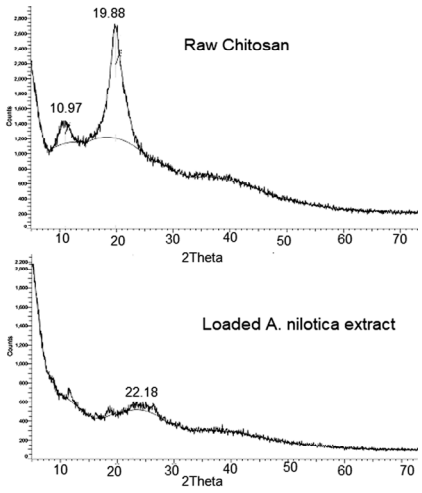

Figure 2. XRD patterns of the loaded A. nilotica extract in compared with raw chitosan

Figure 3 displays Transmission Electron Microscopy (TEM) images of *A. nilotica* leave extract encapsulated in chitosan beads, shown at varying scales (0.5, 1, and 2 µm). The microcapsules formed using chitosan beads via $CaCl_2$ ionic gelation exhibited relatively uniform sizes with smooth, spherical morphologies. Factors such as the viscosity and surface tension of the substrate solution, along with the $CaCl_2$ concentration, were identified as key determinants of the size and shape of the calcium-based beads (Lee *et al*., 2013). The findings suggest that incorporating active compounds from *A. nilotica* into chitosan induces various structural and physical modifications, which depend on the specific type of extract used.

Figure 4 illustrates the analysis of particle size distribution for the loaded *A. nilotica* leaves extract, both prior to and following its storage in a buffer with a pH of 7.4 at ambient temperature for a duration of 21 days, in comparison to the raw extract, utilizing Dynamic Light Scattering (DLS) methodology. It is evident that the average particle size of the loaded extract was determined to be 1299 d.nm, in contrast to the crude extract which measured 771 d.nm. This increase in particle size is attributable to the incorporation of the A. nilotica leaf extract. Conversely, subsequent to the 21-day storage period of the loaded extract in the aqueous buffer at room temperature, a reduction in particle size to approximately 443 d.nm was observed. This phenomenon may be indicative of the release of hydrolyzed phenolic compounds from the beads into the buffer medium.

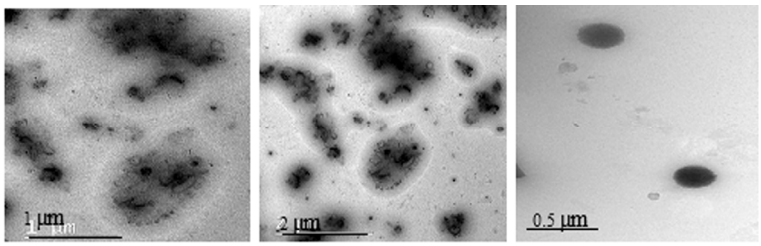

Figure 3. TEM images of the loaded A. nilotica extract onto chitosan beads

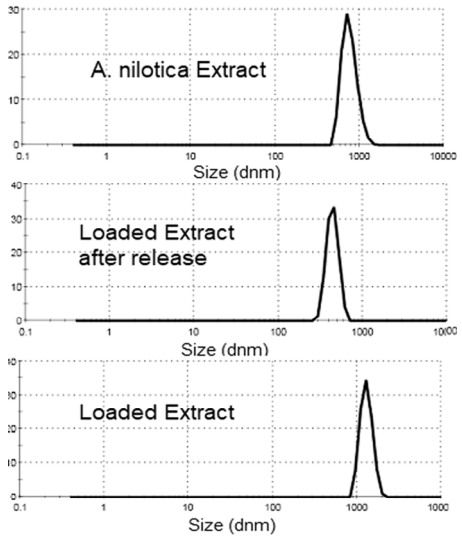

Figure 4. Particle size distribution analysis of the loaded A. nilotica extract before and after released in buffer pH 7.4 at room temperature relative to the crude extract using DLS technique

Figure 5 presents a quantitative analysis regarding the release of *A. nilotica* leaves extract (with respect to catechin content) from chitosan beads, following a 21-day storage period in an aqueous buffer with a pH of 7.4 at room temperature, as determined through High-Performance Liquid Chromatography (HPLC) analysis. It is noteworthy that across the various extract ratios incorporated within the chitosan bead formulations, there was a discernible increase in the concentration of catchin corresponding to the extended storage duration up to 21 days.

In vitro biological activities evaluation

Antioxidant activity evaluation

Table 1 delineates the DPPH scavenging effect observed in chitosan beads integrated with varying concentrations of *A. nilotica* leaves extract, in comparison to both the raw chitosan and the crude extract. The A. nilotica extract exhibited significant antioxidant activity, which was contingent upon the concentration of the extract. As the concentration escalated from 0.01 to 0.05 µg/ml, the antioxidant properties of the extract correspondingly increased from 17% to 40%. All antioxidant activity findings were benchmarked against the standard reference drug, Vitamin C (Ascorbic acid), which demonstrated free radical scavenging activities of 81% and 89% at concentrations of 0.01 and 0.05 µg/ml, respectively.

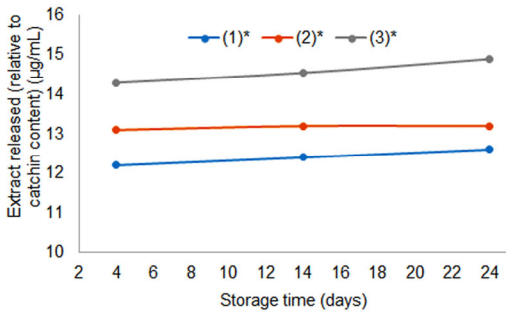

Figure 5. Quantitative HPLC analysis regarding the release of *A. nilotica* leaves extract (with respect to catchin content) from chitosan beads, after 21-day storage period in an aqueous buffer with a pH of 7.4 at room temperature

*Chitosan/extract ratio (1:2),
**Chitosan/extract ratio (1:3),
***Chitosan/extract ratio (1:4).

Table 1. DPPH scavenging (%) of the chitosan beads incorporated with different *A. nilotica* leaves extract concentrations in compared to the raw chitosan and the crude extract

Sample	Free radical scavenging (%) at	
	0.01 µg/ml	0.05 µg/ml
Standard	81±4.1	89±5.9
Chitosan	28.8±0.8	42.5±0.9
Extract	17.5±1.0	40±3.0
(1)*	40±2.2	47.5±2.9
(2)**	42.5±3.9	62.5±4.5
(3)***	45±3.0	69.6±4.9

In other terms, the outcomes of the DPPH scavenging activity revealed a dose-dependent relationship as the concentrations progressed from 0.01 to 0.05 µg/ml, compared to the raw chitosan which exhibited scavenging rates of 28.8% and 42.5%, respectively. The formulation that displayed the most pronounced antioxidant activity, achieving approximately 69.6% at 0.05 µg/ml, was sample 3 (chitosan/extract 1:4), in contrast to samples 1 and 2 which yielded 47.5% and 62.5%, respectively. This observed activity can be ascribed to the phenolic and flavonoid constituents present in the extract, which enhance the antioxidant efficacy. In comparison to prior studies (Maldini *et al*., 2011), which indicated that catechins, a component of *A. nilotica* flowers, could exhibit antioxidative potential, these findings underscore the augmented antioxidant capacity confirmed by the immobilization process.

In vitro antimicrobial evaluation

The inhibitory efficacy of the evaluated samples was assessed against Gram-positive bacteria (*S. aureus*), Gram-negative bacteria (*E. coli*), and the yeast (C. albicans). Table 2 presents the inhibition zones (measured in mm) corresponding to the antimicrobial activity of the encapsulated A. nilotica leaf extract in chitosan beads at varying ratios (1:3 and 1:4), compared to the crude extract. The findings indicated that all samples demonstrated antimicrobial properties against the tested pathogens at elevated concentrations (20-25 mm at 50-150 mg/ml). Additionally, the proliferation of C. albicans was suppressed (10-12.5 mm) at a concentration of 150 mg/ml. In contrast, the crude extract inhibited the growth of all pathogens even at the minimal concentration (12.5 mg/ml).

The minimum inhibitory concentration (MIC) of the synthesized chitosan beads, which were infused with varying concentrations of *A. nilotica* leaves extract, was evaluated against *S. aureus*, *E. coli*, and the yeast (*C. albicans*) utilizing the agar well diffusion method, as depicted in Table 2. Multiple bacterial species, including *Bacillus subtilis*, *B. cereus*, *S. aureus*, and *E. coli*, were adversely affected by *A. nilotica* leaves extracts (Revathi *et al*., 2017; Abdaldafi Sara *et al*., 2024). A crude extract derived from the pods of *A. nilotica* exhibited a bactericidal effect against *S. aureus* and *C. albicans*, with respective MIC values of 200 and 25 mg/ml. Moreover, Table 2 elucidated that the crude extract manifested moderate inhibitory activity, presenting MIC values of 150 and 50 mg/ml regarding the evaluated bacteria and yeast, respectively. Conversely, the *A. nilotica* leaves extract incorporated into chitosan beads at different ratios (1:3 and 1:4) exhibited pronounced antimicrobial activity, with an MIC value of 50 mg/ml against both *E. coli* and *S. aureus*. In terms of the yeast (*C. albicans*), the MIC value was approximately 25 mg/ml. It can be inferred that the MIC values indicate an augmented activity of the crude extract subsequent to its incorporation into chitosan beads, in contrast to the individual constituents, attributable to a synergistic effect. These results are congruent with earlier investigations emphasizing the potent antibacterial characteristics of *Acacia* against resistant strains, with variations ascribed to geographic and methodological differences. The observed discrepancies in MIC values may be attributed to bacterial strains and the protocols employed (Jabaka *et al*., 2019). Nonetheless, additional research is imperative to elucidate the specific

Table 2. Inhibition zones and MIC for antimicrobial activities of the chitosan beads incorporated with different *A. nilotica* leaves extract concentrations in compared to the crude extract using agar well diffusion assay

Concentration (mg/mL)		Inhibition zone (mm)			MIC value (µg/mL)		
		Crude Extract	(2)**	(3)***	Crude Extract	(2)**	(3)***
E. coli	12.5	20	0	0			
	25	22	10	0			
	50	25	25	20	150	50	50
	150	27	25	20			
S. aureus	12.5	10	0	0			
	25	20	0	0			
	50	25	0	0	150	50	50
	150	27	20	20			
C. Albican	12.5	20	0	0			
	25	20	10	10.5		25	25
	50	20	12	11			
	150	35	12.5	11	150		

**Chitosan/extract ratio (1:3),
***Chitosan/extract ratio (1:4).

bioactive compounds accountable for this activity and to comprehend their mechanisms of action.

Table 3. The number of cell forming unit (CFU/mL) after treatment of the different pathogens with the loaded *A. nilotica* leaves extract on chitosan bead with ratio 1:4 in compared to the crude extract and control using cell count method.

Sample	Antimicrobial activity using CFU/ml		
	S. aureus	E.coli	C. Albicans
Control*	1.6×10^5	1.3×10^8	3.4×10^8
Cells treated + raw extract	4×10^4	2.4×10^7	1.2×10^4
Cells treated +Sample (3)***	2.5×10^4	8.8×10^4	4×10^3

*Cells without treatment,
***Chitosan/extract ratio (1:4).

Table 3 illustrates the quantification of cell forming units (CFU/ml) subsequent to the treatment of various pathogens (*S. aureus, E. coli*, and *C. albicans*) utilizing the extract of *A. nilotica* leaves incorporated into chitosan beads at a ratio of 1:4, in comparison to the crude extract. It is evident that a substantial reduction in the viable cell count was observed (2.5×10^4, 8.8×10^4 and 4×10^3, respectively) for the treated cells with the loaded extract, when juxtaposed with the treated cells utilizing the crude extract (4×10^4, 2.4×10^7, and 1.2×10^4, respectively) and the control group (1.6×10^5, 1.3×10^8, 3.4×10^8, respectively). It is apparent that *C. albicans* exhibited the highest sensitivity among the evaluated pathogens in response to the administered sample. This study highlights the significance of the loaded *A. nilotica* leaves extract in chitosan beads as a potential antimicrobial agent. The inhibitory mechanisms of phenolic and flavonoid compounds against pathogenic microorganisms have been elucidated in several scholarly investigations (Campos *et al*., 2009; Khameneh *et al*., 2019).

Acetylcholinesterase inhibition evaluation

Cholinesterase inhibitor pharmaceuticals, which impede AChE activity, sustain the levels of acetylcholine (ACh) by diminishing its degradation rate (Sugimoto *et al*., 2000). Plant-derived compounds have been documented for their utilization in the management of cognitive impairments within the Ayurvedic medicinal framework. Furthermore, the inhibitory activity against AChE is dose-dependent within a concentration range of 50 to 300 µg/ml; although the tested extract demonstrates significant activity, its inhibitory efficacy remains moderate in comparison to the standard Galanthamine (Thangavelu and Ramasamy, 2015). In the present investigation, the AChE activity of both the crude extract and the unmodified chitosan yielded IC_{50} values of approximately 0.22 and 0.20 µg/ml, respectively, as depicted in Figure 6. Conversely, following the loading of the extract at various ratios (1:2, 1:3, and 1:4), the IC_{50} values ranged from 0.13 to 0.053 µg/ml, respectively. The loaded A. nilotica leaves extract at a higher ratio (1:4) exhibited a notable inhibitory activity against AChE, with an IC_{50} value of 0.053 µg/ml, closely paralleling the efficacy of the standard pharmacological agent Donepezil ($IC5_0$ = 0.047 µg/ml). These results indicate potential therapeutic implications for neurodegenerative disorders, particularly in the context of Alzheimer's disease.

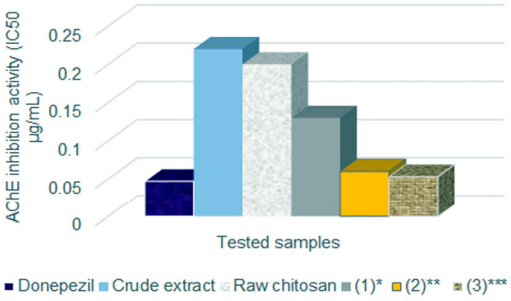

Figure 6. AChE inhibition activity (IC_{50}) of the loaded A. nilotica extract with different ratios in compared with the crude extract and the standard drug (Donepezil) using colorimetric assy.
*Chitosan/extract ratio (1:2),
**Chitosan/extract ratio (1:3),
***Chitosan/extract ratio (1:4).

Conclusions

Chitosan beads loaded with *A. nilotica* leaves extract at varying ratios (1:2, 1:3, and 1:4 w/w) were successfully synthesized using the $CaCl_2$ ionic gelation method. The incorporation of the extract significantly influenced the beads' physicochemical properties, as characterized by FTIR, XRD, TEM, and particle size analysis. The findings revealed the formation of inter- and intra-molecular hydrogen bonds between the phenolic compounds in the extract and the chitosan polymer matrix. Additionally, the *A. nilotica*-loaded beads demonstrated strong antimicrobial activity, notable acetylcholinesterase (AChE) inhibitory effects, and excellent antioxidant properties. This study

highlights an effective approach to enhance the functionality of chitosan-based beads using the ionic gelation technique. The immobilization of *A. nilotica* extract within the chitosan matrix creates a versatile biomaterial with significant bioactivity and multifunctional potential. Future research, including in vivo evaluations and deeper mechanistic studies, is recommended to further develop this innovative biomaterial for potential pharmaceutical applications.

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