



Honeybee Derivatives-Based Nano-Formulations as Effective Alternatives to Control Resistant Pathogenic Microorganisms via Echo-Friendly Approach

Maram I. Nayel¹, Ibrahim E. Mousa¹, Reham M. Abdel-Azeem¹ and Ahmed A. Tayel²

¹Department of Environmental Biotechnology, Faculty of Biotechnology, University of Sadat City, Sadat 32897, Egypt

²Department of Fish Processing and Biotechnology, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University, Kafrelsheikh 33516, Egypt

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ABSTRACT

Bee products, for instance chitosan and propolis (Pro) and bee venom (BV), possess unique features with huge importance in various fields such as antimicrobials and food biopreservation. The worldwide increasing incidence of resistant bacterial pathogens, pose a serious threat to the health of humans. In the present investigation bees' chitosan nanoparticles (BCht), Pro and Pro-mediated AgNPs and their combination with BV have been prepared in order to assess their efficacy as antimicrobial nano-compounds to combat resistant bacterial pathogens. BCht was prepared from the bees' (*Apis mellifera*) corpses with the mean particle diameter of 139.85 nm. Pro was utilized for AgNPs synthesis which contained the mean diameter 6.82 nm whereas Pro-BCht-BV nanoconjugate had the average diameter 182.36 nm. All the compound/composite showed excellent antibacterial activity against drug-resistant *Staphylococcus aureus* and *Salmonella typhimurium* where the highest resistance was observed for *S. Aureus*. BCht/Pro/AgNPs was observed to be the most effective against all the studied strains. The synthesized nano-compounds/nanomaterials (BCht, Pro, Pro/AgNPs, Pro/BCht/AgNPs and BCht/BV/Pro/AgNPs) showed high performance against resistant bacterial pathogens that inhibit and kill the pathogens, where the cells were entirely killed after treatment with these compounds/composites for 10 hours as was revealed by qualitative/quantitative measurements and electron microscopy. Preparation of green nanocomposites using bees' products for the development of promising antimicrobial agents against resistant bacterial pathogens is strongly recommended.

Keywords: Antibacterial, bees chitosan, nanomaterials biosynthesis, propolis, bee venom

1. Introduction

Chitosans are the de-acetylated biopolymers mainly obtained from chitin that possess numerous bioactivities and applications as human compatible compounds (Senica *et al.*, 2016). Chitosans are indeed unique compounds for their use in nutrition, pharmaceuticals, biomedicine, therapeutic, antimicrobial, anti-cancer, food preservation and drug-carrying branches (Senica *et al.*, 2016; Pérez-Burillo *et al.*, 2018; FAO, 2019; Saleem *et al.*, 2022). All the chitosan's bioactivities are importantly reinforced when is transformed to "nano" forms, because of the huge increase of the particles surfaces and the reactivity (Dash *et al.*, 2022; DeMers, 2022). Chitosans have variety of sources such as crustacean waste (the most common sources), fungi biomass or insect skeletons (FAO, 2019; Chen *et al.*, 2021; Felicia *et al.*, 2022). Honeybee (*Apis mellifera* L.) is the gifted insect which can give human multiple beneficial products. Besides the most important product which is honey, there are multiple other high-valued products can be derived from bees, they are propolis, venom and chitosan (Saleem *et al.*, 2020). Because the bees' cuticles are consisted of 30-50% chitin and 60-80% of beehives are replaced every year that could offer 6-10 million kilograms of dead bees' corpus, so honeybees can be

Corresponding Author: Ahmed A. Tayel, Department of Fish Processing and Biotechnology, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University, Kafrelsheikh 33516, Egypt.
E-mail: tayel_ahmad@yahoo.com (AAT)

a promising source for chitosan extraction (Saleem *et al.*, 2020; Chen *et al.*, 2021; Chaudhary *et al.*, 2022; Papagiannopoulos and Sotiropoulos, 2022).

Propolis (bee glue) composed of natural resinous materials, that the worker bees collected from the plant's exudates and buds, then they mix it with pollen, beeswax and enzyme of bees; the source and composition could affect the pharmacologic and therapeutic bioactivities of propolis components (Chaudhary *et al.*, 2020). Bees regularly use propolis to glue and protect their hives from intrusion of other living invaders like insects and microorganisms, so propolis compounds possess active natural antibiotics in fighting infection by bacteria, fungi or viruses (Melro *et al.*, 2020). The main bioactivities and health promotion of propolis were reported to be antibacterial, anthelmintic, antiulcer, antiviral, antifungal, antioxidant, hepatoprotective, antiradiation, antimutagenic, antitumor, cyto- and chemoprotective, anti-angiogenic, wound healing, anti-inflammatory, immune-modulating, anti-diabetic, cardioprotective, cicatrizing, local anesthetic and bio preservative food agent (Kong *et al.*, 2010; Zhang *et al.*, 2022).

Up to now, among the different types of propolis produced in different regions in the world, there were over 850 compounds reported and the phytochemical classes belong to: volatile oil, alcohol, flavonoid, alkan, aromatic acid, terpeneoid, alcohol sugar, aldehyde, chalcon, amino acid, fatty acid, vitamin, carbohydrate, hydrocarbons, waxes esters, ketones, phenols, glycerides derivatives, traces element (Melro *et al.*, 2020; Almutairi *et al.*, 2020; Al-Saggaf *et al.*, 2020). However, the propolis extract (Pro) showed its capacity in reducing metal ions into nanoparticles, which could be due to the excellent reduction and capping ability (Chaudhary *et al.*, 2020; Al-Saggaf *et al.*, 2020).

Biocompatible nanoparticle (NP) formation has drawn intensive research worldwide for its peculiar chemical, physical and biological characteristics (Tayel *et al.*, 2020). Overcoming limitations in NP synthesis (toxic wastes, high energy consumption) due to traditional synthesis routes, the biological method of synthesis (green or bio-synthesis) proves to be one efficient alternative in providing effectively structured electromagnetic and catalytic active nanoparticles with low cost and low toxicity (Raghuraman and Chattopadhyay 2007; Alghuthaymi *et al.*, 2021; Salem *et al.*, 2022).

The defense weapon that is secreted from honeybee (*Apis mellifera*) when attacked is called bee venom (BV). Composite mixture that consists of bioactive peptides is found in BV and could guard bee's colony against various kinds of predators or attackers. It has several bioactives in it; for example, melittin (the main constituent), adolapin, apamin, degranulating peptide (for mast cell), enzymes (hyaluronidase and phospholipase A2) and non-peptides (dopamine, histamine and norepinephrine) (Oršolić *et al.*, 2001; Pak *et al.*, 2017).

The BV or its elements such as melittin have been extensively studied for its use and potential inhibitors to types of tumors. It has several molecular anticancer actions (Oršolić *et al.*, 2001; Logeswari *et al.*, 2013; Aufschnaiter *et al.*, 2020).

The nanometals that is most extensively studied is silver nanoparticles (Ag-NPs) for their high potentials to be applied as powerful antimicrobial, anticancer, antioxidant and wound-healing accelerating agents and these Ag-NPs can be effectively synthesized by the biogenic protocols, increasing its environmentally friendly characteristic, bio-compatibility and safety towards human cells (Logeswari *et al.*, 2013; Bhakya *et al.*, 2016).

Hence, the purpose of this study is to extract bees' chitosan nanoparticles (BCht), Propolis extract (Pro), Pro-mediated AgNPs and composite of BV, and to screen it for use as effective nano-compounds as antimicrobials against food-borne bacterial pathogens.

2. Materials and Methods

2.1. Bee chitosan extraction

Honeybee bodies (*Apis mellifera*) deceased naturally were gathered from 4 apiaries distributed in Kafrelsheikh Governorate, Egypt. Samples were collected from March to October, 2025. Bodies were accumulated from each apiary, washed carefully, then frozen till to be used for chitosan preparation as a raw material. After thawed, bee material was used for oil pressing (to remove majority of the oil and protein part), the skeleton part of the bees' material was consecutively washed using deionized water (DW) and then freeze-dried (Chen *et al.*, 2021). Protocol of the steps for bee chitosan preparation is shown in Figure (1). The DW washing and freeze-dried steps were repeated for each subsequent step.

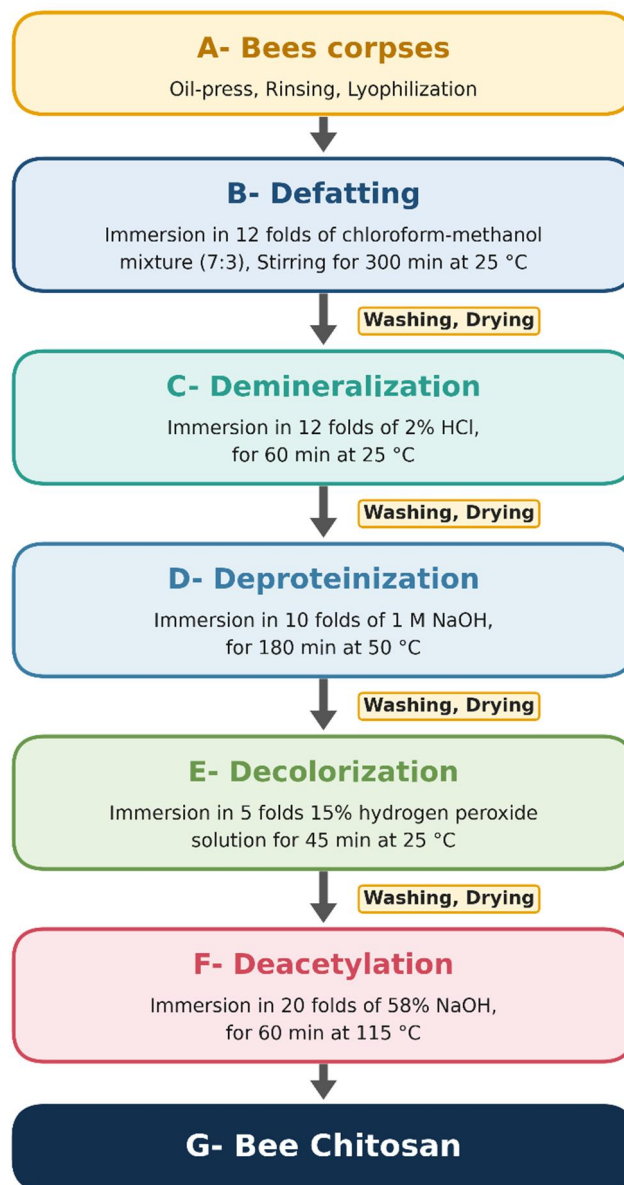


Fig. 1: The applied protocol for bees' chitosan extraction

For the preparation of bee chitosan nanoparticles (BChT), the solutions were prepared in DW and then the pH was adjusted at 5.3 with solutions of Na-tripolyphosphate "NTPP" (concentration of 0.5%; w/v; Sigma-Aldrich; MO; USA) and of chitosan (concentration of 0.1%; w/v; obtained dissolving in 1%acetic acid solution). NTPP was added drop-wise with a syringe needle, at 350 L/min, to a volume of 100 ml of agitated chitosan solution (725-x g) and then agitation was carried out during another 100 min. BChT were recovered by centrifugation (10300- g; 32 min), washed with DW and re-centrifuged, followed by lyophilization (Giannakas *et al.*, 2019).

2.2. Propolis extraction and AgNPs synthesis

The raw propolis collected from the apiaries were processed as stated above. The raw propolis was extracted with 10-fold (v/w) of 72% ethanol at room temperature (RT, 25C) for 42h with the help of shaking (225-x g). The extracted propolis (Pro) solution filtered with Whatman No. 2 paper was left standing for 24h at 4C in order to make wax contents precipitated, then re-filtered (Al-Shmgani *et al.*, 2017). Pro solution was filtered and centrifuged, then the remaining solution was evaporated using vacuum dryer (Buchi, Switzerland) at 40C until dried. Pro was re-dissolved in 2% aqueous Tween 80 solution to achieve 10% (w/v) solution (Khorrami *et al.*, 2019).

Pro/AgNPs biosynthesis was carried out when the fresh-made extract with 1% concentration was dropped into reaction solution containing final concentration of sodium selenite and ascorbic acid 10 mM and 25 mM respectively (Chaudhary *et al.*, 2020). The mixture was then stirred at 220-x g and RT for 8h and it was observed the pale yellow mixture turns to deep brownish orange color. The Pro-mediated AgNPs were then centrifuged at 10500-g for 35 min. After washing with DW and ethanol the Pro-mediated AgNPs were freeze-dried. The blank AgNPs were analysed, composite Pro/AgNPs were washed with DW and ethanol 4 times respectively after being centrifuged for each wash step followed by freeze-dried.

2.3. Products' physiognomies characterization

2.3.1. SPR (Surface plasmon resonance) evaluation

Pro mediated AgNPs spectrum was observed by UV-Visible spectrophotometer (UV-2450, Shimadzu, Japan) taking wavelength in the range 220-550 nm.

2.3.2. FTIR analysis

Detection of biochemical bonding of the molecules thus produced could authenticate their biochemical structure and biochemical interactions with others using infrared analysis. FTIR was used to determine infrared spectra of NCt, RA and NCt/RA/AgNPs, the system used was Fourier transform infrared spectroscopy (FTIR; FTS 45, Biorad, Germany). Transmittance of samples were measured in the range 400-4000 cm⁻¹, each sample was then mixed with 1% KBr and then scanned (Aufschnaiter *et al.*, 2020). Each sample was analyzed at room temperature, 22 C at a resolution of 4 cm⁻¹; each dried sample was grounded finely with anhydrous KBr and then mixture sample was then placed for analysis.

2.3.3. FTIR "Fourier-transform infrared spectroscopy" analysis

The FTIR spectra of the extracted Pro, BChl, BV, Pro/AgNPs and BChl/BV/Pro/AgNPs nanocomposite was investigated by the spectrophotometer (JASCO 4100, Japan) from wavenumber region between 450cm⁻¹ and 4000cm⁻¹. Fine powder of sample was grinded well with KBr and transmission spectrum for every powder was recorded.

2.4. Electron microscopy analysis

The morphology size and structure of Pro-mediated AgNPs and BChl were characterized by TEM "Transmission electron microscopy, JEOL, JEM-2100, Japan" and SEM "Scanning electron microscopy, JSM IT100, JEOL, Japan" respectively. The TEM observation were taken at 180 kV of accelerating voltage. The prepared NP solutions were drop dried on carbon coated copper grid in vacuum before observation. For SEM observation of shape and size the accelerating voltage was set at 20 kV.

Characterization of Zeta potential (ζ) and Particle size (Ps) distribution of Nanoparticles The size and charges (potential) of BNCt, Pro- synthesized AgNPs, and the BNCt/Pro/AgNPs nanocomposites were measured by DLS protocol "Dynamic Light Scattering, Malvern Zetasizer, Malvern Instruments, Malvern, UK". The distribution of D ls and potential were observed using DLS protocol on the dispersed NPs in DW solution after sonication.

2.5. Antibacterial potentiality assessments

Antibacterial activities of BChl, Pro, Pro/AgNPs and the BV-nanocomposites were determined qualitatively and quantitatively using standard bacteria viz; Staphylococcus aureus- ATCC 43300 (MRSA) and Salmonella typhimurium- ATCC 700408 (MDR). The bacteria were cultured, sub-cultured and incubated at 37 C on NA and NB media (Difco Labs, Detroit, MI).

2.5.1 Qualitative Disc diffusion assay

The qualitative antibacterial assay was done based on the recorded appearances of theZOIby using "disc diffusion" method, where bacteria are streaked on the NA plates and paper discs (6.0 mm diameter) that loaded with 28 L solution of the agent (w/v of 10%) were laid on the surface of bacteria lawn. After the plates were incubated for 18-22 hours at 37 C, the inhibition zones around the discs are visually observed and measured. The standard antibiotic ampicillin (Merck, Darmstadt, Germany) was used for the assay by the same challenge.

2.5.2. Quantitative MIC "Minimum inhibitory concentration" assay

The above stated microdilution was performed for estimation of MIC values of nano-composites and pure extracts (37). Triphenyl tetrazolium chloride (TPTC indicator), Merck, Germany was used for estimation of viability of the organism. Series of 10-100 g/mL of all the compounds/composites were serially diluted in NB and taken in 96 well micro plates and inoculated with $\sim 2 \times 10^7$ CFU/mL of all the test bacteria, also 0.5% (w/v) TPTC was added to it. To examine the bactericidal action of all compounds, samples from clear wells were plated on NA plate successively and incubated. MICs were noted down as the lowest concentration that inhibited viable bacteria within micro plates as well as on NA plate.

2.5.3. SEM imaging of antibacterial action

The morphological abnormalities in the structural architecture of *S. Aureus* cells treated with BNCT/Pro/AgNPs nanocomposite solution (at 100mg/L) were assessed using SEM after incubation (aerobically, at 37 °C) for both 5 and 10 h (as discussed previously, (2)). The SEM images recorded were taken at $\times 10000$ magnification with an acceleration voltage of 20 kV and depended on the morphology/structural distortions detected.

2.6 Statistical analysis

All assays were carried out in triplicates and mean $\pm$ S.D ("standard deviations") were computed using MS-Excel- 365. Data significance was calculated using t-test & ANOVA from SPSS software (SPSS V-11.5, Chicago, IL.) at $p < 0.05$.

3. Results

3.1. Bee chitosan

From the bodies of insect bee, chitosan was successfully extracted. Yellow-white powdered polymer was produced, with the molecular weight being 58.7 kDa, deacetylation degree 88.2%, and the solubility being 97.8% in 1.0% acetic acid solution.

3.2. Selenium nanoparticles biosynthesis using propolis extract

Pro extract is able to successfully reduce Ag ions to AgNPs effectively (Fig 2). This can be directly observed from color change of the Ag solution from transparent into blackish brown (Fig 2-1). Maximum UV-Vis's absorbance (max) for the Ag NPs solution is recorded at 423 nm (Fig 2-2).

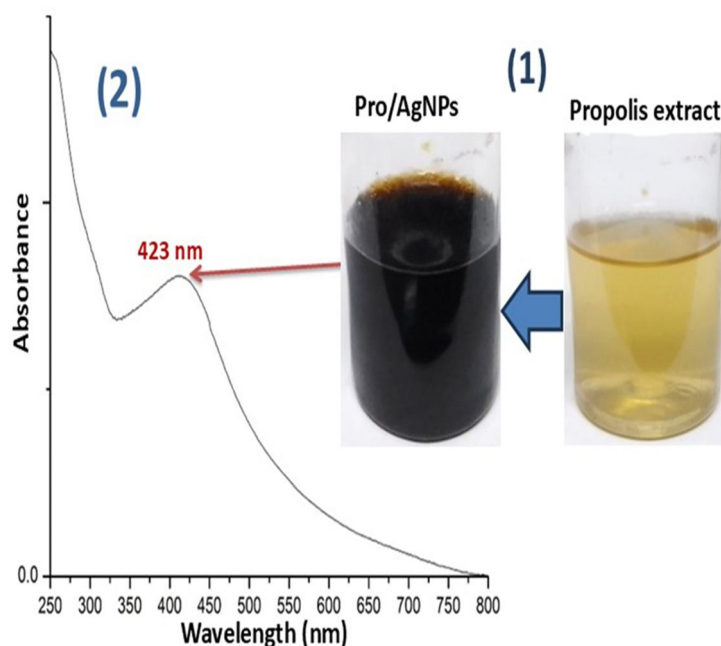


Fig. 2. Visual appearance (1) and UV-Vis spectrum (2) of biosynthesized AgNPs using propolis extract

3.3. Structural analysis of synthesized molecules

3.3.1. FTIR analysis

The infrared characterization for synthesized molecules was carried out to observe the chief biochemical groups/bonds of these molecules, which lead to their activity and potential to form the nanocomposite (Fig 3) (BCht, Pro, Pro/AgNPs and BCht/Pro/AgNPs).

In the BCht spectrum (Fig 3-BCht), the main designative peaks could be seen at 1110.02 cm^{-1} (CO), 1402.91 cm^{-1} (amide III, CN), 1634.32 cm^{-1} (amide I, C=O), 2310.95 cm^{-1} (CH), and 3457.65 cm^{-1} (NH₂) correspondingly. Also, the peaks for C-O bending vibration, and C-H stretched vibration were observed at 1069.08 cm^{-1} and around 2310.95 cm^{-1} respectively. Moreover, the physical interaction between -NH group of chitosan and TPP can be attributed by appearance of a peak at 3244.32 cm^{-1} .

Key designative groups/bonds present in Pro were shown in its FTIR spectrum (Fig 3-Pro). The peaks values in the neighborhood of 2934.55 and 2841.76 cm^{-1} are attributed to lignin polymer. Peak values of carboxyl group at 1466.32 cm^{-1} , phenolic groups at 1027.96 cm^{-1} and 734.33 cm^{-1} .

From the FTIR spectra analysis of Pro/AgNPs spectrum the possible molecules which is responsible for AgNPs synthesis/stabilizing have been determined. The study showed that the compounds in Pro, containing O-H, N-H, C-C and COOH group are mainly responsible for the interactions with the AgNPs. Among them, there is aliphatic saturated C-H stretching was shown in 2931.63 cm^{-1} and peak in 1462.25 cm^{-1} was shown for CH₂/CH₃ of carboxylate group and/or aliphatic compound. The peak at 1256.72 cm^{-1} corresponded to C-N and C-O-S of aromatic amine, while at 1165.55 cm^{-1} for SO₂ and NO₂ of sulphones and the peaks between 1087.65 and 1048.06 cm^{-1} correlated with stretched CH and COC of carbohydrate group and peak at 734.61 cm^{-1} and 552.44 cm^{-1} is shown for CH₂ of hydrocarbons and CC=O of carboxylic acid.

The formed composites BCht and Pro/AgNPs (Fig 3-BCht/Pro/AgNPs) were rich of biochemical bonds/groups of both compounds (blue lines refer to the bonds/groups that comes from BCht and red lines comes from Pro/AgNPs groups). The points colored with blue in Fig 3 represent the groups that are newly appear/immerse after the biosynthesis process of AgNPs in Pro solution, the colored with red points are that disappeared during the process, and that colored with yellow are transferred groups that appear in Pro/AgNPs spectrum after its conjugation with BCht.

In the spectrum of BV can observe a broad absorption band in 3250-3450 cm^{-1} which reflects the free N-H stretching. The amides bonds (amide I (1652.33 cm^{-1}) and amide II (1538.05 cm^{-1}) can also be observed in the FTIR spectrum of BV, as well as the absorption bands at 1110.22 cm^{-1} and 1044.63 cm^{-1} which reflects unsystematic coil conformation (Fig 3-BV).

Conjugation of BV with BCht/Pro/AgNPs seems to have obvious affect to combined FTIR spectrum (Fig 3-BCht/BV/Pro/AgNPs); numerous peaks from BCht/Pro/AgNPs spectrum were transmitted, in contrast, a lot other peaks disappeared or had gone from BCht/Pro/AgNPs spectrum after conjugation with BV which showed the biochemical groups were occupied/destroyed by BV bioactive molecules.

3.3.2. Ultrastructure analysis of synthesized nanoparticles

The physiognomies of synthesized nano particles/nano composites (Pro-synthesized AgNPs and BNct/BV/Pro/AgNPs) were evaluated with electron microscope image (Fig 4) and DLS data (Table 1). The TEM image of NP ultrastructure illustrated that size range and good distribution of Pro-synthesized AgNPs were uniform, having spherical and semi-spherical shape (Fig 4-A). The SEM image of BNct/BV/Pro/AgNPs shown it's a formation of biopolymers nanocomposites, which is heterogeneous in shapes and the average diameter is estimated to be 183 nm (Fig 4-B).

Also, DLS results confirmed the former; mean diameter size of the recorded BCht, Pro/AgNPs, BNct/Pro/AgNPs and BNct/BV/Pro/AgNPs were found to be 139.85, 6.82, 141.58 and 182.36 nm (Table 1) respectively. BCht gave high positive charges (+36.6 mV) but it was reduced slightly to +31.8 mV (+26.5 mV) upon Pro/AgNPs conjugation and BV/Pro/AgNPs conjugation respectively while AgNPs gave negative charges (-23.7 mV) (Table 1).

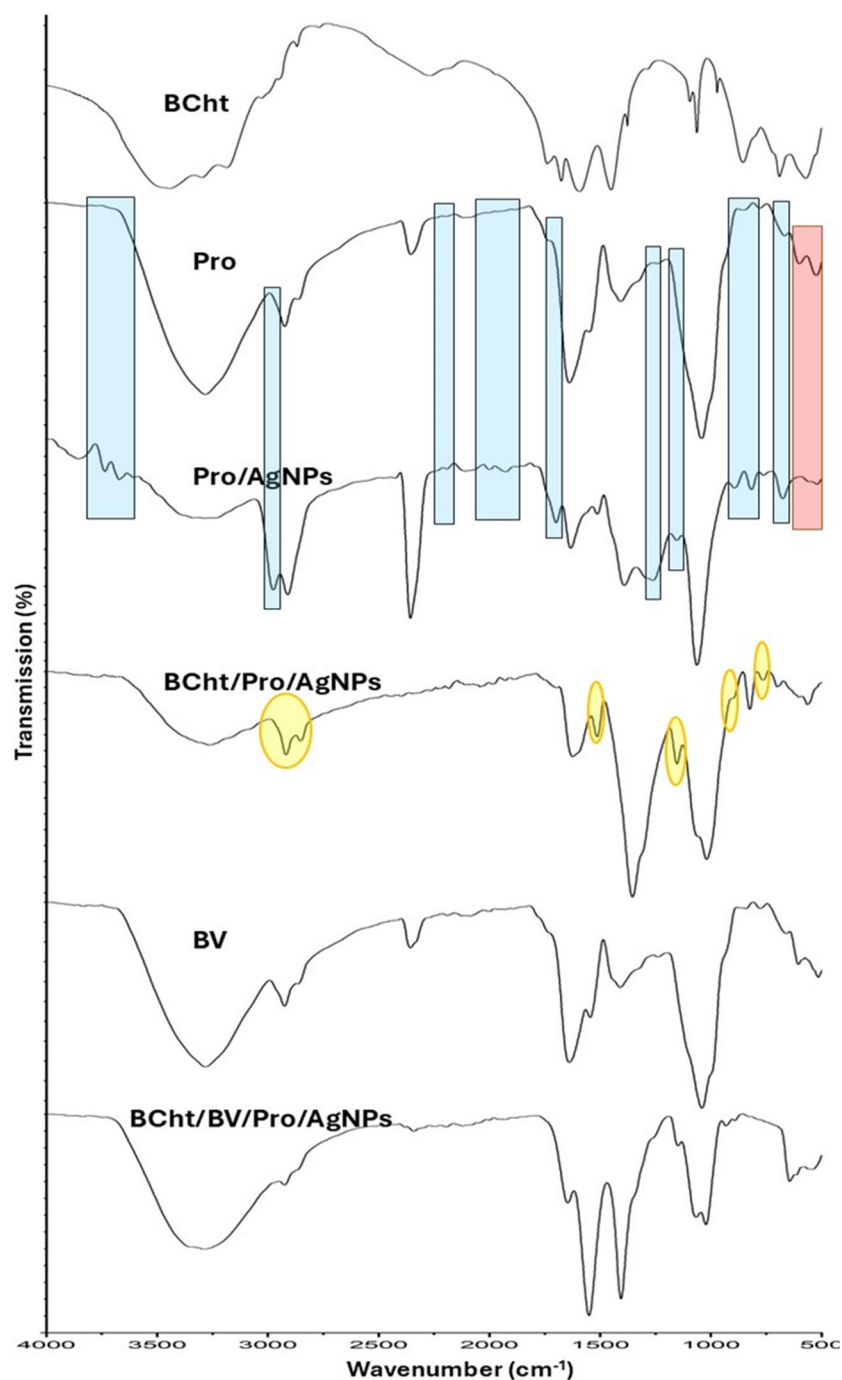


Fig. 3: FTIR spectra of bee chitosan (BCht), propolis (Pro), Pro with synthesized AgNPs (Pro/AgNPs), Bee venom (BV) and BCht/BV/Pro/AgNPs nanocomposite*

*The highlighted parts with blue, represents the emerged/submerged peaks observed post the biosynthesis of AgNPs by Pro, the red zones show the submerged peak post the biosynthesis, and the yellow areas show the shifted peaks from the spectrum of Pro/AgNPs post conjugation by BCht.

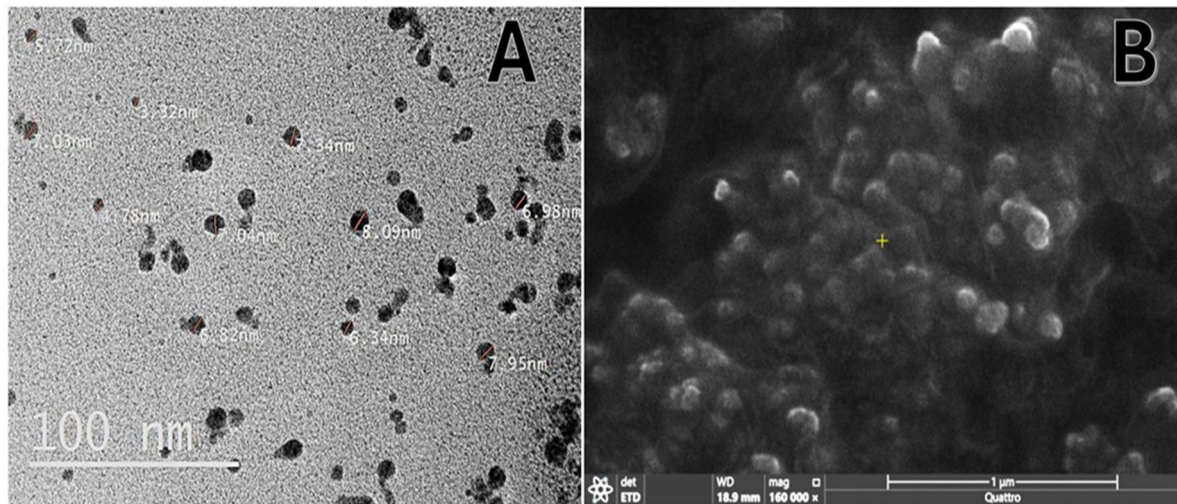


Fig. 4: Ultrastructure of synthesized nanoparticles, including (A): TEM image of Pro-synthesized AgNPs and (B): SEM image of loaded bee chitosan nanoparticles with Pro/AgNPs

Table 1. Nanoparticles' size and charges of fabricated bee nanochitosan (BNCT), propolis-synthesized AgNPs (Pro-AgNPs) and their nanocomposites (BNCT/Pro/AgNPs)

Nanoparticles	Pericles size range (nm)	Pericles size mean (nm)	Zeta potential (mV)
BNCT	41.09 - 247.88	139.85	+36.6
Pro-AgNPs	1.65 – 18.55	6.82	- 23.7
BNCT/Pro/AgNPs	57.15- 325.77	141.58	+31.8
BNCT/BV/Pro/AgNPs	60.91-412.46	182.36	+26.5

3.4. Antibacterial action of nanocomposites

3.4.1 In vitro antibacterial potentialities

The antimicrobial effects that were derived from application of the natural compounds, in terms of both quantitatively and qualitatively, towards the selected bacteria pathogens were also confirmed (Table 2). The whole compounds/ composites such as BCht, Pro, Pro/AgNPs and BCht/Pro/AgNPs, showed significant antimicrobial actions with order BCht < Pro < Pro/AgNPs < BCht/Pro/AgNPs < BCht/BV/Pro/AgNPs. All of the selected strains of bacteria are completely sensitive to the agents examined, the ordering of the bacterial sensitivities towards the created antibacterial agents were found to be Gram-positive < Gram-negative (*S. Aureus* < *S. Typhimurium*). In terms of both ZOI and MICs, BCht/Pro/AgNPs and BCht/BV/Pro/AgNPs nanocomposites demonstrated superior antibacterial action in comparison with the control antibiotic (ampicillin) (Table 2).

Table 2: Antimicrobial activity of bee chitosan nanoparticles (BCht), propolis (Pro), Pro/AgNPs and BCht/Pro/AgNPs

Antimicrobial agents	Antimicrobial activity			
	<i>Staphylococcus aureus</i>		<i>Salmonella typhimurium</i>	
	ZOI**	MIC***	ZOI	MIC
BCht	15.8±0.9 ^a	57.5	16.7±1.3 ^a	52.5
Pro	18.6±1.4 ^b	47.5	21.2±1.7 ^b	27.5
Pro/AgNPs	24.6±2.4 ^c	37.5	27.4±2.4 ^c	32.5
BCht/Pro/AgNPs	27.9±2.2 ^d	32.5	32.9±2.5 ^d	25.0
BCht/Pro/BV/AgNPs	29.1±2.6 ^d	27.5	33.2±2.8 ^d	22.5
Ampicillin	23.5±3.7 ^c	45.0	24.4±2.1 ^b	42.5

Different superscript letters in one column indicate significant difference at $p \leq 0.05$

** ZOI: Mean diameter of inhibition zones in mm ± standard deviation

*** MIC: minimal inhibitory concentrations (mg/L)

3.4.2. SEM analysis of challenged bacteria with nanocomposite

The SEM image of treated resistant bacteria pathogen (*Sal. Typhimurium* and *St. Aureus*) with BChT/BV/Pro/AgNPs nanocomposite can show biocidal effect to destroy bacterial cells (Figure 5). Bacterial cells of control (Fig 5-SL-0 and ST-0) at the start of the treatment, seem with regular shapes and size of its structures/membranes and the contact surfaces look smooth and equal. After 5 hours of incubation in presence of BChT/BV/Pro/AgNPs nanocomposite, *Sal. Typhimurium* and *St. Aureus* cells started swelling and the partial lyses of cell appeared (Figure 5, SL-5 and ST-5); numerous visible NPs were observed at attachment/interaction sites to treated bacterial cells.

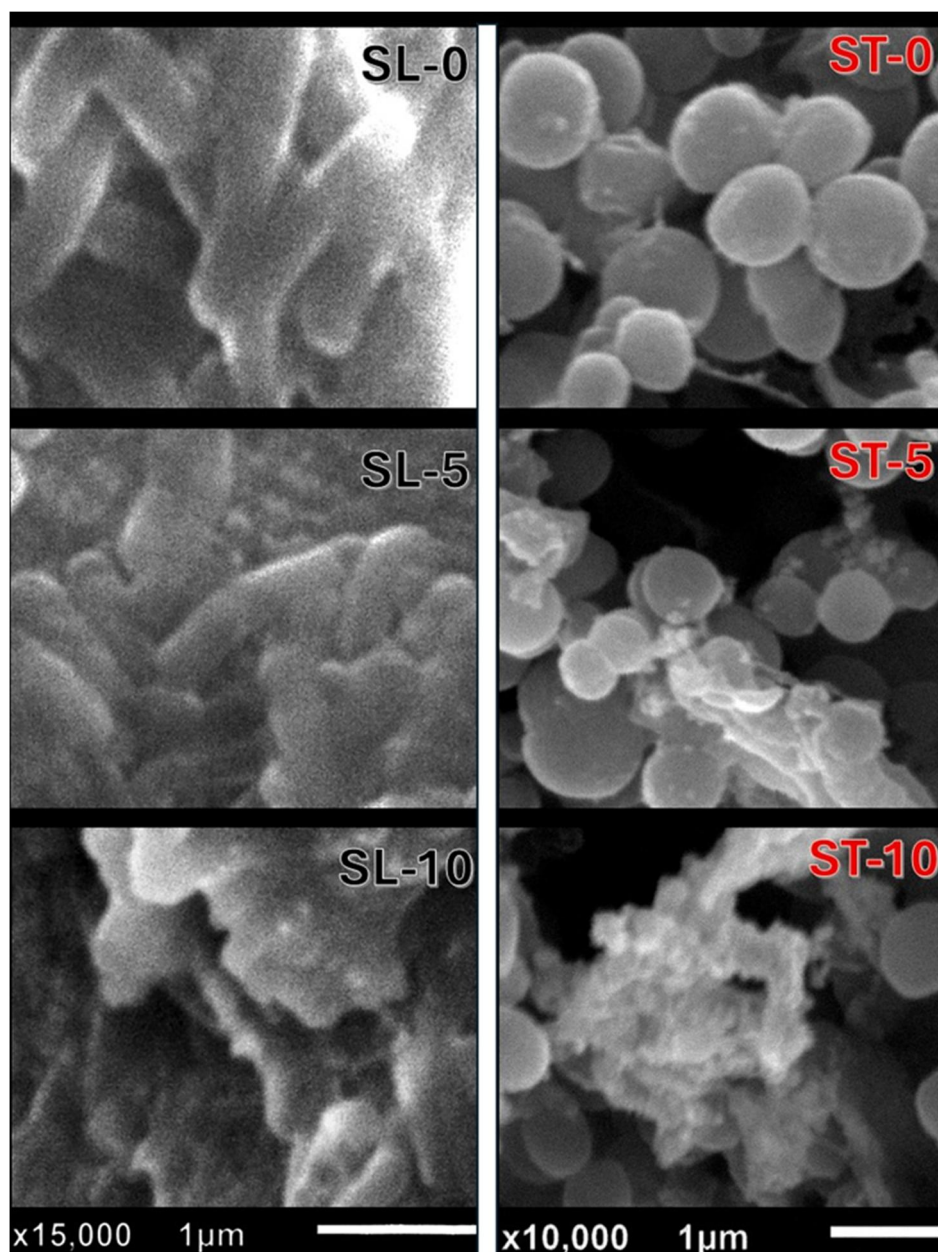


Fig. 5: SEM micrographs of treated *Salmonella typhimurium* (SL) *Staphylococcus aureus* (ST) with BChT/BV/Pro/AgNPs for 5 h and 10 h compared with control cells (0 h)

In time points of cells treated by BChT/BV/Pro/AgNPs nanocomposite 10 hours later, cells were destroyed/killed to the degree which showed a strong punition (as represented by SL-10 and ST-10 in Figure 5), it showed the presence of NPs on the surface of bacteria more clearly and that they contributed to the destruction of bacterial membrane and interior environment.

4. Discussion

It is stated that the characteristics obtained for bee-extracted chitosan are recommendable properties for regular chitosan, for example deacetylation degree of 70% and small relative molecular weight (Senica *et al.*, 2016). In general, the yield and the characters for bee-extracted chitosan were similar to the ones obtained in previously reported works on former insects' chitosan (Felicia *et al.*, 2022; Chaudhary *et al.*, 2022; Papagiannopoulos and Sotiropoulos, 2022).

The characterization of NP's features (e.g. Size, structure and shape) are needed to verify their properties, many techniques were already proposed, the mostly used methods were UV-visible spectrophotometric assessment, FTIR analysis, X-ray diffraction, DLS, electron microscopy imaging (Abdel-Rahman *et al.*, 2020; Huerta-Madroñal *et al.*, 2021). According to the characterization results, it could be proved from UV-vis assessment and visual observation for Pro-synthesized AgNPs that the efficiency of Pro in reduction of Se ions and forming into AgNPs is high, the change of color of AgNPs solution after reduction using Pro depends on SPR which often show theirmax around 265-275 nm (Wazed *et al.*, 2010; Ahmad *et al.*, 2010; Alsharari *et al.*, 2018; Salem *et al.*, 2022).

The typical groups/ bonds of common chitosan (especially from insects) in BCht spectrum (Fig 2- BCht) are listed and were in good agreement with literature (Chen *et al.*, 2021; Dash *et al.*, 2022; Papagiannopoulos and Sotiropoulos, 2022), which indicated that chitosan is successfully extracted from bee skeletons. Interaction between chitosan groups (-NH group etc.) and TPP indicates the synthesis of BCht was successfully performed (Antonioni *et al.*, 2015). Pro spectrum showed many functional groups (-OH, polyphenols etc) are available which can play as reducing/capping/stabilizing agents of AgNPs (Singh *et al.*, 2024; Mancano *et al.*, 2025; Lima *et al.*, 2025; Waktole *et al.*, 2025). The FTIR for Pro/AgNPs was focused on the characterization of main biological constituents in Pro that took part in stabilizing and reducing of AgNPs (Mancano *et al.*, 2025; Lima *et al.*, 2025). Alteration of emergence, disappearance, or shifting peaks corresponding to the groups of Pro can explain the physicochemical interactions between Pro groups and AgNPs (Islam *et al.*, 2025; Waktole *et al.*, 2025; Santos *et al.*, 2025). Also the antioxidant/ reducing ability of Pro molecule can also lead to Se reduction and formation of AgNPs as reported for the synthesis of AgNPs using Pro recently (Al-saggaf, 2021). The size of BCht particles increased a little after encapsulation with Pro/AgNPs and its surface positivity decreases, which verified the effectiveness of nano-biopolymer particles as encapsulating agents of these conjugated molecules (Al-saggaf, 2021; Sharaf *et al.*, 2024; Klebowski *et al.*, 2026). Lower positivity of BCht surface after coupling with Pro/AgNPs also may imply the capping behavior not chemical bonding behavior with them (50). Recorded Zeta potentials (< -30mV & > +30mV) or analyzed NPs' DLS mark revealed the stable dispersion of these particles in solution (Al-saggaf, 2021; Sari *et al.*, 2025). In the current study, these promising results such as increased stability and smaller particle size of BCht-based nanocomposites conform with earlier reported conclusions concerning ability of different types of chitosan to incorporate other bioconstituents and biosynthesized nanomaterials (Zuorro *et al.*, 2021, Liang *et al.*, 2025).

In relation to the antibacterial properties of synthesized nanoparticles/nanocomposites, the Gram-species (*S. Typhimurium*) were more susceptible to their microbicidal activity compared with Gram+ (*S. Aureus*), which confirms earlier research on biosynthesized AgNPs and its combinations with chitosan (Akdaşçi *et al.*, 2025; Marshall and Wattanaphonpinich, 2026). To understand these results, high resistance of Gram+ bacteria to bactericidal nanoparticles are due to the peptidoglycan thick protective layer present in Gram+ bacteria membranes that consist of teichoic/lypoteichoic acids, and presence of some definite proteins (porines) on Gram bacterial surface that facilitates the access of biocidal molecules (like nanopolymer and nanometal) inside the cells, and ROS (reactive oxygen species) generated from the AgNPs diffusion into the interior of the cells are more easily accessed, and the activity and destruction of the intracellular cellular components can occur (Laanoja *et al.*, 2025; Aliero *et al.*, 2025; Selvam *et al.*, 2026).

The results of SEM imaging and DLS analysis would support the high binding and interacting potentiality of BCht/BV/Pro/AgNPs on the *S. Typhimurium* and *S. Aureus* cells thereby resulting in unprecedented killing and incapacitation of the cell viability. Two strains of bacteria namely *S. Typhimurium* and *S. Aureus* are selected for imaging due to their highest susceptibility towards the NPs. It would be due to collective synergistic effects of BCht, Pro, BV and AgNPs that is the instructive actions of NPs occurred at this instance which have been confirmed by DLS analysis which is in accord with earlier findings, showing the capability of chitosan and its nanoparticles to disturb bacterial cell

boundaries and enhance cell membrane permeability (Al-saggaf, 2021; Zuorro *et al.*, 2021; Liang *et al.*, 2025). Bioactivity of chitosan and its NP, particularly antimicrobial efficacy, resulted in positive charge on the particles. Positively charged particles would interact with the cell membrane surface and with crucial internal constituents of microbial cells (RNA, DNA, proteins, enzyme, etc.), consequently resulting in their death (Aliero *et al.*, 2025).

Actions of the phyto-synthesized AgNPs were also confirmed to destruct the bacterial pathogens cells, to inactivate its viability by its penetration into cell, to induce uneven walls' permeability, to trigger ROS, which eventually resulted to bacterial killing (Zuorro *et al.*, 2021; Liang *et al.*, 2025). Antimicrobial effect of AgNPs was shown to be boosted with coupling of it with chitosan NPs (Akdaşçı *et al.*, 2025; Marshall and Wattanaphonpinich, 2026), while it was confirmed that Pro-mediated AgNPs had the highest microbicidal effect more than the effect induced by its composited elements (Al-saggaf, 2021), this is also indicated on other Pro-mediated nanometals (Sari *et al.*, 2025). Moreover, the synergism which is shown between these conjugated antibacterial elements (BChT, Pro, BV, AgNPs) could serve to inhibit the bacteria growth because of the multiple antimicrobial agents which are from different sources and exhibit different effects could not sustain their survival (Liang *et al.*, 2025).

The re-useable applications of AgNPs, especially biogenic NPs in cosmetics, food preservation and processing, are reasonably justified by their fairly low toxicity and high biosafety, which has previously been used to confirm their wide applications and high biosafety in direct human contact (Aliero *et al.*, 2025). Moreover, since the addition of bio-molecules (e.g. Polysaccharides, phytocompounds, biopolymers...) during synthesis and stabilizing processes of AgNPs, may also confer biosafety for the biogenic NPs (Al-saggaf, 2021), it is reasonable to postulate that complexation of BChT, BV with Pro-mediated AgNPs could produce a more competent and high biosafety with less foreseeable toxicity. Meanwhile, the comparatively lower toxicity of AgNPs compared to bulk Ag, and confirmed potent radical scavenging and antioxidant capacities, suggested that AgNPs can serve as chemopreventives (in cancer therapy) and antioxidant agents (in cosmetic applications) (Al-saggaf, 2021; Liang *et al.*, 2025). The above finding suggests that AgNPs agents hold great promise in formulating potent antimicrobial agents.

Pro's free radical scavenging and antioxidant potentials were previously established and their character concentration depended (Islam *et al.*, 2025) and here highly responsible for the preservation of fish fillets from lipid rancidity and protein degradation. The bioactivity of Pro mainly stems from its components with bioactive phytochemicals (e.g., phenolics compounds, volatile aromatics, flavonoids and terpenes) (Waktole *et al.*, 2025; Santos *et al.*, 2025). The antimicrobial property (against various bacteria, yeast, parasites and molds) and antioxidant activity of many Pro polyphenols has been reported (Islam *et al.*, 2025; Santos *et al.*, 2025). Chrysin flavone, is present in the phytochemicals of Pro which possess strong anti-microbial, anti-oxidative, and anti-cancer capabilities (Waktole *et al.*, 2025); the antibacterial potentiality of chrysin is related to its activity on the bacterial cell walls/membranes disruption. Many other Pro polyphenols (e.g., p-coumaric acid, caffeic acid and ferulic acid) have demonstrated their interference in the DNA synthesis pathway of microbic and cancer cells (Santos *et al.*, 2025). Genistein is another important isoflavonoid component found in Pro which is highly effective against microbic infection (from mycotic and bacterial origin), cancers and inflammation by means of their strong anti-oxidative and chemotherapeutic potentiality (Zuorro *et al.*, 2021; Liang *et al.*, 2025). Abundantly present also in Pro, pinocembrin is another flavonoid which has demonstrated to possess several pharmacological activities like anti-inflammatory, anti-oxidative and widely effective antibacterial characteristics (Islam *et al.*, 2025; Waktole *et al.*, 2025). Also contained in Pro are the glycolic acid, glycerol and vanillin which are used in the formulation of various food and cosmetic products as anti-bacterial, anti-aging, and anti-oxidative agent respectively (Al-saggaf, 2021; Zuorro *et al.*, 2021). Pro and its compounds could be capable of eliminating bacteria through various means such as; by direct interaction with the cell wall and constituents of bacteria or by altering the host cell's immunity (Santos *et al.*, 2025). Among the identified pathways for Pro activity, cell division, proteins and nucleic acids synthesis inhibition, disruption of cytoplasmic membrane function and permeability, bacteriolysis, decreasing bacteria resistance and biofilm formation, and inhibition of energy production pathway (Islam *et al.*, 2025). It was also found that Pro-AgNPs exhibit other antibacterial and antioxidative properties as compare to those pure substances (Santos *et al.*, 2025).

Polymeric structure and decrease in particles size of BChT enhance its antibacterial and antioxidative action to fabricate formulations, particularly after combination of Pro, BV and AgNPs. It

has been found that CS NPs are beneficial in developing potent antimicrobials in conjugation with different plant extracts and nanomaterials due to enhancement of both microbicidal, anti-oxidative and surface limiting activities (Al-saggaf, 2021; Zuorro *et al.*, 2021; Liang *et al.*, 2025). Food spoilage factors either internal (like lipid oxidation, microbiological load, and enzymatic action) and external (like free radicals and O₂, external contamination) were protected from spoilage caused by these factors due to preparation of nanocomposited formulations of CS NPs that were recommended for food products. It was also discovered that this would give more reasons to utilize this nature based and biosafe nano components especially after conjugation with Pro and BV substances (Akdaşçı *et al.*, 2025; Marshall and Wattanaphonpinich, 2026).

5. Conclusions

The fabricated BCht from bees' wastes, and the biosynthesized AgNPs by Pro and BV were obtained. The size of BCht, Pro mediated AgNPs and BNCT/BV/Pro/AgNPs was 139.85 nm, 6.82 nm and 182.36 nm respectively. All synthesized agents/ composites were convincingly demonstrated effective in vitro antibacterial activities against resistant foodborne pathogens (*Salmonella typhimurium* and *Staphylococcus aureus*). The most active was toward all bacterial strains that synthesized compound was BNCT/BV/Pro/AgNPs. The fabricated nanocomposites made by the above produced materials showed effective bacteria destroying and inhibition on bacterial cells. This work shows that it can be excellently proposed that it can be successfully developed in to new kinds of therapeutics for healthcare as antimicrobial approaches.

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