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ANTI INFLAMMATORY AND ANTIMICROBIAL ACTIVITY OF HALLOYSITE NANOTUBES INCORPORATED BROMELAIN NANOCOMPOSITE AGAINST WOUND PATHOGENS

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Abstract

Biomedical science has long been on the lookout for new diagnostic and therapeutic mediums. The most recent development is the use of nanomaterials in such applications, which has given rise to the field of nanomedicine. Halloysite nanotubes (HNTs) are tubular clay nanomaterials that are formed by rolling aluminosilicate kaolin sheets many times. The aluminol and siloxane groups on the surface of HNT help to generate hydrogen bonds with the biomaterials that adhere to it. These qualities make HNT useful in a wide range of fields, including environmental sciences, waste-water treatment, dye removal, nanoelectronics and nanocomposites fabrication, catalytic research, glass coatings or anticorrosive coatings, cosmetics, stimuli response, and forensic sciences. Drug delivery, gene delivery, tissue engineering, cancer and stem cell separation, and bioimaging are just some of the few applications of HNT's unique features in biomedicine and nanomedicine. Bromelain is a compound of proteolytic enzymes found in all pineapple tissues (*Ananascomosus*). In burn treatment, it is recognised to be an effective debriding agent. The efficacy of bromelain-halloysite nanotubes for wound healing was studied in this study in-vitro.

Keywords: Halloysite, Halloysite nanotube, Halloysitenanocomposite, bromelain, wound pathogens

INTRODUCTION

Nanotechnology is an emerging field with numerous applications in innovation and technology, industry, the environment, energy, and other fields. Because this domain has bright future prospects, substantial research is being done to further its capabilities [1]. Halloysite nanotubes (HNTs) are a resourceful nanomaterial that can be used in a wide range of biomedical applications [2–4]. Halloysite is a commercially available, highly efficient clay nanomaterial derived from naturally available sources. HNTs are Halloysite tubular structures that chemically mimic kaolin. They come in a variety of shapes and sizes where short tubular and spheroidal halloysite particles with elongated tubes are the most commonly seen HNTs [5]. They are layered aluminosilicates (Al2Si2O5 (OH)4nH2O) having a hollow tubular geometry. The external diameter is projected to be between 40 and 70 nm, the internal diameter to be between 10 and 20 nm, and the length to be between 500 and 1500 nm. They have become a promising material for a spectrum of uses because of their lumens, high aspect length–diameter ratio, and low hydroxyl density on their surface. HNTs can also interact with a wide range of synthetic and biological components due to their enhanced surface area, positively entrusted interior surfaces with Al–OH groups, and negatively entrusted external surfaces with Si-OH and Si–O–Si groups [6].

HNT is commonly characterized using scanning electron microscopy (SEM), transmission electron microscopy (TEM), Fourier transform infrared spectroscopy, and X-ray diffraction (XRD). The multifunctional groups on the surface of HNTs have shown to aid in the loading of negatively charged macromolecules into the positive inner lumen of the nanotube, such as DNA encapsulation. The interactions of DNA and the HNT have been used to evaluate DNA damage using HNT gold and silver nanoparticle composites [7]. HNT covered with polyethylene glycol seemed to show increased biocompatibility, lengthen circulation time, and prevent protein adsorption and accumulation in biological environments [8]. This makes them perfect for modern biomedical applications such as the development of innovative medicine and gene delivery vehicles, tissue engineering, wound dressings, the isolation of malignant cells, and superior human cell adhesion [9]. A nanopore serves as the active core for drug entrapment in the HNT. To improve its persistence, various studies have been done where the HNT drug has been coated with different polymers [10]. This study focuses on the release of an active medicinal compound, Bromelain, from HNT.

Bromelain is a crude, aqueous extract from the stems and immature fruits of pineapples (*Ananascomosus*Merr., mainly var. Cayenne from the family of *bromeliaceae*). Bromelain is an incredibly complex mixture of several thiol-

endopeptidases and other not yet completely characterized components such as phosphatases, glucosidases, peroxidases, cellulases, glycoproteins, and carbohydrates, among others [11, 12]. Bromelain also contains a number of proteinase inhibitors [13, 14]. Fruit-bromelain (EC. 3.4.22.33), previously known as bromelin, is distinct from stem-bromelain (EC. 3.4.22.32). The proteolytic activity is the basis of assessment of commercial bromelain formulations. The protease activity appears to be linked to the platelet aggregation inhibitory and antiinflammatory effects. Additional non proteolytic components of bromelain, however, have other actions such as inhibiting tumor cell growth and metastasis, as well as debridement of burns [15].

Bromelain is a phytotherapeutic agent that may be used orally and has few adverse side effects, making it easier for patients to accept. Bromelain is absorbed orally and has a variety of pharmacological systemic effects, including anti-inflammatory effects, stimulation of monocytes to secrete cytokines like Il-1b and TNF-a, induction of phagocytosis and cytotoxicity by granulocytes, inhibition of platelet aggregation and stimulation of fibrinolysis, immunomodulatory effects that promote antigen-unspecific tumor cytotoxicity, and much more. Bromelain may act as a prophylactic medication to prevent metastases, according to in vitro and in vivo research. However, clinical evidence to back up this claim is still inadequate. These findings may suggest bromelain as a suitable model drug for further scientific research into the proteinase class to which it belongs [15].

This study therefore aims to synthesize and evaluate the anti-inflammatory and antimicrobial activity of halloysite nanotubes incorporated bromelainnanocomposite against wound pathogens.

MATERIALS AND METHODS

Preparation of Extract

A clean beaker was taken and 0.294 g of halloysite clay was added and dissolved in 100 mL of distilled water. The solution was filtered by using Whatman no. 1 filter paper. The filtered extract was collected and stored at 4°C for further use.

Synthesis of HNT

100 mg of Bromelain dissolved in 2 ml of distilled water was added to 30 ml of the prepared halloysite extract and kept in a magnetic stirrer for nanoparticle synthesis. The color change was observed visually and photographs were recorded (Figure 1). The solution was centrifuged using a lark refrigerated centrifuge at 8000 rpm for 10 minutes and the pellet was collected and washed with distilled water twice. The final purified pellet was collected and dried at 60°C for 2 hours and stored in an airtight eppendorf tube.

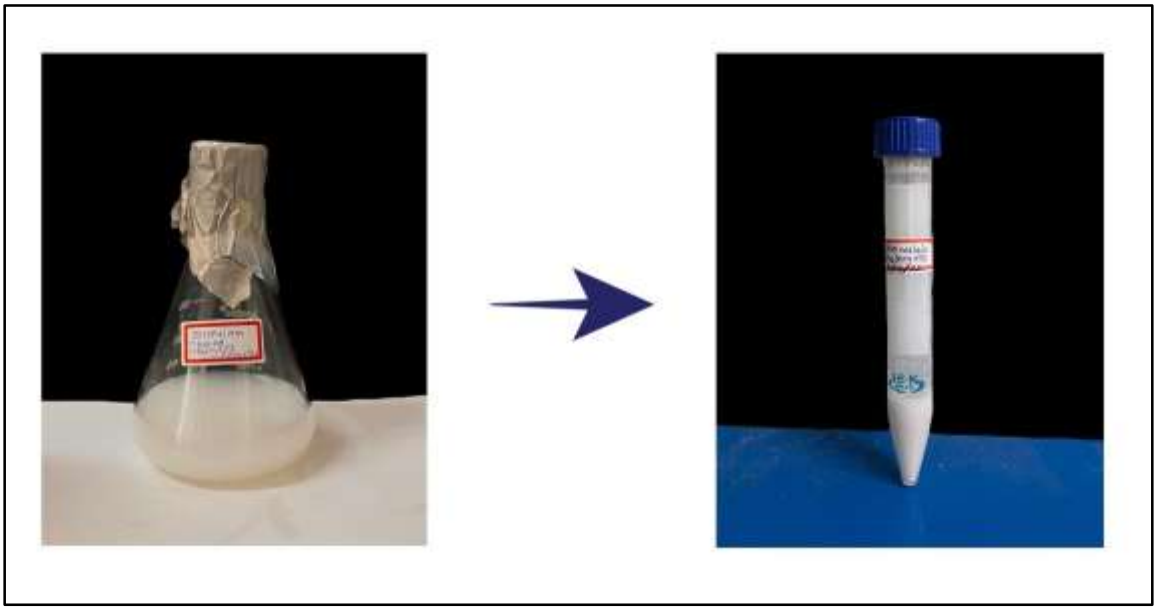

Figure 1: Visual observation of formation of SeNPs

Characterisation of HNT

The synthesized solution was preliminarily confirmed by using UV-visible-spectroscopy. 3 mL of the solution was taken in a cuvette and scanned in double beam UV-vis-spectrophotometry from 300 nm to 700 nm wavelength. The results were recorded for graphical analysis (Figure 2).

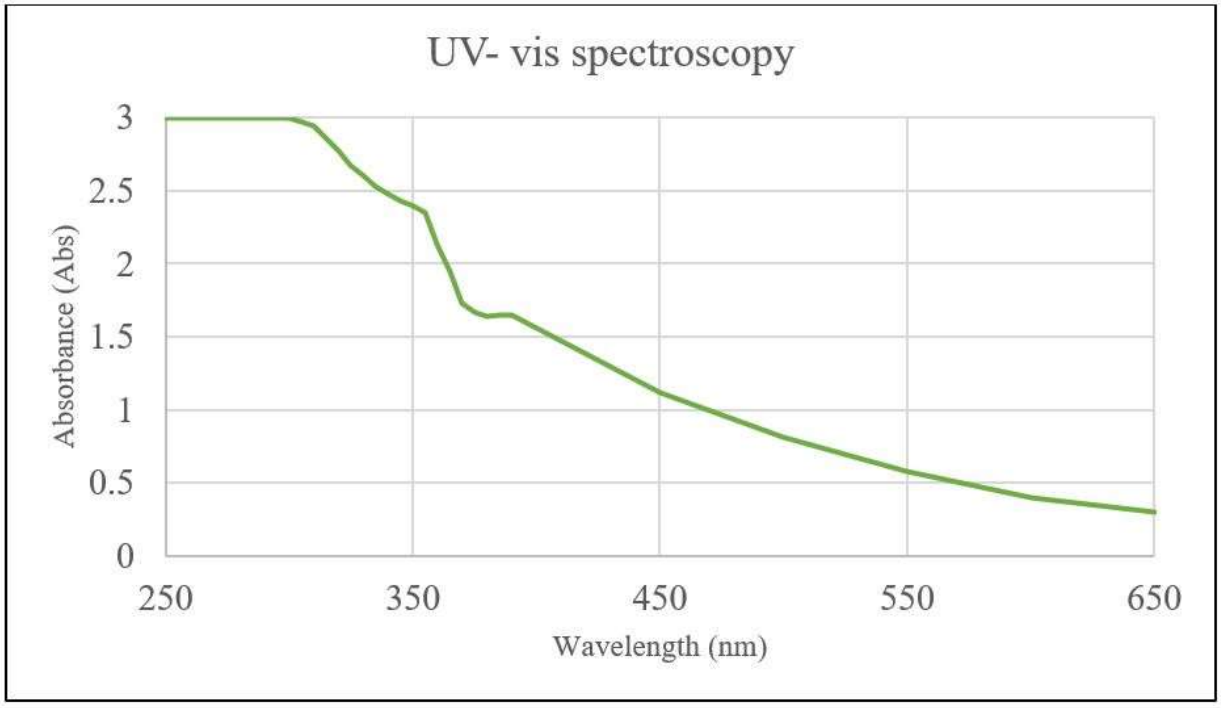

Figure 2: UV-vis spectroscopy. X-axis shows the different wavelength (in nm) and Y-axis shows the absorbance (in Abs). UV-vis spectroscopy revealed a peak at 385 nm.

Cytotoxicity Analysis (BRINE SHRIMP LETHALITY ASSAY):

2g of iodine free salt was weighed and dissolved in 200ml of distilled water. 6 well ELISA plates were taken and 10-12 ml of saline water was filled. To that 10 nauplii were slowly added to each well (20µL, 40µL, 60µL, 80µL, 100µL). Then the nanoparticles were added according to the concentration level. The plates were incubated for 24 hours (Figure 3). After 24 hours, the ELISA plates were observed and noted for number of live nauplii's present and calculated by using following formula,

% death = Number of dead nauplii / Number of dead nauplii + number of live nauplii × 100

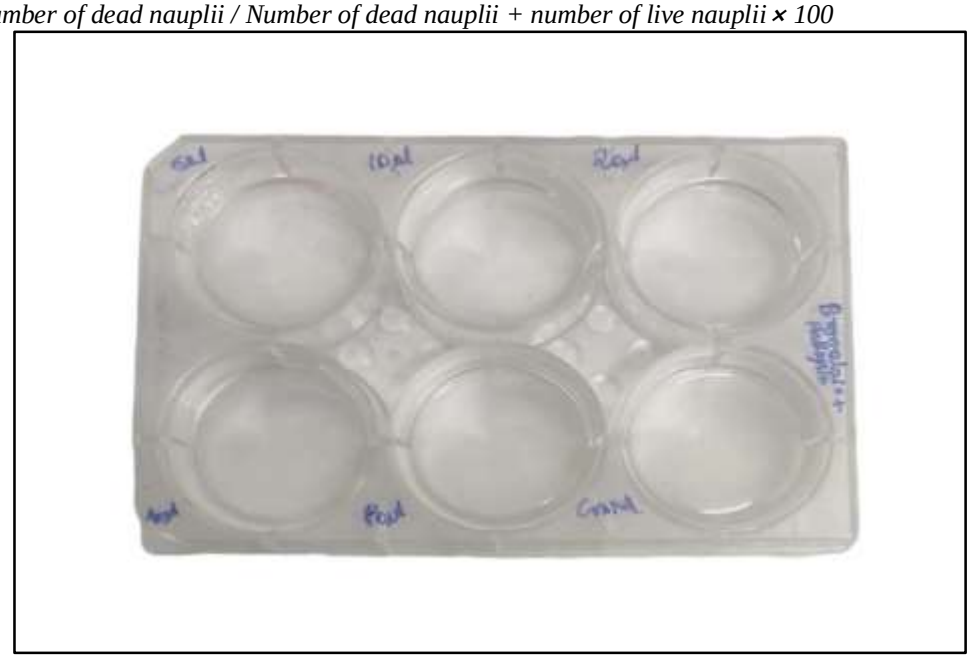

Figure 3: Brine Shrimp Lethality Assay

Antibacterial Activity

Antibacterial activity of respective nanoparticles against the strain *Staphylococcus aureus*, *Pseudomonas*, and *E.coli*. MHA agar was utilized for this activity to determine the zone of inhibition. Muller Hinton Agar was prepared and sterilized for 45 minutes at 120 lbs. Media was poured into the sterilized plates and let to stabilize for solidification. The wells were cut using a well cutter and the test organisms were swabbed. The nanoparticles with different concentrations were loaded and the plates were incubated for 24 hours at 37°C. After the incubation time, the zone of inhibition was measured (Figure 4).

Figure 4: Anti-bacterial activity of Bromelain incorporated HNT

Anti-inflammatory activity (ALBUMIN DENATURATION ASSAY):

The anti-inflammatory activity was tested by the following convention proposed by Muzushima and Kabayashi with specific alterations [16]. 0.05 mL of Bromelain incorporated HNT of various fixation (10µL, 20µL, 30µL, 40µL, 50µL) was added to 0.45 mL Bovine Serum Albumin (1% aqueous solution) and the pH of the mixture was acclimated to 6.3 utilizing a modest quantity of 1N hydrochloric acid. These samples were incubated at room temperature for 20 min and then heated at 55 °C in a water bath for 30 min. The samples were cooled and the absorbance was estimated spectrophotometrically at 660 nm. Diclofenac Sodium was used as the standard. DMSO is utilized as a control. Percentage of protein denaturation was determined utilizing following equation,

% inhibition= Absorbance of control- Absorbance of sample×100 /Absorbance of control

RESULTS

Table 1 depicts the cytotoxicity of Halloysite Nanotubes reinforced Bromelain extract. At 5 µL concentration there was a death of 30% of nauplii and at 10µL, 20µL, 40µL and 80µL there was a death of 40% of nauplii. It was seen that as the concentration increased, the cytotoxicity of the nanoparticles increased and remained to be a constant of 40% death from 10µL to 80µL.

Concentration (μL)	Viable Nauplii	% Death
5 μL	7	30
10 μL	6	40
20 μL	6	40
40 μL	6	40
80 μL	6	40

Table 1: Cytotoxicity of Bromelain infused HNT

Table 2 and Figure 5 describe the anti-microbial activity of the Bromelain incorporated HNTs against wound pathogens. It was found that the HNTs didn't show comparable antimicrobial activity against *E. coli* and *S. aureus* when compared to the control antibiotic irrespective of the concentration. However, the zone of inhibition obtained against *Pseudomonas sp* was constant in all three concentrations (25µL, 50µL and 100µL) and similar to that shown by the control antibiotic (9 mm).

Concentration of NP solution (µL)	Zone of inhibition (mm)		
	E. coli	*S. aureus*	*Pseudomonas sp*
25 µL	9	9	9
50 µL	9	9	9
100 µL	9	9	9
Control	30	40	9

Table 2: Anti-microbial Activity

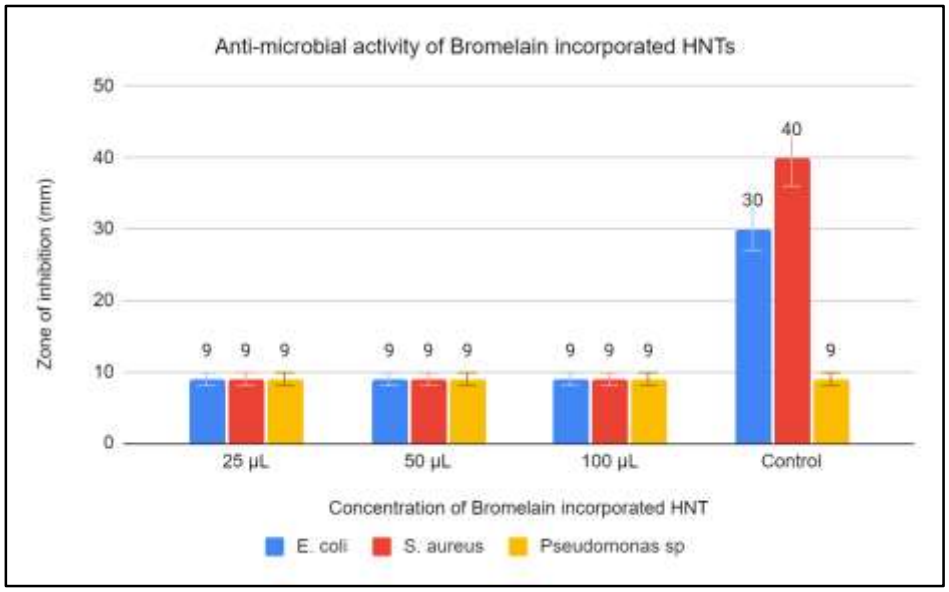

Figure 5: Anti-microbial activity of Bromelain infused HNTs

Table 3 and Figure 6 shows the anti-inflammatory activity of the HNTs. It was observed that the Bromelain infused HNTs showed better anti-inflammatory effect than the control. Percentage of inhibition was found to be 62% at 10µL, 67.6% at 20µL, 73.5% at 30µL, 82.4% at 40µL and 88.32% at 50µL. It was seen that as concentration of the HNTs was increased, greater was the anti-inflammatory effect.

Concentration	Standard	Absorbance
10 µL	46.52	62
20 µL	54.65	67.6
30 µL	63.85	73.5
40 µL	72.52	82.4
50 µL	83.65	88.32

Table 3: Anti-inflammatory activity

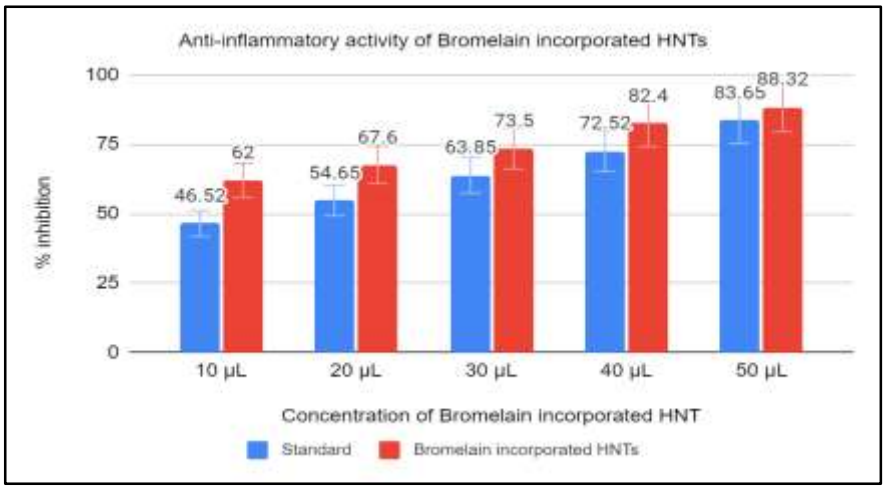

Figure 6: Anti-inflammatory effect of Bromelain infused HNTs

DISCUSSION

Nanoparticle production has been advancing at a rapid pace recently. Nanoparticle synthesis used to be done via physio-chemical approaches earlier. Despite the fact that traditional physical and chemical processes take less time to synthesize vast quantities of nanoparticles, hazardous compounds are necessary as capping agents to maintain stability, resulting in environmental toxicity [17, 18]. Thus, incorporating plant derived extracts into nanodrug synthesis has been an upcoming trend in recent years.

A study done by Jayashri et al. on the cytotoxicity of Bromelain extract showed that the maximum percentage of nauplii death was 20% noted at both 20 µl and 30 µl respectively, which was poor [19]. In this study, we observed the efficacy of bromelainwhen incorporated with HNTs was better in showing cytotoxic effects. As the concentration increased, the cytotoxicity activity of the Bromelain incorporated HNTs increased. The highest percentage of death of nauplii was constantly 40% in 10µL, 20µL, 40µL and 80µL concentration of HNTs reinforced with Bromelain extract. Bromelain's efficacy as an anticancer drug, either alone or in combination with other medicines, has been limited to a few anecdotal evidence [20]. Bromelain was shown to inhibit the growth of three mouse tumour cell lines in a study by Taussig et al. [21]. Bromelain has also been shown to suppress the invasive capabilities of glioma cells in a reversible manner according to another study by Tysnes et al. [22]. In a study by Beuth et al, mice implanted with murine sarcoma L-1 cells were given bromelain therapy, which resulted in a considerable reduction in tumour growth [23]. Baez et al. demonstrated in vivo antitumoral and antimetastatic efficacy of bromelain against a panel of murine cancer cell lines [24]. Bromelain is a complex mixture of proteolytic enzymes. Its therapeutic value may be attributed to glycoprotein, the potent ingredient in bromelain[25].

Anti-microbial activity of the Bromelain incorporated HNTs in the current study didn't show any significant zone of inhibition when compared to the control. However, the zone of inhibition obtained against *Pseudomonas sp* was constant in all three concentrations (25µL, 50µL and 100µL) and similar to that shown by the control antibiotic (9 mm). Bromelain's antimicrobial function is not well understood, although it is thought that it inhibits bacterial growth by hydrolyzing certain peptide bonds in the bacterial cell wall [26]. The cell wall is destroyed when bromelain digests the surface proteins, allowing the cell to leak, enlarge, and open [26, 27]. Bromelain also prevents bacteria from adhering to certain glycoprotein receptors on the surface, which restricts their growth [28]. Bromelain also inhibits the synthesis of enterotoxins by *Escherichia coli* (*E. coli*) and prevents diarrhoea induced by *E. coli*[29]. Bromelainhas antibacterial activity against *E. coli*, *Aggregatibacteractinomycetemcomitans* (*A. actinomycetemcomitans*), *Porphyromonasgingivalis* (*P. gingivalis*), *Streptococcus mutans* (*S. mutans*) [29, 30], *Bacillus subtilis* (*B. subtilus*), *S. aureus*, *Pseudomonas aeruginosa*[31]. Furthermore, when bromelain and antibiotics are used together, the antibacterial activity is enhanced due to higher antibiotic absorption induced by bromelain, resulting in improved drug distribution in microorganisms [29].
The Bromelain infused HNTs in the present study showed higher anti-inflammatory effect than the control. Percentage of inhibition was found to be 62% at 10µL, 67.6% at 20µL, 73.5% at 30µL, 82.4% at 40µL and 88.32% at 50µL. It was seen that as concentration of the HNTs was increased, the anti-inflammatory effect also increased significantly. Bromelain's anti-inflammatory activity may be attributed to Bromelain mediated increased serum fibrinolytic activity, decreased plasma fibrinogen levels, and decreased bradykinin levels (resulting in reduced vascular permeability), reducing edema and pain; modulating the formation of pro-inflammatory prostaglandins (by lowering levels of prostaglandin E2 (PGE2) and thromboxane A2 (TXA-2)), enhancing anti-inflammatory mediators, and increasing levels of prostaglandin I2 (PGI2) [32–34]. Various studies have been conducted where Bromelain has shown promising results as a potent anti-inflammatory agent [35–40].

Based on the findings of the current study, we can state that Bromelain incorporated Halloysite Nanotubes may be used as an effective alternative to commercially available anti-inflammatory agents.

CONCLUSION

The present study revealed that Halloysite nanotubes can be synthesized in a simple, eco-friendly method and infused with Bromelain extract. These Bromelain loaded HNTs have the potential to be used as an effective antibacterial agent against *Pseudomonas sp* and an effective anti-inflammatory agent which is more potent than the current commercially available drugs for wound healing. Hence, it may be employed in commercial large scale production to be used for targeted drug delivery in accelerating wound healing.

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CONFLICT OF INTEREST

There exists no conflicts of interest as defined by the authors.

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