ANTIMICROBIAL AND PROTECTIVE EFFECTS OF PURIFIED CHITOSAN AND A STANDARDIZED PROPOLIS FRACTION AGAINST *Staphylococcus aureus* INFECTION IN WISTAR RATS

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Abstract: The increasing risk of antibiotic-resistant *Staphylococcus aureus* strains has fueled the quest for new and sustainable antimicrobial therapies. In this work, the *in vivo* antimicrobial activity of chitosan obtained from the shell of crustaceans, and propolis alone and combined, against *Staphylococcus aureus* (ATCC 6538) using a Wistar rat infection model was explored. A total of 35 female Wistar rats (three months old) were randomly assigned to seven groups (n = 5), including untreated controls, infected controls, and groups treated with various concentrations of chitosan (0.5%, 1%, 2%), propolis (0.5%, 1%, 2%), and their combination. Following a two-week acclimatization period, all treatments were administered orally via gavage for 15 days. Quantitative microbiologic analysis indicated a marked reduction in bacterial load after as early as 48 hours of treatment, particularly in the chitosan-propolis combination group, which showed enhanced antimicrobial activity compared with the other treated groups. Hematologic and histopathologic analysis also indicated improvement in immune parameters and healing of tissue in the treatment groups. These findings underscore the therapeutic potential of chitosan and propolis as natural bioactive compounds. The combined treatment may offer a promising strategy for combating bacterial infections, including drug-resistant strains, and warrants further mechanistic and clinical investigations

Keywords: antibiotic resistance; chitosan; natural antimicrobials; propolis; *Staphylococcus aureus*; Wistar rat model.

INTRODUCTION

Food safety is a major public health issue for all countries. Food-borne diseases, caused by microbial pathogens, biotoxins or chemical pollutants present in food, represent a serious threat to the health of millions of people around the world. Numerous epidemics linked to the consumption of contaminated food have been recorded on every continent in recent decades, highlighting their considerable impact on public health and society as a whole.

These epidemics, although alarming, are often only the visible part of a much wider problem. Food-borne diseases not only affect the health and well-being of populations, but also have significant economic consequences for individuals, families, communities, businesses and nations. Despite the progress made in the field of food safety, millions of cases of food-borne illnesses are still reported every year.

The situation is all the more worrying given that the growing emergence of antimicrobial resistance among the bacteria responsible for these infections is complicating conventional therapeutic strategies. This problem has prompted the scientific community to explore natural alternatives to traditional antibiotics. These promising alternatives include chitosan and propolis, two natural substances with well documented antimicrobial properties. Chitosan, a linear polymer produced by partial deacetylation of chitin, consists of D-glucosamine and N-acetyl-D-glucosamine units linked by β-(1→4) bonds, a structure similar to cellulose [27, 30]. Chitosan is renowned for its biocompatibility, biodegradability, safety, and broad spectrum of antimicrobial activity [12, 21]. Various studies have reported its cell wall disruption ability against bacteria and therefore its serious potential as an alternative to conventional antibiotics [10, 30, 33].

The mechanism of action of chitosan against bacteria and fungi has been well described in the literature. Liu *et al.* [19], Raafat *et al.* [29], and Ramya *et al.* [31]demonstrated its interaction with microbial membranes. Following studies confirmed these effects and also detailed the molecular mechanisms [15, 29, 34] and its activity against a broader range of microorganisms [11, 15, 40].

Propolis is a resinous compound collected by bees from tree buds and utilized for consolidation of the hive structure. Propolis is well known for its various biological activities, including antibacterial and anti-inflammatory properties [3, 4, 13, 35]. Propolis, being rich in bioactive molecules, is a strong natural antimicrobial agent [17, 26, 28]. The combination of chitosan and propolis is a particularly promising synergistic approach for combating resistant pathogens [36]. The combined effect of the membrane disrupting action of chitosan and the bioactive properties of propolis makes it possible to develop a multifunctional defence strategy against microbial infections. The aim of this study is to assess the therapeutic effect of this natural combination against *Staphylococcus aureus* (ATCC 6538), a bacterial strain responsible for serious food poisoning. Based on the antimicrobial and hepatoprotective effects of chitosan and propolis, this research offers a potential alternative to conventional antibiotic treatments.

MATERIALS AND METHODS

Materials

The shrimp wastes originating from multiple fisheries in Tlemcen province (Algeria), which we have collected, consists of shrimp shells that served as the raw material for chitin and chitosan extraction, Samples of raw propolis were gathered from beekeepers in Tlemcen

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(Bnisnous region). The samples were collected during autumn and were stored at a temperature of -4°C.

Methods

Preparation of propolis extracts

One gram of powdered propolis was sonicated with 100 mL of 70% ethanol in the dark. The mixture was sonicated using a probe sonicator (UltraAutosonic, India; model Ultrasonic Atomizer Probe Sonicator, capacity 100-500 mL) with a titanium probe at a frequency of 20 kHz. Sonication was performed for 15 minutes in a water bath of 25±2 °C (cold extraction condition). Following sonication, the extract was centrifuged for 10 minutes at 4000 rpm. The supernatant, containing the bioactive compounds, was carefully separated from the sediment and subsequently filtered through Whatman No. 4 filter paper to remove residual solid particles and obtain a purified solution. The propolis sample was re-extracted two more times using the same conditions to maximize the recovery of bioactive compounds from the raw propolis [23].

LC-MS/MS analyses

Samples of propolis extracthaving 50 mg were transferred into Eppendorf having 2 mL. The resulting solution was mixed with 2 mL of ethanol and stirred. The formed mixturewas extracted using hexane. There after, the last mixture was centrifuged and stirred at 9000 rpm for 10 min.LC–MS/MS analysis was carried out by (Agilent Technologies 1260 Infinity II, 6460 Triple Quad Mass spectrometer). Poroshell 120 SB-C18 (3.0 × 100 mm, I.D., 2.7 µm) column was employed. A 100 µL sample was taken from methanol phases in the resulting solution and diluted to 900 µL as 450(water)/450(methanol). In the end, the resulting last sample was filtered and analyzed by LC/MS-MS operating at 5.12 µL injection volume, 0.400 mL/min flow rate 30 min method time and at 40°C.

Composition and physical and chemical characteristics of propolis

Propolis usually contains a variety of chemical compounds. A complete inventory of its substances would be tedious. However, we should mention polyphenols (flavonoids, phenolic acids, and their esters), terpenes, steroids, and amino acids [9, 21]. Their abundance is influenced by geographic factors, as well as the collection season. [18]. Propolis from the Tlemcen region is similar to standard Nigra poplar propolis, with the presence of flavanones (pinocembrin, pinostrobin, etc.), flavones (chrysin, apigenin, etc.) and flavonols (galangin, kaempferol, etc.) with high percentages [32].

Extraction of chitosan from shrimp waste

The extraction of chitosan from crustacean shells involves a complex process of 3 steps which are demineralization, deproteinization, and deacetylation [41].Demineralization 100g of dry sample (weighed through assay balance) was treated with 2N HCl solution. The solid-to-solvent ratio is 1:15 (w/v) at 50°C under constant agitation for 30 minutes. The demineralization step was tailed by rinsing with distilled water until all the neutrality was reached deproteinization. The demineralized sample was treated with 50% NaOH 1:10 (w/v) alkaline solution for 1 hour at a high temperature of 90°C. The deproteinized chitin product was rinsed many times to achieve a neutral pH. Deacetylationchitosan is obtained through the deacetylation of chitin. This process is typically carried out in a 50% NaOH solution at a temperature of 121°C for 30 minutes using an autoclave. After being acquired, the chitosan is filtered to remove it from the mixture. The sodium hydroxide (NaOH) is then rinsed away with distilled water until the pH of the wash water is neutral. Then washing and filtration are conducted to eliminate any residual impurities or acid residues.The molecular weight of chitosan was found to be 2.81×10^4 g/mol, and the degree of deacetylation (DD) was 75%. These physicochemical parameters are known to strongly influence the biological and antimicrobial properties of chitosan.

Propolis -chitosan mixture preparation

The chitosan solution was prepared by dissolving 2 g of the chitosan powder in 100 mL of 1% (v/v) acetic acid with continuous stirring. The final concentration of the chitosan solution was then mixed with the ethanolic propolis extract in a ratio of 1:1 (v/v) to obtain the chitosan-propolis mixture. Ethanol was completely removed under reduced pressure before application, and the resulting solution was kept at 4 °C. For *in vivo* application, the blend was equivalent to end concentrations of 1% (w/v) propolis and 1% (w/v) chitosan.

Animals

This study was conducted with ethical clearance obtained from the Health Research Ethics Commission, Faculty of Nature and Life Sciences, and Earth and Universe Sciences, Algeria. Healthy rats were sourced from the Pasteur Institute of Algiers, Algeria. The breeding of these animals took place at the animal facility of the Department of Biology, Faculty of Natural and Life Sciences, Earth and Universe Sciences, Abou Bekr University (Tlemcen, Algeria).

The animals were kept under controlled conditions: the temperature ranged from 18°C to 22°C, ambient humidity was maintained between 40% and 70%, and there was sufficient ventilation. The animals were kept on a 12 hour light/dark cycle, and they had free access to food and water.

Experimental groups

A total of 35 female Wistar rats were randomly assigned into seven groups (n = 5 per group). After a two-week acclimatization period, treatments were administered daily by oral gavage using a feeding probe for 15 days. Body weight was recorded daily throughout the experimental period.

- **G1 (chitosan toxicity):** rats received chitosan solutions at concentrations of 0.5%, 1%, and 2%.
- **G2 (propolis toxicity):** rats received propolis solutions at concentrations of 0.5%, 1%, and 2%.

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- **G3 (infected + chitosan):** rats were inoculated intragastrically with *Staphylococcus aureus* (ATCC 6538) at a dose of 0.5 mL of 1×10^9 CFU/mL suspension in sterile saline. Treatment with chitosan was initiated 24-h post-infection.
- **G4 (control):** rats received no treatment.
- **G5 (infected + propolis):** rats were inoculated as in G3 and treated with propolis, starting 24-h post-infection.
- **G6 (infected + chitosan + propolis):** rats were inoculated as in G3 and treated with a combination of chitosan and propolis, starting 24-h post-infection.
- **G7 (infected untreated):** Rats were inoculated as in G3 and left untreated to evaluate the pathogenic effect of *Staphylococcus aureus* (ATCC 6538).

The chosen inoculum 1×10^9 CFU/mL, 0.5 mL per rat, via oral gavage was selected based on previous reports where oral administration of *S. aureus* at this level successfully induced reproducible gastrointestinal/systemic colonization while maintaining animal survival [22]. The 24-h interval before treatment was applied to allow establishment of infection.

Toxicity test

The subacute oral toxicity of chitosan, propolis, and their combination was evaluated following the OECD guideline 407 (repeated dose 28 day oral toxicity study in rodents). Female Wistar rats (n = 5 per group) received daily oral gavage of the test solutions (0.5%, 1%, and 2%) for 28 consecutive days. During the experiment, animals were observed daily for mortality and clinical signs including changes in general appearance, fur, posture, locomotor activity, piloerection, salivation, tremors, diarrhea, and abnormal behaviors. Body weight and food consumption were recorded every third day. At the end of the study, the rats were anesthetized and blood samples taken from the abdominal aorta in EDTA tubes were subjected to hematological analysis, which comprised white blood cell count, red blood cell count, hematocrit, and hemoglobin. Immediately after blood collection, organs such as the liver, heart, kidneys, and intestines were excised, washed with 0.9% NaCl physiological saline, and fixed in 10% formalin for histological examination. Histopathological examination was performed at the "Dr. Tidjani Damerdji" University Hospital Center of Tlemcen (CHU) Department of Pathological Anatomy, Algeria.

Statistical analysis

Microbiological results

Data were analyzed using SPSS software (SPSS 3.0; SPSS, Inc., Chicago, IL, USA). Results are expressed as mean ± standard deviation (SD). Comparisons between groups were performed using one-way ANOVA, followed by Dunnett's post hoc test to compare each treatment group with the control. A value of $p < 0.05$ was considered statistically significant.

Rat body weight evolution

The data were analyzed using a repeated-measures analysis of variance (ANOVA) to evaluate the effect of different treatments on rat body weight over time. A significance level of $P < 0.05$ was considered statistically significant.

RESULTS

Identification and quantification of phenolic compounds by LC-MS/MS analysis

LC-MS/MS analysis of the propolis extract exhibited a broad range of bioactive molecules, including phenolic acids, flavonoids and other polyphenols. They include, among others salicylic acid (0.651 mg/g), trans-ferulic acid (3.766 mg/g), vanillic acid (0.323 mg/g), and quercetin (0.192 mg/g). Catechin, caffeic acid, coumarin, chrysin, and other traces were also present, testifying to the complex chemical nature of the extract. Table 1 summarizes the contents, where the presence and relative amounts of 21 various compounds based on their retention times and concentrations are reported.

Table 1. Composition of propolis extractsby LC-MS/MS (mg/g).

Compound	RT	Amount (mg/g EP)
Gallic acid	3.718	0.006
Protocatechuic acid	6.077	0.021
Catechin	7.379	0.047
Hydroxybenzaldehyde	8.227	0.002
Vanillic acid	7.775	0.323
Caffeic acid	8.314	0.013
Caffeine	8.810	0.028
Vanillin	9.052	0.158
p-coumaric acid	9.756	0.006
Salicylic acid	9.821	0.651
trans-ferulic acid	10.302	3.766
Coumarin	11.266	0.089
Rutin	11.963	0.018
Isoquercitrin	11.561	0.006
Hesperidin	11.963	0.056
Kaempferol-3-glucoside	12.853	0.001
trans-cinnamic acid	13.946	0.022
Quercetin	14.327	0.192
Naringenin	14.472	0.043
Luteolin	17.833	0.015
Chrysin	17.699	0.034

Rat body weight evolution

Our findings (Table 2) show that chitosan mono therapy and chitosan plus propolis treatments helped to ensure body weight stability in infected rats. While all treatments failed to cause weight gain, they effectively prevented significant weight loss. GroupG3(chitosan) and group G5 (propolis) rats exhibited less weight loss compared with the untreated infection control group (G7), which implies a protective function. The group with combination (G6) maintained the most consistent body weight throughout the period of the study. The control (G4) and infection-only (G7) rats declined in weight consistently, which proved the impact of infection when there was no treatment.

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Table 2. The body weight measured every days during test period G1)5 rats treated with (0.5\1\2) % of chitosan,G2) 5 rats treated with (0.5\1\2) of propolisG3) 5 rats got contaminated by *Staphylococcus aureus* and treated with chitosan to test its antimicrobial activity,G4)control group,G5) 5 rats got contaminated by *Staphylococcus aureus* then treated with propolis to test its antimicrobial activity.G6) 5 rats got contaminated by *Staphylococcus aureus* then treated with chitosan/propolis,G7) 5 rats got contaminated by *Staphylococcus aureus.*

Day	G1 (Chitosan)	G2 (Propolis)	G3 (*S. aureus* + Chitosan)	G4 (Control)	G5 (*S. aureus* + Propolis)	G6 (*S. aureus*+ Chitosan+ Propolis)	G7 (*S. aureus* only)
0	245 ± 2	246 ± 3	247 ± 2	246 ± 2	247 ± 2	246 ± 3	248 ± 2
3	246 ± 2	247 ± 3	245 ± 2	247 ± 2	245 ± 3	245 ± 3	247 ± 3
5	248 ± 3	248 ± 3	243 ± 3	248 ± 2	244 ± 3	243 ± 3	245 ± 3
7	249 ± 2	249 ± 3	242 ± 3	249 ± 2	242 ± 3	241 ± 3	242 ± 3
9	250 ± 2	250 ± 3	241 ± 3	250 ± 3	240 ± 3	239 ± 3	238 ± 3
12	252 ± 2	252 ± 2	240 ± 3	252 ± 3	239 ± 3	238 ± 3	235 ± 3
14	253 ± 2	253 ± 2	239 ± 3	253 ± 3	238 ± 3	237 ± 3	234 ± 3

Results are expressed as mean ± standard deviations (SD) (n = 5).

Histopathological studies

Histopathological examination of *S. aureus* infected rats revealed several changes (fig.1, 2, 3). The liver had dilated centrilobular veins in certain sections and none in others. Edema, vascular congestion, and inflammatory infiltration with lymphocytes predominantly and a few polymorphonuclear cells were observed. Hepatocytes exhibited mild hydropic changes and necrosis of varying intensity, but atrophy was not observed. Kidney tissue also showed inflammation that was marked by lymphocytic infiltration, vascular congestion with thickened vessel walls and irregular tubular structures with narrowed lumens. The renal glomeruli had dilated spaces.

There were notable histological improvements in rats treated with chitosan. The liver tissue lacked edema, and the inflammatory infiltrate was minimal. The hepatic architecture and trabecular organization were maintained, without any necrosis or atrophy. Similarly, the renal tissue had reduced inflammation and less tubular lesions, but the cortical capsule and glomeruli remained unaffected. The structure of the intestine was also preserved, without atrophy or intense inflammation.

Rats treated with propolis post-infection also showed the same protection. The overall architecture of the liver, kidney, and intestine was maintained, with minimal infiltration but without congestion, edema, or atrophy. The status was slightly better than that of the chitosan alone group. Finally, the combination treatment of chitosan-propolis produced the best histopathology profile, with no noticeable inflammation and well-preserved structural integrity in all the organs examined.

These microscopic observations were corroborated by the semi-quantitative lesion scoring presented in Table 3. The table provides a comparative overview of the histopathological alterations across groups using a "–/ +" grading system. The infected untreated group showed the highest severity of lesions in all examined organs (+++ to ++++), whereas treatment with chitosan or propolis alone reduced the scores substantially (+/++). The combined chitosan-propolis treatment completely prevented lesion development (–), confirming its superior protective effect against *Staphylococcus aureus* induced tissue damage.

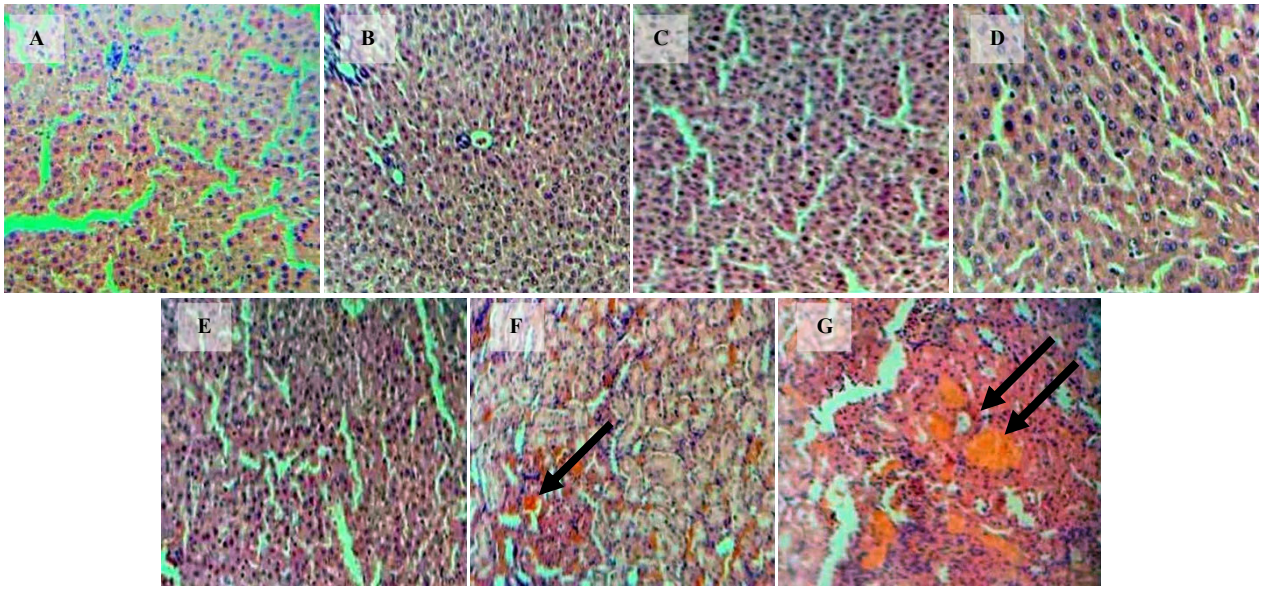

Figure 1. Histological structure of rat liver 10x view the 7 rats treated group: (A) rats treated by (0.5%, 1%, and 2%) of chitosan; (B) rats treated with (0.5%, 1%, and 2%) of propolis; (C)rats got contaminated by *Staphylococcus aureus* and treated with chitosan;(D) control group;(E) rats got contaminated by *Staphylococcus aureus* then treated with propolis; (F) rats got contaminated by *Staphylococcus aureus* and then treated with chitosan/propolis;(G) rats got contaminated by *Staphylococcus aureus*.

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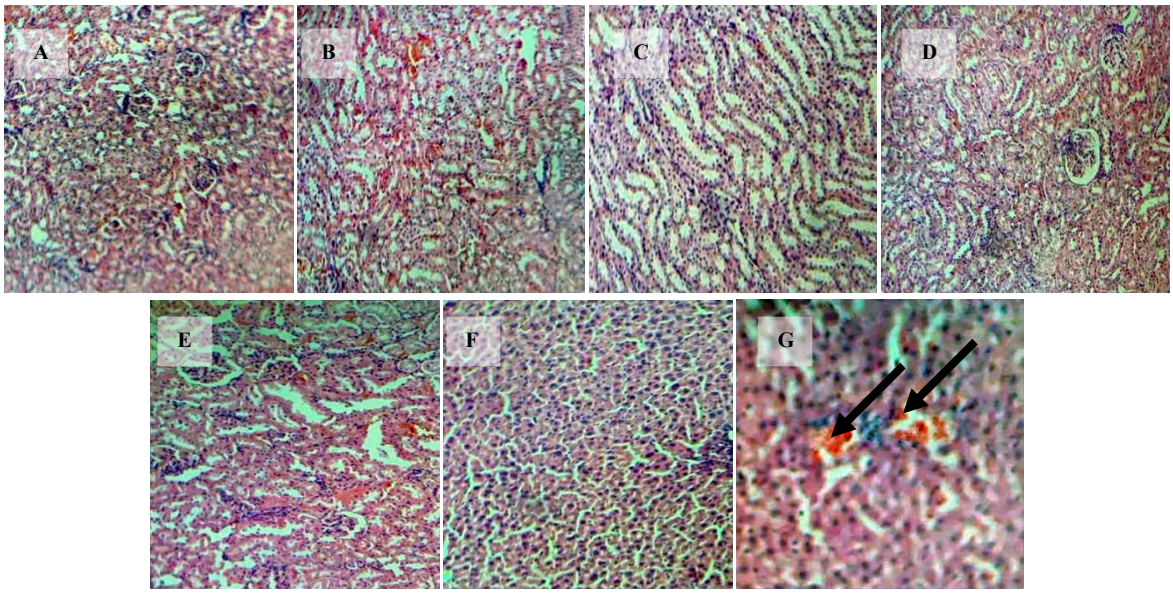

Figure 2. The histological structure of rat kidneys 10x view the 7 rats treated group: (A) rats treated by (0.5%, 1%, and 2%) of chitosan; (B) rats treated with (0.5%, 1%, and 2%) of propolis; (C)rats got contaminated by *S. aureus* and treated with chitosan;(D)control group;(E) rats got contaminated by *S. aureus* then treated with propolis;f) rats got contaminated by *S. aureus* and then treated with chitosan/propolis; (G)rats got contaminated by *S. aureus*.

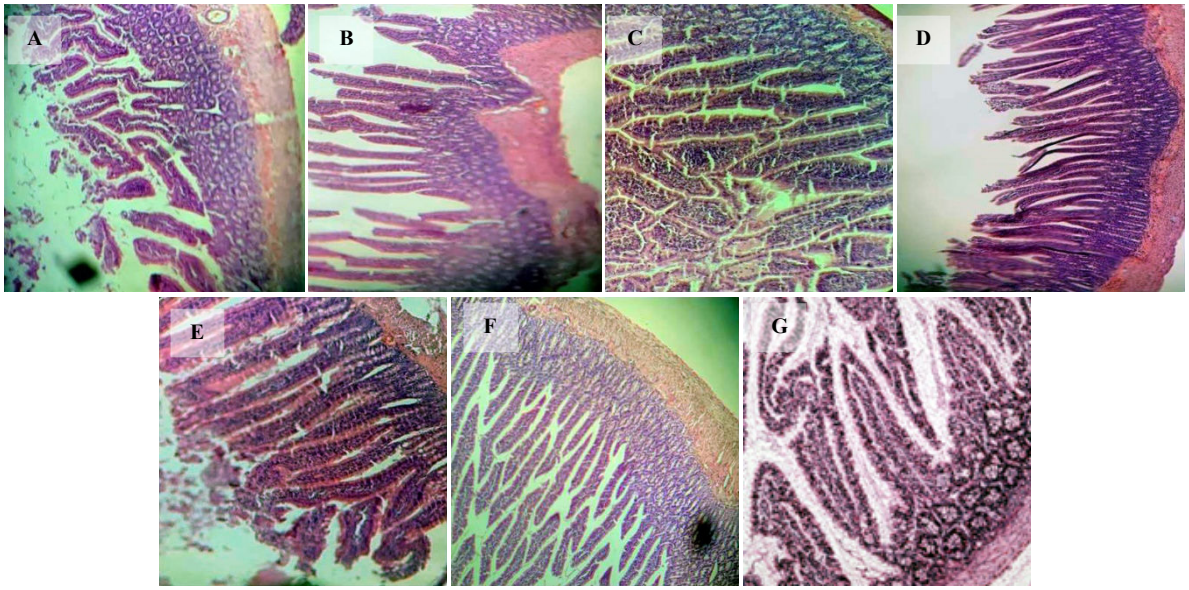

Figure3. The histological structure of rat intestine 10x view the 7 rats treated group: (A) rats treated by (0.5%, 1%, and 2%) of chitosan; (B) rats treated with (0.5%, 1%, and 2%) of propolis; (C)rats got contaminated by *S. aureus* and treated with chitosan; (D) control group;(E) rats got contaminated by *S. aureus* then treated with propolis;(F) rats got contaminated by *S. aureus* and then treated with chitosan/propolis;(G) rats got contaminated by *S. aureus*.

Table3. Semi-quantitative histopathological grading of liver, kidney and intestinal lesions in *Staphylococcus aureus*-infected rats following different treatments.

Group	Liver: congestion/edema	Liver: necrosis	Kidney: tubular inflammation	Kidney: glomerular dilatation	Intestine: villous atrophy	Global profile
G1 – Chitosan (0.5–2%)	-	-	±	-	-	-
G2 – Propolis (0.5–2%)	-	-	-	-	-	-
G3 – Infected + Chitosan	+	+	+	+	±	+
G4 – Control (healthy)	-	-	-	-	-	-
G5 – Infected + Propolis	±	-	-	-	-	-
G6 – Infected + CS + PP	-	-	-	-	-	-
G7 – Infected untreated	+++	++	+++	++	++	+++

Scoring was done as follows: none (-), mild (+),minimal alteration(±), moderate (++)severe (+++). The scoring is semi-quantitative and descriptive and was not subjected to statistical analysis.

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The antibacterial activity of *S. aureus*

The evolution of *Staphylococcus aureus* counts during the 10-day treatment period is summarized in Table 4. Bacterial loads were determined from fecal suspensions and expressed as CFU/mL. On day 1, all groups showed similar bacterial loads (≈ 5 log CFU/mL), confirming successful infection. In the untreated infected group (S), bacterial counts remained consistently high throughout the experiment, reaching 4.89 ± 0.12 log CFU/mL on day 6 and 4.60 ± 0.11 log CFU/mL on day 10.

In contrast, all treated groups (chitosan, propolis, and chitosan-propolis) exhibited a gradual reduction in bacterial counts starting from day 2. By day 6, the control infected group without treatment (S) had significantly higher bacterial levels (4.89 ± 0.12 log CFU/mL) compared with the chitosan group (4.39 ± 0.14 log CFU/mL, $P<0.05$), the propolis group (4.47 ± 0.14 log CFU/mL, $P<0.05$), and the chitosan-propolis group (4.43 ± 0.13 log CFU/mL, $P<0.05$).

At day 10, this trend persisted: the S group maintained a high bacterial load (4.60 ± 0.11 log CFU/mL), whereas the chitosan (3.77 ± 0.09 log CFU/mL), chitosan-propolis (3.82 ± 0.09 log CFU/mL), and propolis groups (4.17 ± 0.13 log CFU/mL) showed significantly lower counts indicating enhanced bacterial clearance. Among treatments, chitosan alone produced the strongest reduction, followed by chitosan-propolis, while propolis showed a moderate but still significant effect compared with untreated animals.

These results confirm the antimicrobial potential of chitosan and propolis, with chitosan being the most effective in reducing bacterial burden *in vivo*.

DISCUSSION

The presence of trans-ferulic acid, salicylic acid, and quercetin in propolis extract confirms its widely reported biological activities, including antioxidant, anti-inflammatory, and anticancer effects. These findings concur with previous research that attributed the health enhancing activity of propolis to its rich flavonoid and phenolic acid content [3, 24, 35, 37]. The presence of kaempferol-3-glucoside, even in traces (0.001 mg/g), also demonstrates the pharmacological significance of the extract due to its already proven anticancer and anti-inflammatory action.

The predominance by trans-ferulic acid (3.766 mg/g) would signify that it holds a central position in the therapeutic activity pattern of the extract, particularly for its antioxidant and anti-inflammatory actions. Similarly, the presence of salicylic acid (0.651 mg/g), a compound utilized widely in analgesic and anti-inflammatory medication [8, 24]explains the potential of propolis to manage inflammation and pain. Compounds such as vanillin, caffeine, and vanillic acid, present in notable amounts, also contribute to the antioxidant properties of the extract, similar to what has been described previously by Machado *et al.* [20].

Additionally, the discovery of flavonoids like quercetin, kaempferol, and chrysin confirms the anti-inflammatory and antimicrobial activity of the extract [26, 35]. The presence of various lignans and other polyphenolic compounds provides added depth to the therapeutic significance of the propolis extract. Overall, the comprehensive chemical profile revealed through this study positions propolis as a highly efficacious natural molecule with a wide range of health benefits, confirming and substantiating prior findings on its bioactive composition and pharmacological diversity.

Body weight stabilization of treated groups indicates that chitosan and propolis are working primarily as infection protection agents, rather than as weight gain agents. The results are in accordance with those found in the literature, Dai *et al.* [7],and Zhang *et al*. [42], where they reported that chitosan has no influence on weight gain in healthy animals but is able to reduce weight loss when the animals are under stress. In our study, chitosan (G3) slightly reduced weight, while propolis (G5) showed a slight increase in body weight which are supported by Kean & Thanou [16] and Punarvasu & Harish Prashanth [25]. Notably, the combined treatment group (G6) showed the most stable weight, with a possible synergistic effect being implied, as seen in work by Correa-Pacheco *et al.* [6]. The weight loss in control (G4) and infection-only (G7) groups also depicts the adverse effect of infection, augmenting the findings of Misawa *et al.* [22]. The capacity of both propolis and chitosan to avert infection induced weight loss also further cements their roles as protective agents. Overall, our results are consistent with the literature, supporting the fact that even if neither chitosan nor propolis has a growth stimulating effect, both are effective in antagonizing the negative effect of infection, possibly due to their antimicrobial and anti-inflammatory actions.A limitation of the present study is the small group size (n = 5), which restricts statistical power for detecting small to moderate effects. Post-hoc calculations indicate the study is powered mainly to detect very large effects (minimal detectable Cohen's d ≈ 1.77 for 80% power, $\alpha = 0.05$). For the key comparison at day 14 (G6 vs G7), the observed mean difference in

Table 4. Evolution of the microbial load of *S. aureus* during treatment for 10 days *In vivo* C: rats contaminated by *S. aureus* and treated with chitosan 1% , P/C: rats contaminated by *Staphylococcus aureus* and treated with chitosan /propolis(1%, 1%) , P: rats contaminated by *S. aureus* and treated with propolis 1% , S: rats contaminated by *S. aureus*.

Group	Day1	Day2	Day4	Day6	Day8	Day10
C	5.00 ± 0.150	4.68 ± 0.120	4.56 ± 0.130	4.39 ± 0.140	4.30± 0.100	3.77± 0.090
P/C	5.00± 0.170	4.65± 0.110	4.58 ± 0.115	4.43 ± 0.130	4.25± 0.105	3.82 ± 0.085
P	5.00 ± 0.180	4.74 ± 0.130	4.68 ± 0.140	4.47 ± 0.135	4.39 ± 0.115	4.17 ± 0.125
S	5.00 ± 0.140	4.95 ± 0.100	4.92± 0.110	4.89± 0.120	4.69 ± 0.105	4.60 ± 0.110

Results are expressed as mean ± standard deviations (SD) of three determinations.

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body weight was 21.0 g (95% CI 19.03–22.98 g), indicating a substantial and biologically meaningful effect.

The histopathological findings clearly demonstrate that infection by *S. aureus* creates appreciable structural damage and inflammation in kidney and liver tissues, which are consistent with bacterial pathogenesis. However, treatment with chitosan showed significant tissue recovery due to most importantly, the reduction in inflammatory infiltrates and liver and kidney architecture preservation. The findings are supported by previous research that has established the biocompatibility and low toxicity of chitosan [2, 16, 25, 39, 40]. Propolis treated rats also exhibited protective effects with minimal organ damage and preservation of structure, validating its long-standing anti-inflammatory and hepatoprotective properties. Nonetheless, the literature also shows that long term administration of high doses of propolis may lead to liver toxicity [4, 14], hence the need for control of dosage. Variability of chemical composition of propolis by geography and season may also influence therapeutic action.Interestingly, the combination of chitosan and propolis had the most favorable histopathological profile, with nearly no histological dysfunctions and intact tissue architecture in all organs investigated. These findings suggest a potential interactive effect between chitosan and propolis, likely involving complementary biological mechanisms that may contribute to improved protection against infection-induced tissue damage, particularly against kidney and liver infections.

The sizeable reduction in *S. aureus* count from the treated groups further suggests the antimicrobial property of chitosan, previously documented by Youcefi *et al.* [41].The mechanism of antibacterial action of chitosan has been attributed to the polycationic nature of the compound, enabling it to bind to the surface of bacteria, disrupt metabolism, and influence cell wall permeability [2, 5].Additionally, low molecular weight chitosan (<5000 Da) is able to penetrate into microbial cells and suppress DNA/RNA transcription and consequently enhance its antibacterial effect. In contrast, propolis alone exhibited a weaker antibacterial effect, which aligns with the earlier findings that propolis in general exhibits a greater antibacterial effect against Gram-positive than Gram-negative bacteria owing to differences in membrane structures[1, 11, 18, 28, 38]. However, the combination of chitosan with propolis presented a positive outcome, indicating a possible complementary antimicrobial interaction. This is consistent with work by Correa-Pacheco *et al.* [6],in which they proved that the incorporation of propolis into chitosan films enhances their antimicrobial activity. The antibacterial action of propolis is attributed primarily to its flavonoids rutin, quercetin, and naringenin whose cell membrane disruption, inhibition of nucleic acid biosynthesis, and denaturation of microbial proteins endow them with the above mentioned characteristics. Together, these findings show that combining chitosan and propolis can improve antimicrobial efficacy, likely due to the combined antimicrobial mechanisms. This combination may be a useful strategy for combating bacterial infections, particularly those by Gram-positive organisms like *Staphylococcus aureus.*

This study demonstrated the effective antimicrobial properties of chitosan and propolis, both individually and in combination against *Staphylococcus aureus* strain ATCC 6538. After 6 days of treatment the bacterial count in treated rats decreased to 2.5 log/mL compared to 4.3 log/mL in the untreated group. Chitosan showed a strong antibacterial effect consistent with previous studies, while propolis had a moderate effect, particularly against Gram-positive bacteria. The combined treatment of chitosan and propolis produced the most beneficial results, suggesting a enhanced combined effect.

In addition to its antimicrobial activity, the treatments helped stabilize body weight during infection. While none of the treatments promoted weight gain, they successfully prevented significant weight loss, supporting their protective role. These findings confirm the potential of chitosan and propolis as promising natural antimicrobial agents, with strong antimicrobial and protective properties. Further research is needed to optimize their use and explore additional benefits.

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