European Journal of Molecular & Clinical Medicine
ISSN 2515-8260 Volume 09, Issue 08, 2022

MECHANISM OF ANTIBACTERIAL ACTIVITY OF HESPINDIN INCORPORATED HALLOSITE NANOTUBES AGAINST STREPTOCOCCUS HALLOSITE

Type of Manuscript: Original study

Running Title: MECHANISM OF ANTIBACTERIAL ACTIVITY OF HESPINDIN INCORPORATED HALLOSITE NANOTUBES AGAINST STREPTOCOCCUS HALLOSITE 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.Hesperidin is a plant chemical that is classified as a "bioflavonoid." It is found primarily in citrus fruits.Hesperidin is most often used for blood vessel conditions such as hemorrhoids, varicose veins, and poor circulation (venous stasis).The efficacy of hesperidin-halloysite nanotubes for wound healing was studied in this study in-vitro.

Keywords: Halloysite, Halloysite nanotube, Halloysite nanocomposite, hesperidin, wound pathogens

INTRODUCTION

Nanotechnology is a rapidly developing science that has a wide range of uses in business, industry, the environment, energy, and other sectors. There is a lot of study being done to enhance the skills of this sector since the future seems promising [1]. A versatile nanomaterial with a variety of biological uses is halloysite nanotubes (HNTs) [2-4]. A highly effective clay nanomaterial that is readily accessible on the market is called halloysite. It comes from naturally occurring sources. HNTs are tubular Halloysite structures that resemble kaolin chemically. They appear in a range of sizes and forms, but the most frequent types of HNTs are short tubular and spheroidal halloysite particles with elongated tubes [5].They are layered aluminosilicates with a hollow tubular shape (Al2Si2O5 (OH)4nH2O). The interior diameter is predicted to be between 10 and 20 nm, the length to be between 500 and 1500 nm, and the exterior diameter to be in the range of 40 to 70 nm. Due to its lumens, high aspect length-to-diameter ratio, and low hydroxyl density on their surface, they have emerged as a potential material for a variety of applications. Due to their increased surface area, positively entrusted inner surfaces with Al-OH groups, and negatively entrusted exterior surfaces with Si-OH and Si-O-Si groups, HNTs can also interact with a variety of synthetic and biological components [6].

Scanning electron microscopy (SEM), transmission electron microscopy (TEM), Fourier transform infrared spectroscopy, and X-ray diffraction are frequently used to analyse HNT

(XRD). It has been demonstrated that the HNTs' multifunctional groups help load negatively charged macromolecules like DNA encapsulation into the positive inner lumen of the nanotube. HNT gold and silver nanoparticle composites have been utilised to assess DNA damage by interacting with DNA [7]. Polyethylene glycol-coated HNT appeared to be more biocompatible, prolong the duration in circulation, and avoid protein adsorption and accumulation in biological settings [8]. Because of this, they are ideal for current biomedical applications such as the creation of novel medicinal treatments and gene delivery mechanisms, tissue engineering, wound dressings, the isolation of cancerous cells, and more[9]. The HNT's active core for drug trapping is a nanopore. Numerous experiments have been conducted using the HNT medication coated with various polymers to increase its persistence [10]. The aim of this investigation is the release of bromelain, an active pharmaceutical ingredient, from HNT.

Citrus fruits including lemon, grapefruit, and sweet orange (Citrus sinensis) are rich in hesperidin ($C_{28}H_{34}O_{15}$), a flavanone glycoside. Unripe sour oranges, Ponderosa lemons, Citrus unshiu, and C. mitis have all been shown to contain this substance [7,41,42]. It may also be found in several plant families and species, including Fabaceae [43], Papilionaceae [7], and It has anti-inflammatory, anti-oxidant, and anti-carcinogenic properties [48]. The extrinsic and intrinsic apoptosis of several malignant cells was significantly mediated by hesperidin (Bartoszewski et al. 2014; Park et al. 2008; Etcheverry et al. 2008). It has been discovered that hesperidin and its aglycon, hesperetin, are beneficial against a variety of malignancies, including lung cancer (Park et al. 2008), colon cancer (Park et al. 2008), and gastric cancer (Park et al. 2007). Additionally, it was discovered that some viruses could not replicate when hesperidin was present (Mojzer et al. 2016) By reducing capillary permeability and raising capillary resistance, hesperidin administration alleviated blood vessel diseases such oedema, bleeding, and pleurisy [7,62,63]. Hesperidin treatment has a number of advantages, including safety, non-accumulativeness, and few side effects, even during pregnancy.After 13 weeks, it had no mutagenic, toxic, or carcinogenic effects when administered to mice at dosages up to 5% [7]. Clinical support for this assertion is currently insufficient, though. Hesperidin may be a viable model medication for future scientific study, according to these findings. Therefore, the objective of this work is to synthesise and assess the anti-inflammatory and antibacterial properties of a hesperidin nanocomposite with halloysite nanotubes against wound pathogens.

MATERIALS AND METHODS

Preparation of Extract

0.294 g of halloysite clay was introduced to a clean beaker and dissolved in 100 mL of purified water. Whatman no. 1 filter paper was used to filter the solution. The filtered extract was collected and kept for future use at 4°C.

Synthesis of HNT

In order to create nanoparticles, 30 ml of the produced halloysite extract was mixed with 100 mg of bromelain that had been dissolved in 2 ml of distilled water. The colour shift was seen visually, and pictures were taken (Figure 1). Using a Lark chilled centrifuge, the solution was centrifuged at 8000 rpm for 10 minutes before the pellet was recovered and twice cleaned with distilled water. An airtight eppendorf tube was used to hold the final purified pellet after it had been collected and dried at 60°C for two hours.

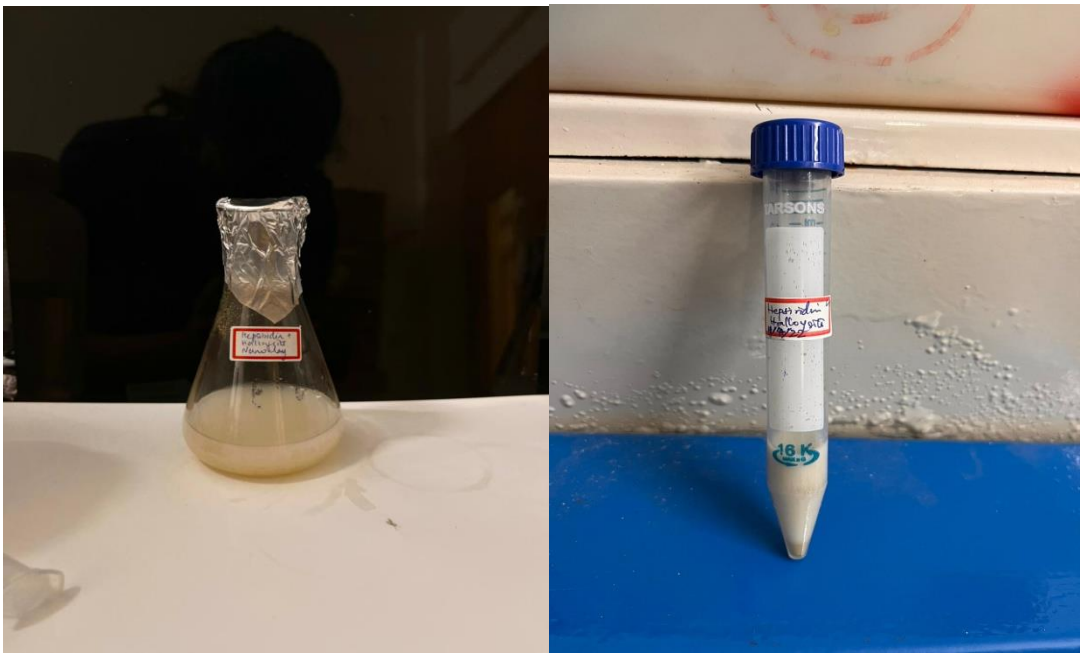

Figure 1: Visual observation of formation of SeNPs

Characterisation of HNT

UV-visible spectroscopy was initially used to confirm the produced solution. A cuvette containing 3 mL of the solution was used to scan it using a double beam UV-vis spectrophotometer at wavelengths ranging from 300 nm to 700 nm. For graphical analysis, the findings were recorded (Figure 2).

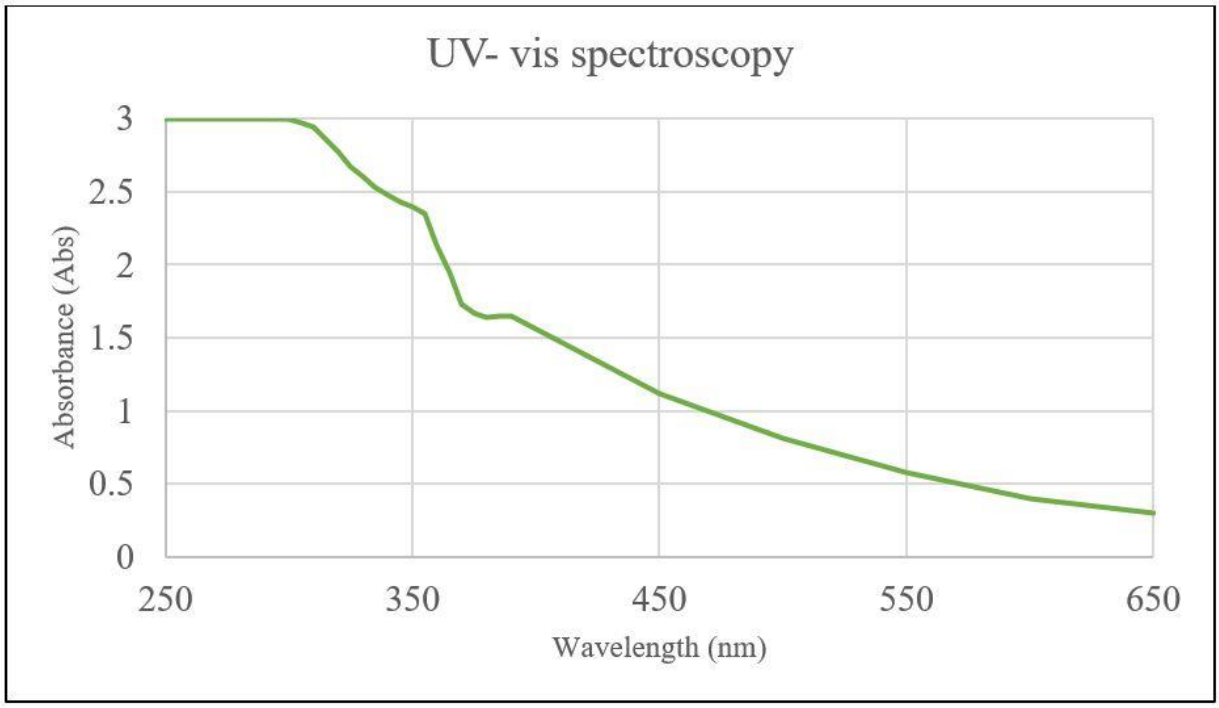

Figure 2: UV-vis spectroscopy. X-axis shows the different wavelength (in nm) and Y-axis shows the absorbance (in Abs). UV-vis spectroscopy revealed a peak at 385 nm.

Cytotoxicity Analysis (BRINE SHRIMP LETHALITY ASSAY):

200ml of distilled water was used to dissolve 2g of iodine-free salt. 10–12 ml of saline water were added to 6 well ELISA plates. Each well received 10 nauplii, which were introduced gradually (20 mL, 40 mL, 60 mL, 80 mL, and 100 mL). The nanoparticles were then introduced in the appropriate concentrations. The plates underwent 24-hour incubation (Figure 3). The ELISA plates were examined after 24 hours to count the living nauplii present. The percentage of death was then estimated using the following formula: % death = Number of dead nauplii / Number of dead nauplii + Number of live nauplii.

Figure 3: Brine Shrimp Lethality Assay

Antibacterial Activity

Relative nanoparticles' antibacterial efficacy against the strains of Staphylococcus aureus, Pseudomonas, and E. coli. For this experiment, MHA agar was used to identify the zone of inhibition. Mitchell Hinton Agar was prepared and heated to 120 lbs. for 45 minutes. The disinfected plates were filled with media, which was then allowed to settle and solidify. The test organisms were swabbed after the wells were cut with a well cutter. Different concentrations of nanoparticles were added, and the plates were then incubated for 24 hours at 37°C. The zone of inhibition was assessed following the incubation period (Figure 4).

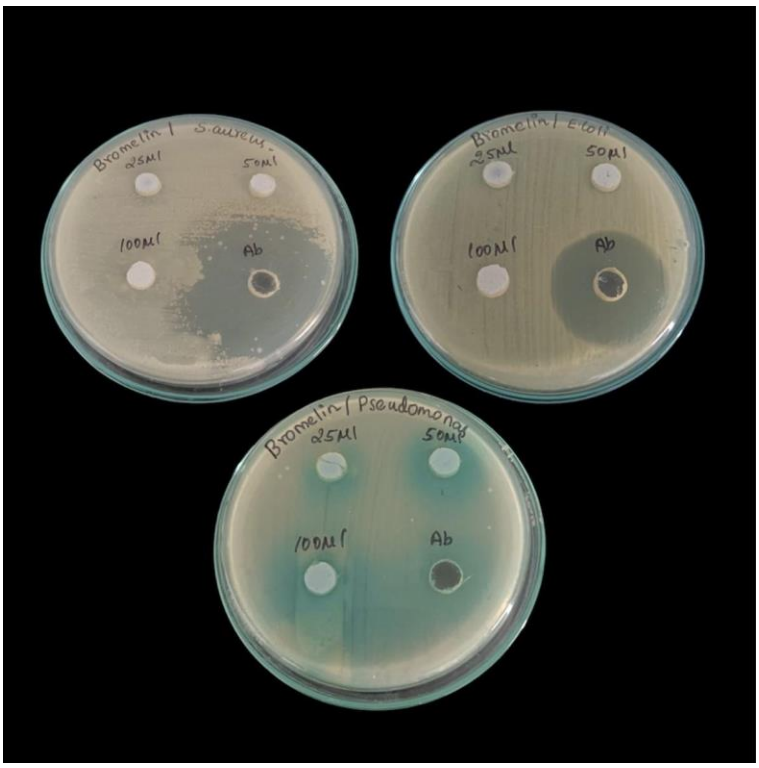

Figure 4: Anti-bacterial activity of Bromelain incorporated HNT

Anti-inflammatory activity (ALBUMIN DENATURATION ASSAY):

The following convention, with particular modifications from Muzushima and Kabayashi, was used to investigate the anti-inflammatory activity [16]. Bovine serum albumin (1% aqueous solution) and 0.05 mL of Bromelain-incorporated HNT of varied fixation (10, 20, 30, 40, and 50 mL) were combined, and the pH of the resulting mixture was adjusted to 6.3 using a little amount of 1N hydrochloric acid. These samples were heated to 55 °C in a water bath for 30 minutes after being incubated at room temperature for 20 min. After cooling the samples, the absorbance at 660 nm was calculated spectrophotometrically. The benchmark was diclofenac sodium. The control used is DMSO. The following equation was used to calculate the percentage of protein denaturation:

% 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.

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

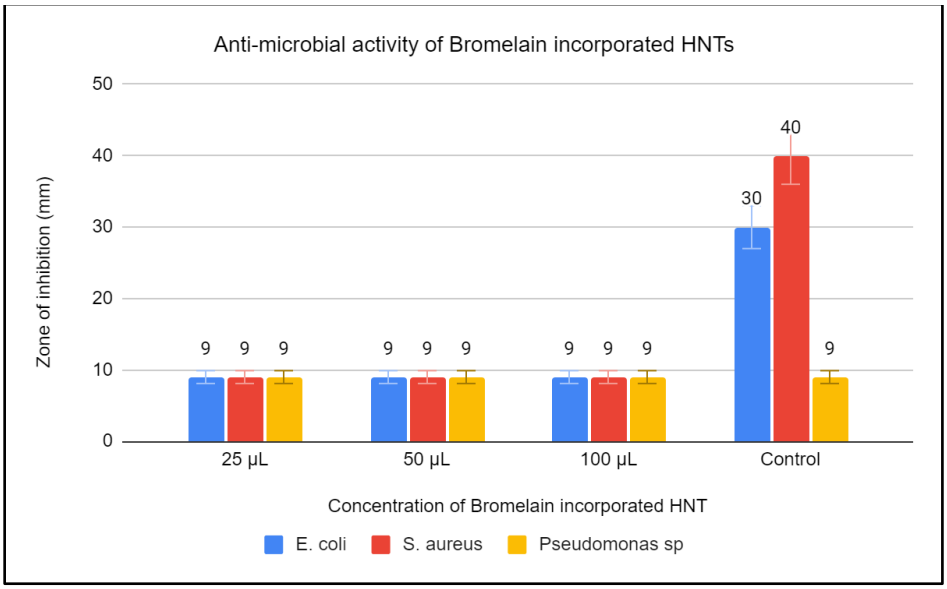

Figure 5: Anti-microbial activity of Bromelain infused HNTs

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

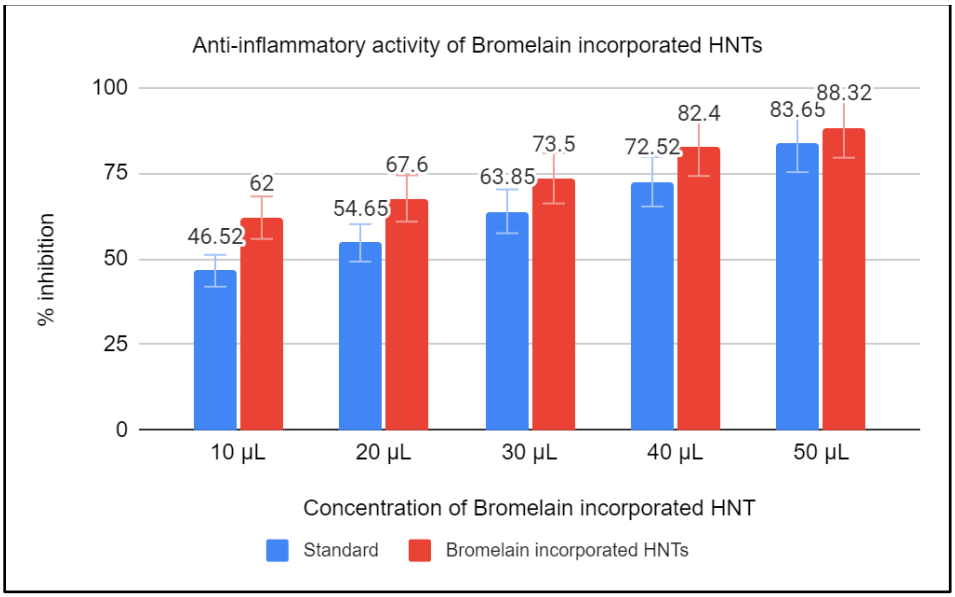

Figure 6: Anti-inflammatory effect of Bromelain infused HNTs

DISCUSSION

Production of nanoparticles has lately advanced quite quickly. Physio-chemical methods used to be employed in the past to synthesise nanoparticles. Even if it takes less time to produce enormous amounts of nanoparticles using conventional physical and chemical methods, toxic substances are still required as capping agents to ensure stability, which causes environmental toxicity [17, 18]. Thus, a growing trend in recent years has been the use of plant-derived extracts in the manufacture of nanodrugs.

A research on the cytotoxicity of bromelain extract by Jayashri et al. revealed that the greatest percentage of nauplii mortality was 20%, which was poor at both 20 l and 30 l, respectively [19]. In this work, we found that bromelain was more effective in producing cytotoxic effects when combined with HNTs. The cytotoxicity activity of the bromelain-incorporated HNTs rose as the concentration did. In concentrations of HNTs reinforced with bromelain extract of 10L, 20L, 40L, and 80L, the maximum percentage of nauplii mortality was consistently 40%. There are just a few anecdotal reports that bromelain is effective against cancer, either on its own or in conjunction with other drugs [20].In a research by Taussig et al. [21], it was discovered that bromelain inhibits the development of three mouse tumour cell lines. Another work by Tysnes et al. [22] demonstrated that bromelain can reversibly inhibit the invasive potential of glioma cells. In a research by Beuth et al., bromelain treatment significantly slowed the formation of tumours in mice implanted with murine sarcoma L-1 cells [23]. The effectiveness of bromelain against a variety of murine cancer cell lines was established in vivo by Baez et al. [24]. A complex blend of proteolytic enzymes makes up bromelain. Glycoprotein, a powerful component of bromelain, may be responsible for its therapeutic benefits [25].

In the current investigation, the bromelain-incorporated HNTs' anti-microbial efficacy failed to exhibit any discernible zone of inhibition when compared to the control. However, the zone of inhibition against Pseudomonas sp found was continuous at all three concentrations (25 l, 50 l, and 100 l), and it was comparable to that displayed by the control antibiotic (9 mm). Although the exact mechanism by which bromelain prevents the development of bacteria is unknown, it is believed to do so by hydrolyzing a few peptide bonds found in the bacterial cell wall [26]. When bromelain breaks down the surface proteins, the cell wall is damaged, which causes the cell to leak, expand, and open [26, 27]. Additionally, bromelain limits the development of bacteria by preventing them from sticking to certain glycoprotein receptors on the surface [28].
Bromelain also inhibits the synthesis of enterotoxins by *Escherichia coli* (*E. coli*) and prevents diarrhoea induced by *E. coli* [29]. E. coli, Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Streptococcus mutans, Bacillus subtilis, S. aureus, and Pseudomonas aeruginosa are all susceptible to bromelain's antibacterial properties [29, 30]. Additionally, when bromelain and antibiotics are combined, the antibacterial effectiveness is increased because

bromelain causes better antibiotic absorption, which improves medication dispersion in microorganisms [29].

In the current investigation, the bromelain-infused HNTs had a greater anti-inflammatory impact than the control group. At 10 L, 67 L, 30 L, 40 L, and 50 L, the percentage of inhibition was determined to be 62%, 67 L, 30 L, 73 L, 82 L, and 88 L, respectively. It was shown that the anti-inflammatory impact considerably increased as the HNT levels rose. The anti-inflammatory effects of bromelain may be attributed to its modulation of the production 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 (). These effects reduce edoema and pain, decrease plasma fibrinogen levels, decrease bradykinin levels, and reduce vascular permeability Bromelain has demonstrated encouraging results as a powerful anti-inflammatory drug in a number of investigations [35–40].

According to the results of the current study, Bromelain-incorporated Halloysite Nanotubes may be employed as a successful substitute for anti-inflammatory drugs that are readily accessible on the market.

CONCLUSION

The current work shown that Bromelain extract may be added to Halloysite nanotubes during their easy, environmentally friendly synthesis. These bromelain-loaded HNTs have the potential to be employed as more powerful anti-inflammatory and antibacterial agents for wound healing than the currently marketed medications against Pseudomonas sp. Therefore, it might be utilised in large-scale commercial production to distribute drugs more precisely and speed up the healing of wounds.

ACKNOWLEDGEMENT

The authors express their sincere gratitude to Saveetha Dental College and Hospitals, Chennai for their support in enabling us to carry out this study.

CONFLICT OF INTEREST

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

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