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Determination of Biological Activities of Encapsulated Bee Venom

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Abstract

Bee venom is an important bee product used directly in apitherapy applications. Once harvested, bee venom crystallizes rapidly upon contact with air. Furthermore, bee venom that is not produced and stored under optimal conditions deteriorates rapidly. For this reason, there is a need for new methods that can extend the shelf life of bee venom. Encapsulation techniques are widely used to extend the shelf life of bioactive components by immobilizing them within a biocompatible biopolymer. In this study, the potential of encapsulating bee venom using ionic gelation with chitosan, a biocompatible polymer, was investigated. It was found that chitosan had a 91% encapsulation efficiency for bee venom. It has been determined that bee venom inhibits the acetylcholinesterase enzyme by 84%±2.1, whilst its total protein content is 64.2%±1.8. The antimicrobial activity of the encapsulated bee venom was evaluated against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Bacillus subtilis*. The inhibition zones ranged from 11 to 12 mm. The results showed that the encapsulated bee venom retained its biological activity without significant loss.

Keywords: Antimicrobial activity, Bee venom, Encapsulation, Ionic gelation

INTRODUCTION

Bee venom is an important hive product that has been the subject of research since the 19th century for its therapeutic properties (Abd El-Wahed et al. 2019, İkiz et al. 2025). Bee venom is secreted into a sac located in the bees' abdominal cavity. The therapeutic potential of bee venom was first recognized when beekeepers, whilst tending to their hives, were naturally stung by bees and subsequently observed a reduction in their joint pain and bodily inflammation (Hellner et al. 2008, Khalil et al. 2021). As a result, Hippocrates began practicing apitherapy with bee venom, and its use in apitherapy became widespread (Kim 2013). Today, bee venom is used both directly and in apitherapy treatments combined with acupuncture. Generally, when the chemical structure of insect venoms was examined, they were found to consist of many different proteins, enzymes, and smaller molecules (Khalil et al. 2021). It has been observed that bee venom contains molecules similar to those found in other insect venoms but has a more complex structure (Abd El-Wahed et al. 2019, Pucca et al. 2019).

This complex structure was reported to be rich in proteins, enzymes, sugars, amines, volatile components, phospholipids, and pheromones. Furthermore, over 80% of bee venom consists of water. The two major components of bee venom were melittin and phospholipase A2 (PLA2) (Abd El-Wahed et al. 2019). Melittin is a peptide constituting approximately 50–60% of the dry weight of bee venom (Carpena *et al.* 2020). Although melittin is the most toxic component of bee venom, it has significant effects on many diseases (Pacakova et al. 1995). Phospholipase A2, on the other hand, is the most abundant enzyme in bee venom. It accounts for approximately 10–12% of the dry weight (Carpena et al. 2020). Although PLA2 plays a role in numerous biological activities, it is also described as the most allergenic component (Abd El-Wahed et al. 2019). Although bee venom is an allergenic toxin, its lethal dose is quite high. The lethal dose for an adult is 2.8 mg/kg (Carpena et al. 2020). For example, a 70 kg individual would require 196 mg of bee venom to reach a lethal dose. However, this amount is quite substantial. This is because a single bee can produce only between 0.15 and 0.30 mg of bee venom (Khalil et al. 2021). Consequently, although bee venom is allergenic, there is no risk associated with its use in therapeutic applications. The components of bee venom and their synergistic interactions can be used as supportive treatments in modern medicine to manage various diseases.

A study by Sobral *et al*. (2016) indicated that bee venom possesses antioxidant, anti-inflammatory, and cytotoxic effects. In a study by Lee et al. (2016), it was noted that bee venom has antimicrobial and anti-apoptotic effects, whilst in a study by Zhang *et*

al. (2018), it was stated that melittin has muscle-regenerating and anti-cancer effects, and is effective against rheumatoid joint inflammation, Parkinson's disease, and multiple sclerosis. Bee venom, whose effects on numerous diseases have been identified in studies, could be administered to patients either directly via live bees or, after being harvested from the hive using specially developed equipment (in crystallized form).

However, due to factors such as the non-standardized composition of bee venom administered via either method, the inability to maintain live bees in a hospital setting, and the difficulty of ensuring that directly administered bee venom reaches the target site, there is a need to develop standardized bee venom products. Encapsulation techniques are used to encapsulate materials in solid, liquid, and gaseous forms whilst preserving all their properties under specific conditions, and to ensure the controlled release of the encapsulated materials (Nedovic et al. 2011). The material being encapsulated is referred to as the core, whilst the coating material is termed the carrier, shell, or encapsulant (Gharsallaoui et al. 2007). Capsules obtained by encapsulation are classified by size. If the size of the resulting capsules is 200 nm (0.2 µm) or less, it is termed nano-encapsulation; if between 0.2 and 5000 µm, it is termed micro-encapsulation; and if the capsule size is greater than 5000 µm, it is termed macro-encapsulation (Kordas 2023). The resulting capsules may have different structures and morphologies. These differences vary depending on the method used, the coating material employed, and the material being encapsulated. By using encapsulation techniques, the interaction of a material within a system can be restricted. The resulting capsules facilitate the separation of the material from the system. Furthermore, encapsulation can reduce or prevent functional loss, unpleasant odors or aromas, structural degradation, and loss of activity during storage or production, whilst also providing moisture control, protection against oxidation, preservation of active reducing agents, and increased bioavailability (Gharsallaoui et al. 2007).

Encapsulation techniques are used in many fields, including food, chemistry, agriculture, medicine, pharmacy, veterinary science, and biotechnology (Zabot et al. 2022). These techniques are widely used in the production of food additives. Vitamins, minerals, enzymes, proteins, organic acids, probiotics and prebiotics, essential oils, sweeteners, preservatives, colorings, flavorings, fatty acids (ω-3, conjugated linoleic acid), carotenoids (β-carotene, lycopene), and antioxidants (tocopherol, flavonoids, polyphenols) can be cited as examples of materials used in encapsulation studies.

The encapsulation process can be carried out using various techniques such as freeze-drying, co-precipitation, ionic gelation, or spray-drying. The ionic gelation method is a practical and economical approach (Nedovic et al. 2011). The active ingredient is homogeneously mixed with the encapsulant, then dripped or injected into a dispersing phase (such as $CaCl_2$) using a syringe to produce spherical gel particles. This method is primarily used to produce calcium alginate capsules. The fundamental principle of the method is the formation of cross-links between calcium ions in $CaCl_2$ and sodium alginate (egg-box model). Among the advantages of this method is the ability to encapsulate virtually all components—whether hydrophilic or hydrophobic, heat-sensitive, fluid or viscous, solid or liquid (Nedovic et al. 2011). The choice of coating materials varies depending on the capsule's size, shape, stability, and permeability properties.

Generally, the capsules used are expected to have high stability, appropriate permeability, dimensions at the desired level, and high environmental compatibility. An ideal coating material should be non-toxic, easy to apply, and provide complete protection. Alginate, arabic gum, and chitosan are edible, biocompatible biopolymers that exhibit distinct release behaviors across pH ranges and are frequently used in encapsulation studies (Rajabi et al. 2019). A review of the literature reveals that studies on bee venom encapsulation are limited; the effects of the resulting capsules have primarily been assessed in cancer cells, and their antimicrobial activity has been detected against only a few microorganisms. In this study, bee venom was encapsulated in chitosan, and the inhibitory effect of the resulting capsules on acetylcholinesterase, which is associated with Alzheimer's disease, was evaluated. Furthermore, the antimicrobial activity of the encapsulated bee venom was identified.

MATERIAL AND METHODS

The bee venom used in the study was obtained commercially from a beekeeping business operating in the province of Balıkkesir. The obtained bee venom samples were stored in amber bottles at -20°C. All chemicals were used in analytical grade and purchased from Sigma-Aldrich.

Protein Assay

Bee venom was dissolved in 0.9% NaCl. The total protein content was determined spectrophotometrically using the Bradford method with a BSA standard (Bradford 1976). In this technique, the negatively charged Coomassie Brilliant Blue G-250 reagent turns blue upon binding to positively charged groups in the protein, resulting in absorbance at 590 nm. The determination was performed by plotting a calibration curve of absorbance versus concentration, using bovine serum albumin (BSA) as the standard.

Preparation of Bee Venom-Loaded Microcapsules

The encapsulation process was carried out using the ionic gelation technique. Chitosan was used as the

encapsulant. A chitosan stock solution (2 mg/mL) was prepared by dissolving chitosan in 100 mL of dilute acetic acid. A 100 mL solution of tripolyphosphate (TPP) (1 mg/mL) was prepared by dissolving sodium tripolyphosphate in deionized water (Keskin et al. 2019). Bee venom, dissolved in 0.9% NaCl to a final concentration of 20 mg/mL, was added to the prepared TPP solution. The encapsulation process was carried out by adding the TPP solution, containing bee venom, dropwise to the chitosan solution. After the addition of TPP was completed, the solution was stirred for 1 hour at a constant speed of 200 rpm using a magnetic stirrer. The resulting microcapsules were obtained by centrifugation at 12000 rpm. The supernatant was stored for protein quantification (Keskin 2020). The encapsulation efficiencies of the obtained microcapsules were calculated using the following equation, based on protein quantifications of the pre-encapsulation bee venom samples and the resulting filtrates.

% EE = ((A – B) / A) × 100
A: Total protein content of the bee venom sample
B: Total Protein Content of Supernatant/Filtrate

Acetylcholinesterase inhibition

The measurement of acetylcholinesterase enzyme activity is based on the color reaction of DTNB (5,5'-dithio-bis-[2-nitrobenzoic acid]) with thiocoline, which is released from the hydrolysis of acetylthiocoline by the enzyme (Baltaş *et al*. 2016). The enzyme solution was prepared in 1% gelatin to a final concentration of 2.5 units/mL. 50 µL of the enzyme solution was added to 3 mL of 0.01 M phosphate buffer (pH 8). Subsequently, 20 µL of acetylthiocholine substrate (0.25, 0.5, 0.75, 1.0, and 1.5 mM) and 100 µL of DTNB reagent were added to the solution to initiate the enzymatic reaction. Enzyme activity was calculated from absorbance at 412 nm after a 10-minute reaction. To determine the inhibition rates of the microcapsules, activity assays were performed by mixing the enzyme solution with the sample (re-extracted bee venom in acetate buffer (pH 5.2)) in equal volumes, followed by a 5-minute pre-incubation at room temperature. The enzymatic reaction was then initiated by adding the necessary reagents. Activity was calculated from absorbance measurements. Inhibition values were calculated using the activity values obtained in the absence of an inhibitor and in the presence of an inhibitor. A positive control was performed using donepezil hydrochloride. All analyses were replicated in triplicate.

Antimicrobial activity: Microorganisms used in the study and antimicrobial activity tests

In this study, *Escherichia coli (E. coli,* ATCC® BAA-2523™*), Staphylococcus aureus (S. aureus,* ATCC 43300*), Bacillus subtilis (B. subtilis,* ATCC 23857*),* and *Pseudomonas aeruginosa (P. aeruginosa,* ATCC 27853) were used. Mueller-Hinton agar (MHA) and Mueller-Hinton broth (MHB) were used to incubate the bacteria. The overnight culture was diluted in 0.9% w/v saline to achieve a 0.5 McFarland standard. 100 µL of this suspension was spread onto agar plates. Sterile paper discs (Oxoid, CT09988, 6 mm in diameter) were also placed on the agar, and 100 µL of the sample (1000 µg/mL) was impregnated onto each disc. The inhibition diameter formed in the medium was measured in millimeters (mm) after 24 hours of incubation at 37°C (Karlıdag et al. 2021). All experiments were performed separately for each microorganism and repeated three times.

Statistical analysis

All experiments were conducted in triplicate, and the results were presented as the mean ± standard deviation (SD) of three replicates. Statistical analyses were performed using SPSS 20.0 for Windows (SPSS Inc., Chicago, IL). Statistical significance was assessed at P≤0.05, and mean differences were evaluated using Duncan's multiple range test (DMRT) (Güneri and Keskin 2026). To compare the quantified variables, one-way analysis of variance (ANOVA) was conducted, and Tukey's test was applied to determine significant differences.

RESULTS

Bee venom-loaded chitosan microcapsules were prepared using the ionic gelation technique. The encapsulation efficiencies of the prepared microcapsules were calculated by determining the protein content in the supernatants before and after encapsulation. The calculations revealed that the encapsulation efficiency for the chitosan microcapsules was 91% (Table 1).

Table 1. The total protein amounts

Sample	Total protein amounts (%)
Bee venom solution	64.2±1.8
Supernatant	5.80±1.5

The measurement of acetylcholinesterase enzyme activity is based on the color reaction of DTNB (5,5'-dithio-bis-[2-nitrobenzoic acid]) with thiocoline, which is released during the hydrolysis of acetylthiocoline by the enzyme (Baltaş et al. 2016). In the inhibition study, microcapsules were incubated with the enzyme solution at a concentration of 10 mg/mL for 5 minutes, after which the activity assays were performed. Inhibition rates were calculated from the activity assay results. The findings are presented in Table 2. It was determined that microcapsules loaded with bee venom exhibited significant inhibitory activity.

Table 2. The inhibitory effect of the microcapsule on the acetylcholinesterase enzyme

Sample	Inhibition (%)
Bee venom (BV)	84±2.1[a]
Encapsulated BV	80±1.8[b]
Donepezil	99±2.4

Data were presented as means±SD from three replicates. Different letters indicate a significant difference, while the same letter denotes no significant difference according to Duncan's multiple range test at P≤ 0.05.

The results of in vitro antimicrobial activity tests on *Escherichia coli (E. coli), Staphylococcus aureus (S. aureus), Bacillus subtilis (B. subtilis),* and *Pseudomonas aeruginosa (P. aeruginosa)* are presented in Table 3 as zone diameters. The findings indicate that the bee venom-loaded microcapsules exhibited moderate antimicrobial activity against the tested organisms.

Table 3. The antimicrobial activities of microcapsules

Microorganism	Bee venom (BV) (mm)	Encapsulated BV (mm)	Ampicillin (mm)
E. coli	13±1.0[a]	12±0.5[a]	13 ± 1.0
S. aureus	12±1.1[b]	11±1.0[b]	14 ± 1.0
B. subtilis	12±1.0[c]	12±1.0[c]	13 ± 1.0
P. aeruginosa	13±1.1[d]	12±0.9[d]	13 ± 1.0

Data were presented as means ± SD from three replicates. Different letters indicate a significant difference, while the same letter denotes no significant difference according to Duncan's multiple range test at P≤ 0.05.

DISCUSSION

Bee venom is a specific mixture containing various classes of biomolecules—such as polypeptides, proteins, lipids, and sugars—secreted by the venom glands of honeybees to defend their colonies and injected into their enemies via their stingers. Like all other bee products, bee venom does not differ in the components it contains, but it does vary in the relative quantities of these components. For example, bee venom produced in one region may contain 45% melittin, whilst venom obtained from another region may contain 56% melittin. Due to its important bioactive components, bee venom is used in various fields, including traditional and complementary medicine, new drug development, and cosmetic products (Samancı and Kekeçoğlu 2019). However, bee venom also exhibits toxic effects due to its components, such as melittin, phospholipase A2, apamin, and hyaluronidase. The uncontrolled use of bee venom at high doses can cause pain, edema, inflammation, and, in allergic individuals, severe allergic reactions (Park et al. 2015, Yoo and Lee 2022). For this reason, bee venom must be administered in low concentrations and in a controlled manner.

Bee venom was produced using venom collection devices. Bees release their venom onto the glass surfaces of these devices when they sting them. The venom crystallizes rapidly upon contact with air. The crystallized venom accumulated on the glass surface is scraped off with a suitable scraper and harvested. Venom obtained in this manner can be stored in a deep freezer for a short period. For long-term storage, however, the crystallized venom must be freeze-dried. In this case, the shelf life can be up to one year. Consequently, shelf life is a significant issue for bee venom-containing products intended for use in various fields. Furthermore, producing a standardized product is the first step towards manufacturing products with a defined composition, enabling their use in various applications and yielding comparable results. In this study, bee venom was encapsulated using the ionic gelation technique with chitosan. The inhibitory effect of the microcapsules on acetylcholinesterase and their antimicrobial activity against certain microorganisms was investigated.

In their study, Hassan et al. (2019) encapsulated honeybee venom in chitosan capsules and evaluated their antimicrobial activity. According to this study, the encapsulation efficiency of the capsules ranged from 86.5% to 91.3%. It was reported that the capsules exhibited an antimicrobial effect against *Aspergillus flavus*. In their study, El-Dek et al. (2019) encapsulated honeybee venom in chitosan capsules and found that the capsules induced apoptosis in various cell lines. In their study, Saber *et al*. (2017) found that bee venom-loaded chitosan capsules were effective against Entamoeba histolytica. In their study, Park et al. (2018) produced bee venom-loaded PLGA capsules. According to this study, the encapsulation efficiencies of bee venom-loaded capsules produced in different formulations ranged from 45.9% to 85.8%. In a study by Alalawy et al. (2020), bee venom-containing nanospheres were produced using fungal chitosan, with an encapsulation efficiency of 92%. In another study, the encapsulation efficiency of nanoparticles obtained using chitosan was reported to be 93.3% (Saber et al. 2017). The results were consistent with the literature.

When the inhibitory effect of the microcapsules on acetylcholinesterase was examined, chitosan microcapsules at a concentration of 10 mg/mL were found to cause 80% inhibition of the enzyme. The standard donepezil caused 99% inhibition. The findings suggest that microcapsules containing active components of bee venom could be a significant inhibitor of acetylcholinesterase. Although few studies in the literature have examined the inhibitory effect of bee venom on acetylcholinesterase, melittin, one of its main components, has been reported to act as a non-competitive inhibitor of the enzyme (Mitchell et al. 1971).

When the antimicrobial activity of bee venom-loaded microcapsules was evaluated, they exhibited strong activity against the tested microorganisms. It has been reported that melittin and phospholipase A2, the main components of bee venom, possess high antimicrobial activity (Carpena et al. 2020). It has

also been stated that both melittin and phospholipase A2 exert this effect by disrupting biological membranes. Furthermore, minor components of bee venom, such as the peptides secapin and vitellogenin, also exhibit antimicrobial activity. It can be said that the high antimicrobial activity of the bee venom-loaded microcapsules obtained is due to the synergistic effect of the bioactive components mentioned above.

Conclusion

Bee venom is widely used in apitherapy to treat various conditions due to its bioactive components. However, when bee venom is not administered directly by bees, it crystallizes upon exposure to air and is prone to spoilage under unsuitable storage conditions. Consequently, there is a need for alternative administration methods that preserve the properties of bee venom. In this study, bee venom was encapsulated with a chitosan biopolymer using the ionic gelation technique. The enzyme-inhibitory properties and antimicrobial activity of the obtained bee venom capsules were determined. It was found that the encapsulated bee venom exhibited good activity against the infection-causing microorganisms *E. coli, S. aureus, P. aeruginosa, and B. subtilis*. In subsequent studies, this preliminary study will be evaluated for physicochemical characterization, stability, release properties, toxicity, *in vivo* studies, allergic reactions, and biosafety parameters. After obtaining stable, controlled capsules, the researchers will focus on developing new products containing encapsulated bee venom, such as creams and shampoos.

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