Supplementary Information

Antibacterial Efficacy of Low-Dosage Silver Nanoparticle-Sodium Alginate-Chitosan Nanocomposite Films Against Pure and Clinical Acne Strains

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Supplementary Methods

1. Identification of bacterial strain

Identification of bacterial strains was conducted according to the Vietnam government's guidelines for clinical microbiology techniques and the specific microbiological testing characteristics of *C. acnes* [1, 2]. Clinical bacteria were collected and stored in 20 mL of Brain Heart Infusion (BHI) to maintain an anaerobic environment for *C. acnes*, while other bacteria were stored aerobically [1]. All microbiological testing adhered strictly to these government guidelines.

Gram staining procedure [3]**:**

1. Smear bacteria onto a slide and tilt it at a 45° angle.
2. Coat the bacterial smear with gentian violet for 10 seconds, then rinse with gently running water.
3. Fix the smear with Lugol's iodine for 20 seconds, and wash briefly with 90% alcohol for 5 seconds.
4. Rinse the slide again with water to remove any remaining alcohol.
5. Cover the smear with safranin red for 10 seconds, rinse with water, and blot the slide dry.
6. Observe the slide under a microscope at 4x magnification. Then, add one drop of mineral oil to the slide and observe under the 100x objective lens.
7. Record the type of bacteria and their Gram stain results.

Catalase Test [1]**:**

1. Take a few bacterial colonies and place them on a slide.
2. Add a drop of 3% H_2O_2 (hydrogen peroxide) onto the bacterial colonies.
3. Observe for the formation of bubbles, which indicates a positive catalase reaction (+).

Coagulase 10s-Test [1]**:**

1. Apply a drop of sterile water onto a slide.
2. Collect a few bacterial colonies and mix them with the sterile water to create a bacterial suspension.
3. Add a small amount of plasma to the suspension and mix well.
4. Observe within 10 seconds. The appearance of white particles indicates a positive coagulase reaction (+).

Cytochrome Oxidase Test [1]**:**

1. Use a sterile loop to collect a bacterial colony.
2. Spread the colony on an oxidase test strip or paper plate impregnated with 1% Tetramethyl-p-phenylenediamine.
3. A positive test is indicated if the paper turns purple-black within 10-20 seconds.

Biochemical Activity Test on Kligler Iron Agar (KIA) Medium [4]:

1. Use a sterilized straight inoculation rod to collect bacteria from a prepared Eppendorf tube (0.5 McFarland standard).
2. Inoculate the agar by streaking the slant surface in a zig-zag pattern.
3. Insert the inoculation rod vertically into the agar to the bottom.
4. Incubate the inoculated agar at 37°C.
5. Read the results:
   - Lactose Fermentation (-): The slant is red and the butt is yellow.
   - Lactose Fermentation (+): Both the slant and butt are yellow.
   - No Fermentation: Both the slant and butt are red.
   - H_2S Production (+): Black precipitate appears in the agar.
   - Gas Production (+): Formation of bubbles or cracks in the agar indicates gas production.

Activity Test on Sulfur, Indole, Motility (SIM) Medium [5]:

1. Use a sterilized straight inoculation rod to collect bacteria from a prepared Eppendorf tube (0.5 McFarland standard).
2. Insert the inoculation rod straight into the center of the SIM agar without touching the bottom.
3. Incubate the inoculated medium at 37°C.
4. Read the results:
   - H_2S Production (+): Black precipitate appears in the test tube.
   - Indole Production (+): A red ring forms at the surface of the medium after adding Kovac's reagent.
   - Motility (+): Growth radiates from the inoculation line, indicating bacterial movement.

Gelatin Liquefaction Test [6]:

1. Use a sterilized straight rod to collect bacteria from a prepared Eppendorf tube (0.5 McFarland standard).
2. Insert the inoculation rod straight into the center of the gelatin medium without touching the bottom.
3. Incubate the inoculated gelatin medium at 37°C.
4. Determine the result: Gelatin liquefaction is positive (+) if the gelatin medium has liquefied after incubation.

Nitrate Reduction Test [1]:

1. Grow the bacteria in a medium containing 0.1% potassium nitrate.
2. After incubation, add 0.1 ml of a reagent solution prepared by mixing solution A (8g Sulfanilic acid in 1 liter of acetic acid) and solution B (6ml N,N-dimethyl-1-naphthylamine in 1 liter of acetic acid) in a 1:1 ratio.
3. A positive result (+) is indicated by the solution turning red within a few minutes.

Other Biochemical Tests [1, 7]:

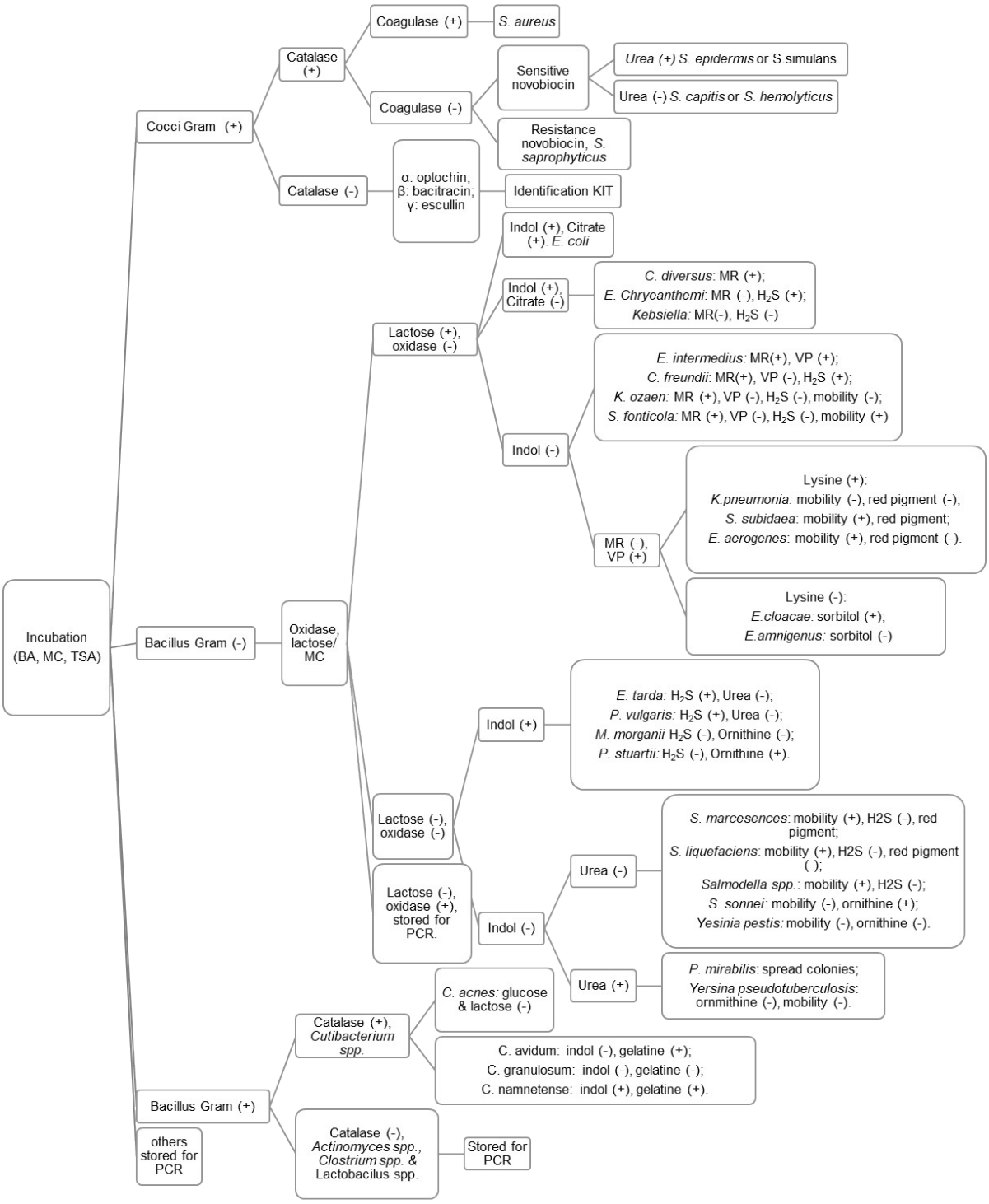
- Citrate Test: Inoculate the bacteria onto citrate agar. A positive result (+) is indicated by a color change from green to blue.

- Urea Test: Inoculate the bacteria into urea broth. A positive result (+) is indicated by a color change from yellow to red.
- Voges-Proskauer (VP) Test: Conduct the VP test to detect acetoin production. A positive result (+) is indicated by the development of a red color.
- Methyl Red (MR) Test: Inoculate the bacteria in MR-VP broth and add methyl red indicator. A positive result (+) is indicated by a color change from yellow to red.
- Lysine Decarboxylase Test: Inoculate the bacteria in lysine decarboxylase broth. A positive result (+) is indicated by the retention of the purple color.

Antibiotic Disk Diffusion Tests:

- Perform the agar disk diffusion method using antibiotic disks for Novobiocin, Bacitracin, and Optochin.
- Measure the diameter of the zone of inhibition around each disk.
- Sensitivity criteria:
- Novobiocin: Sensitive if the zone diameter is ≥ 16 mm.
- Optochin: Sensitive if the zone diameter is ≥ 14 mm.
- Bacitracin: Sensitive if a zone of inhibition is present.

Graph S1 below shows the implementation instructions of the biochemical tests to identify the bacterial strains.



Graph S1. Biochemical identification of bacterial strains.

2. Qualify phase of antibacterial activity test

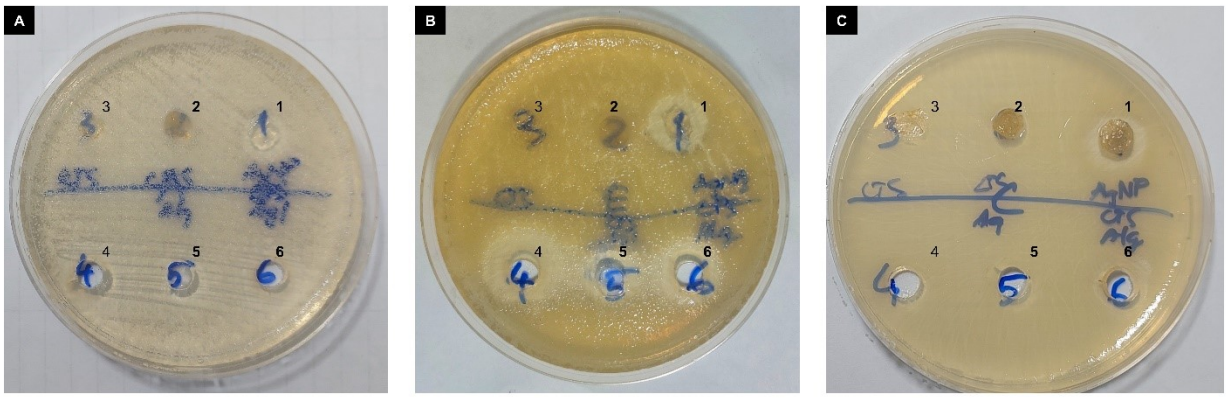


Figure S1. Quantify the antibacterial activity of the nanomaterials on bacterial strains.

Quantify the antibacterial activity of the nanomaterials on bacterial strains of (A) *S. aureus*, (B) *E. coli*, (C) *C. acnes* at (1) Silver nanoparticles-sodium alginate-chitosan film (2) Sodium alginate-chitosan film, (3) Chitosan film, (4) Chitosan solution, (5) Sodium alginate-chitosan solution, (6) Silver nanoparticles-chitosan solution.

Before determining the minimum inhibitory concentration (MIC) for application in antibacterial activity, it is essential to ensure that the material can inhibit bacterial growth during incubation [1]. Figure S1 demonstrates that the synthesized nanomaterial exhibits significant antibacterial activity. Building on these initial results, we proceeded with further testing to determine the MIC of the silver nanoparticles, the silver nanoparticle-chitosan solution, and the silver nanoparticle-sodium alginate-chitosan nanocomposite film.

3. Quantify phase of antibacterial activity test

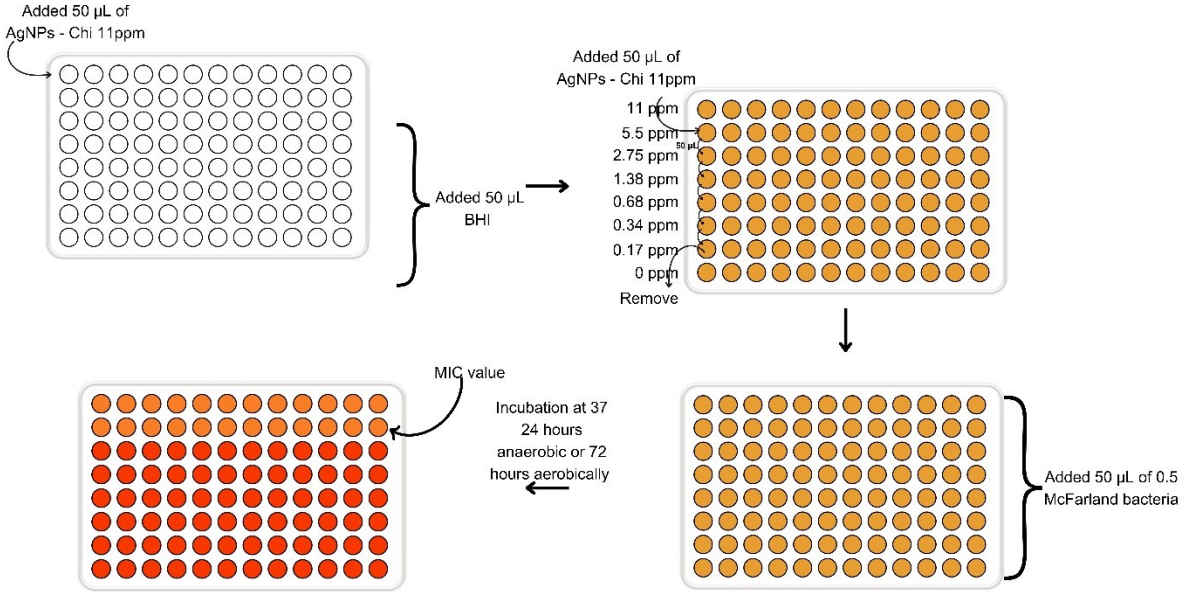


Figure S2. The serial dilution method uses 96-well agar plates to determine the minimum inhibitory concentration (MIC).

The serial dilution method on 96-well agar plates [8] to determine the minimum inhibitory concentration (MIC) was conducted in four main steps:

1. Preparation of Positive Control and Broth Addition:

- Added 50 µL of 11 µg/mL silver nanoparticles or silver nanoparticle chitosan solution into the first row (Row A) of all 12 columns (positive control).
- Added 50 µL of BHI broth for bacterial growth to each well in Row A.

2. Serial Dilution:

- Added 50 µL of 11 µg/mL silver nanoparticles or silver nanoparticle chitosan solution into the second row (Row B).
- Mixed the solution, then removed 50 µL from Row B, creating a 5.5 µg/mL mixture (2-fold decrease in concentration).
- Repeatedly transferred 50 µL of the mixed solution from each subsequent row to the next, up to Row G, ensuring each row has a 2-fold decrease in concentration.
- Row H served as the negative control.

3. Bacterial Inoculation: Added 50 µL of a 0.5 McFarland concentration of bacteria into all wells.

4. Incubation: Incubated at 37°C for 24 hours under anaerobic conditions or 72 hours aerobically.

After incubation, the MIC value was determined as the lowest concentration at which no turbidity increase was observed in the wells.

4. General and clinical characteristics of the study population

Our study involved 65 acne patients, and 26.15% of the patients had C. acnes isolated (Table S1).

Table S1. Clinical characteristics of the study population.

Patient characteristic		Total (n = 65)		Patient samples with *C. acnes* growth (n = 17)		Patient samples without *C. acnes* growth (n = 48)		p[a]
		n	%	n	%	n	%	
General characteristics								
Aged, mean ± SD (years)		19.86 ± 4.05		19.24 ± 3.15		20.08 ± 4.33		0.346[b]
Female		36	55.4	9	52.9	8	47.1	0.814
Residence	City	30	46.2	10	58.8	20	41.7	0.223
	Countryside	35	53.8	7	41.7	28	58.3	
Clinical characteristics								
Length of illness (month), median (IQR)		12 (4 – 24)		6 (4 – 42)		12 (3 – 24)		0.844[c]
Symptoms	None	15	23.1	6	35.3	9	18.8	0.164
	Pain	32	49.2	7	41.2	25	52.1	0.440
	Hot	18	27.7	4	23.5	14	29.2	0.655
	Itchy	38	58.5	8	47.1	30	62.5	0.267
	Others	2	3.1	0	0.0	2	4.2	0.393
Number of symptoms	0	15	23.1	6	35.3	9	18.8	0.595
	1	19	29.2	5	29.4	14	29.2	
	2	23	35.4	4	23.5	19	39.6	
	3	7	10.8	2	11.8	5	10.4	
	4	1	1.5	0	0.0	1	2.1	
Skin condition	Oily	36	55.4	10	58.5	26	54.2	0.825
	Dry	15	23.1	3	17.6	12	25.0	
	Sensitive	0	0.0	0	0.0	0	0.0	
	Mixture	14	21.5	4	23.5	10	20.8	
Acne type	Whiteheads	53	81.5	14	82.4	39	81.3	0.920
	Blackheads	32	49.2	9	52.9	23	47.9	0.722
	Papules	30	46.2	24	50.0	6	35.3	0.296
	Pustules	48	73.8	36	75.0	12	70.6	0.722
	Nodules	7	10.8	1	5.9	6	12.5	0.449
	Pseudocysts	1	1.5	1	5.9	0	0.0	0.090
Number of acne-type	1	6	9.2	2	11.8	4	8.3	0.925
	2	28	43.1	8	47.1	20	41.7	
	3	15	23.1	3	17.6	12	25.0	
	4	15	23.1	4	23.5	11	22.9	
	5	1	1.5	0	0.0	1	2.1	
Acne location	Face	64	98.5	17	100.0	47	97.9	0.549
	Chest	18	27.7	7	41.2	11	22.9	0.148
	Neck	8	12.3	3	17.6	5	10.4	0.436
	Dorsum	23	35.4	8	47.1	15	31.3	0.241
Sequelae	None	38	58.5	12	70.6	26	54.2	0.238
	Pigmentation changes	22	33.8	4	23.5	18	37.5	0.296
	Scar	4	6.2	2	11.8	2	4.2	0.263
	Keloids	7	10.8	0	0.0	7	14.6	0.096

Karen McCoy degree	Mild	38	58.5	10	58.8	28	58.3	0.588
	Moderate	19	29.2	6	35.3	13	27.1	
	Severe	8	12.3	1	5.9	7	14.6	
Medical history	None	23	35.4	10	58.8	13	27.1	0.019*
	Self-treatment	7	10.8	2	11.8	5	10.4	0.878
	Privacy Healthcare	16	24.6	1	5.9	15	31.3	0.037*
	Public Healthcare	17	26.2	4	23.5	13	27.1	0.774

*SD: Standard deviation, IQR: Interquartile range; p: 2-tailed significant; [a]: Pearson Chi-squared test; [b]: Independent Samples T-test; [c]: Mann-Whitney U test; *: $p < 0.05$.*

Our study population primarily consisted of females aged 13-24, with an average age of 19.28 ± 4.05 years (Table S1). The general characteristics observed were similar to those reported by Giavina-Bianchi *et al.* (2022) [9] and Skroza *et al.* (2018) [10]. In Skroza *et al.*'s (2018) study of 1,167 patients, the majority population was female (85%). Giavina-Bianchi *et al.* (2022) [9] assessed 2,459 acne patients, with the majority being female (69.3%) and aged between 13-24 (67.2%). In our study, patients from urban areas accounted for 46.2%, while those from rural areas made up 53.8%. Regarding previous medical treatment, 35.4% had no prior treatment, 10.8% practiced self-treatment, 26.2% received public healthcare, and 24.6% used private healthcare. The medical history of previous treatment differed from Giavina-Bianchi *et al.* (2022) [9], likely due to differences in healthcare access and population characteristics.

The most common clinical symptoms in our study were itching (58.5%) and pain (49.2%) (Table S1), with an average acne duration of approximately 12 months. According to Karen McCoy's acne severity scale, the majority of patients had mild acne (58.5%), and 98.5% of the patients had facial acne (Table S1). Oily skin was the most prevalent (55.4%), followed by dry skin (23.1%) and combination skin (21.5%). The types of acne observed were diverse, with whiteheads being the most common (81.5%), followed by pustules (49.2%), papules (46.2%), nodules (10.8%), and cysts (1.5%) (Table S1). The present clinical characteristics were consistent with those reported by Giavina-Bianchi *et al.* (2022) [9] and Skroza *et al.* (2018) [10]. Giavina-Bianchi *et al.* (2022) noted that the majority of acne was located on the head and neck (86.1%), with mild (28.1%) and moderate (53.5%) severity [9]. Similarly, Skroza *et al.* (2018) found that the majority of their 1,167 patients had mild acne [10]. Thus, our study population shares similar characteristics with these previous studies, supporting its use as a reference demographic for other acne research.

5. Antibacterial activity of silver nanoparticles-sodium alginate-chitosan nanocomposite films

Figure S3 illustrates examples of the quantified antibacterial tests of the AgNPs and Ag-Chi solutions conducted using the serial dilution method. All MIC values have been calculated, and analyzed, and are presented in Figure 8 of the manuscript.

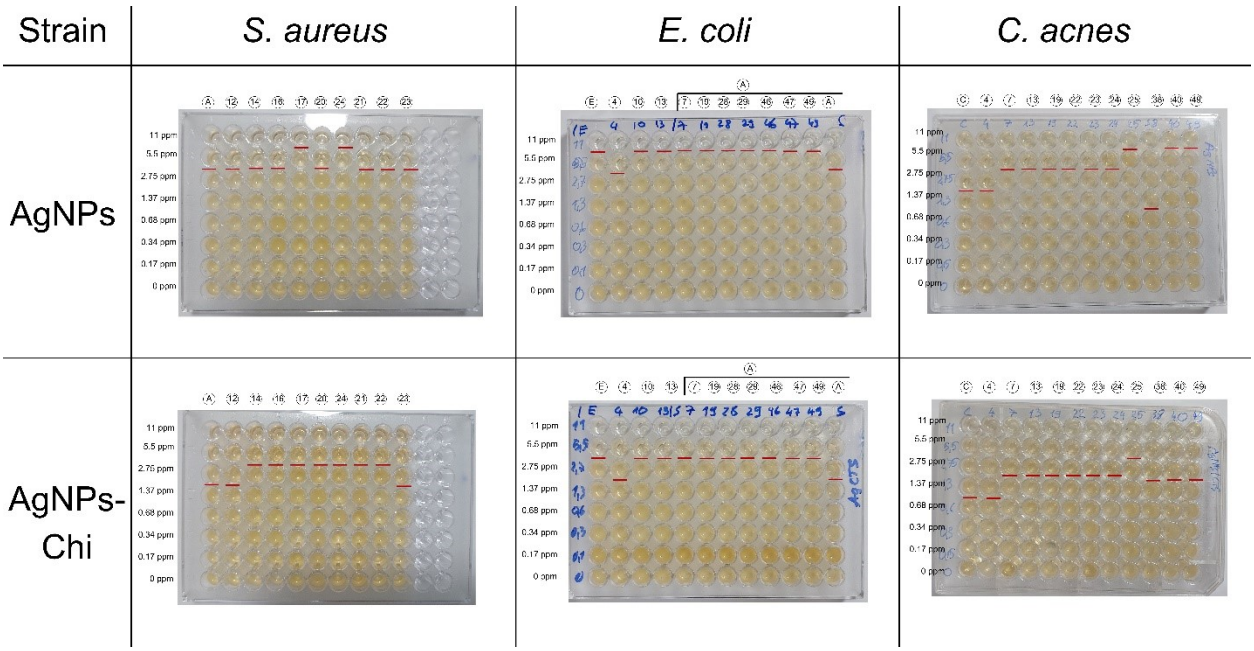


Figure S3. Quantify the minimal inhibitory concentration (MIC) of silver nanoparticles-, and silver nanoparticles-chitosan- solutions through the serial dilution method.

AgNPs: silver nanoparticles solution, AgNPs-Chi: silver nanoparticles chitosan solution, A: pure strain of S. aureus, E: pure strain of E. coli, C: pure strain C. acnes, No.: patient's number that collected the bacterial, redline: MIC value of samples.

Figure S4 showcases the quantified antibacterial tests of silver nanoparticle-sodium alginate-chitosan nanocomposite films using the Kirby-Bauer disc diffusion method. The MIC values have been recorded, analyzed, and are presented in Table 2 of the manuscript.

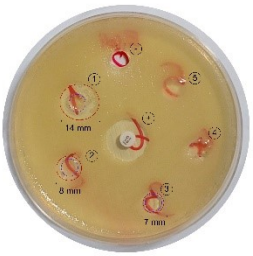
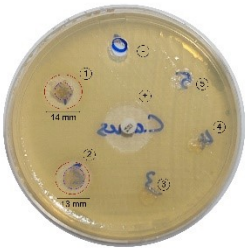
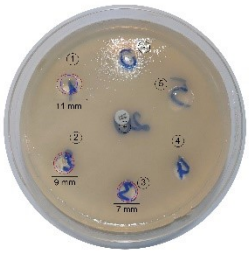
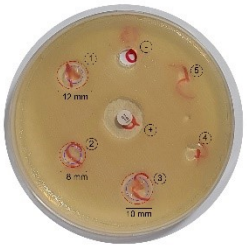
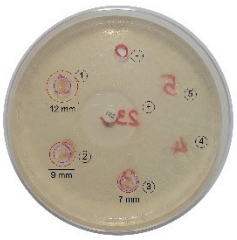
Strain	*S. aureus*	*E. coli*	*C. acnes*
Pure			
Clinical			

Figure S4. Quantify the minimal inhibitory concentration value of silver nanoparticle-sodium alginate-chitosan nanocomposite films through the Kirby-Bauer disc diffusion method.

S. aureus: Staphylococcus aureus, E. coli: Escherichia coli, C. acnes: Cutibacterium acnes, +: positive control, -: negative control, blue circles: 6mm cut of the films, red circles: MIC value and the sterile diameter ring.

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