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Original Article

Propolis Silver Nanoparticles as an Adjuvant in Immunization of Rats with *Citrobactor Freundii* Antigens

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Abstract

This study aimed to examine the impact of *Citrobacter freundii* killed whole cell sonicated antigen (KWCSAg) alone and in combination with propolis nanoparticles on humoral immunoglobulin (IgG) and cellular immune responses of rats. The ELISA interleukin 4 (IL4) and IgG, delayed-type hypersensitivity (DTH) skin test, and phagocytosis activity tests were used in this study. In total, 45 rats were divided into five groups of 9 rats. The first group received a 1,000 µg\ml dose of KWCSAg-CF. The second group received an injection of 1,000 µg/ml of KWCSAg-CF antigen and 30 mg/ml of propolis AgNPs. The third group received an injection of 1,000 µg/ml of KWCSAg-CF antigen along with 10 mg/ml of propolis AgNPs. The fourth group was subjected to 30 mg/ml of propolis AgNPs. One ml of phosphate-buffered saline (pH 7.2) was injected into the fifth group (the negative control group). The rats received booster injections of the same antigens after 14 days. Blood was obtained from them to detect immunoglobulin and interleukin 4 (IL-4) on days 21, 28, 32, 46, 50, and 60 following the injection. The second group showed the most significant rise in IL-4 and IgG concentration, followed by the third group, the first group, and the fourth group, compared to the negative control group (fifth group). In all immunized groups, the DTH test results demonstrated an increase in the means of induration with significant differences (P<0.05) of the concentrated antigen after 24 and 48 h, and subsequently a decrease after 72 h, compared to the negative control group. At 48 h after the concentrated antigen was indurated, the second group displayed the most significant increase in diameter.

Keywords: Propolis sliver nanoparticales, Adjuvant, Immunization, Citrobactor Freundii

1. Introduction

A facultative anaerobic gram-negative bacillus, known as *Citrobacter freundii* has been found in soil, water, food, and the environment (e.g., hospitals). It belongs to the *Enterobacteriaceae* family and leads to a number of disorders (e.g., pneumonia, meningitis, sepsis, bacteremia, and urinary tract infections) (1-3). Some *C. freundii* isolates have developed virulence features, resulting in human cases of food poisoning or diarrhea (4, 5). In the present study, *C. freundii* was isolated from the diarrheal sheep sample in Baghdad City, Iraq (6). *Citrobacter spp*. produce potent toxins (Shiga-like toxin), lipopolysaccharide (LPS), and outer membrane proteins. Their antigenic structure is closely related to many Salmonella and Escherichia coli. These three species also share the flagellum H, somatic O, and capsular antigen K (7) that induce hemorrhagic colitis, hemolytic uremic syndrome, and pediatric diarrhea. Toxins, including Shiga-like toxins, heat-stable toxins, and a homolog of the cholera toxin B component, are the primary virulence factors discovered in diarrhea-associated *C. freundii* (4).

Silver nanoparticles (AgNPs) are increasingly used in several domains, including optical, electrical, thermal, high electrical conductivity, and biological features (8). The most recent advancements in metal nanotechnology have shown a wide range of potential

uses. As a result, concentration on antigen-presenting cells can be employed for antigen delivery and immune system stimulation (APC). The enhancement of vaccination efficacy depends critically on how the antigen is presented to the APCs. Immune cells can now receive regulated antigen delivery thanks to adjuvanted nanoparticle vaccinations (9).

2. Materials and Methods

2.1. Bacterial Isolate

In total, 100 sheep fecal samples were obtained from various regions of Baghdad. The size, form, and color of colonies were all visually inspected. Following that, they underwent a gram-stain microscopy examination and biochemical testing for urease, gelatinase, oxidase, and catalase (10). The Vitek2 system (Bio Mérieux, France) was chosen to identify the isolates that were found to be positive using conventional morphological and biochemical tests.

2.2. Antigen Preparation (Sonicated Crude Antigen)

The killed whole cell sonicated antigen of *C. freundii* (KWCSAg-CF) was prepared according to NM (11) with some modification, and stored in a freezer at -20 ^{o}C until used. The supernatant was filtered by a Millipore filter (0.45 µm), considered SSP Ags, and stored at 4 ^{o}C till used in a skin test.

2.3. Preparation of Silver Nanoparticles

2.3.1. Preparation of Propolis Aqueous Extract

The extract was prepared according to a method previously described by Priyadarshini, Sivakumari (12).

2.3.2. Preparation of Propolis Silver Nanoparticles

A magnetic stirrer at room temperature was used to blend 1 mL of 10% propolis extract and 9 mL of 5 mM AgNO3 for the production of AgNPs (13).

2.4. Laboratory Animal Immunization

In total, 45 albino rats were selected, which were randomly divided into four equal groups of nine animals:

1. Day 1: The first group received the KWCSAg-CF S/C. The second group received the KWCSA-CF vaccine together with propolis nanoparticles (30 mg/ml S/C). The third group received (KWCSA-CF) + propolis nanoparticles (10 mg/ml S/C) vaccination. The fourth group was injected with 30 mg/ml S/C of propolis nanoparticles. Phosphate Puffer Saline pH 7.2 S/C was injected into the fifth group (control).

1. Day 14: the same immunization-related antigens were administered in a booster dose (1 ml) to the first, second, and third groups on day 14.

2. On day 21, all injected animals got a delayed-type hypersensitivity (DTH) skin test.

3. On day 28, three animals from each group were subjected to oral challenge tests.

4. On days 21, 28, 35, 49, and 53, blood samples (2 ml) were obtained from all animal groups to calculate the interleukin 4 (IL-4) and immunoglobulin (IgG) concentration.

5. Three mice from each animal group were sacrificed one week after the challenge test, and internal organs, like the liver, lung, intestine, and brain were removed for histological analysis.

6. Rat Immunoglobulin G, IgG ELISA Kit (SunLong Biotech Co., LTD/china) and Rat Interleukin 4, IL-4 ELISA KIT (SunLong Biotech Co., LTD/china) were used to estimate the IL-4 and IgG concentration.

2.5. Delayed Type Hypersensitivity Footpad

According to Hudson, Hay (14), this test was carried out by injection of 0.1 ml of soluble *C. Freundii* antigen intradermally into the right hind footpad of all groups while injecting 0.1 ml of sterile phosphate-buffered saline (PBS, PH 7.2) into the left hind footpad of all immunized groups. The thickness of the skin was measured using a Vernier caliper before and 24, 48, and 72 h after the injection.

3. Results and Discussion

3.1. Characterization of Silver Nanoparticles

3.1.1. Characterization of AgNPs

3.1.1.1. Silver Nanoparticles Produced through Green Synthesis

In the current study, the synthesized changes in color initially identified propolis silver nanoparticles. After 24 h of incubation in a dark room, the color of the mixture changed instantly to dark brown. This result was in with those of a study performed by Ali and Khudair (15).

Some of the measurements used and compiled are as follows: digital data and UV-visible absorption spectra

of the materials were captured using the UV-visible spectrometer (16) (Figure 1). A scanning electron microscope is a type of microscope that makes use of electrons. A thin layer of AgNPs powder was created and this microscope was used to examine the stability of nanoparticles and their size distribution (17) (Figure 2). The characteristics of the various nanomaterial can easily be identified in transmission electron microscopy (TEM). The TEM image indicates the properties of the various nanomaterials. The concentration of the Nano extract can determine the shape and size of the resulting nanoparticles. In this research, the results showed that a layer of organic matter surrounded the surface of Gaps, where the layer appeared as a strong crystalline shell that stabilized the AgNPs (Figure 3). This result is in line with those of a study performed by Khan, Tareq (18).

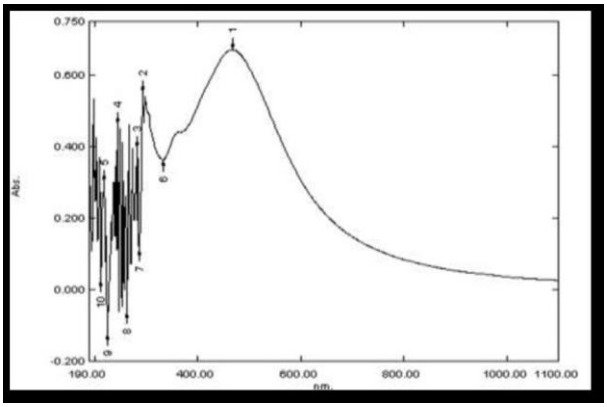

Figure 1. UV-Visible spectral analysis of AgNPs

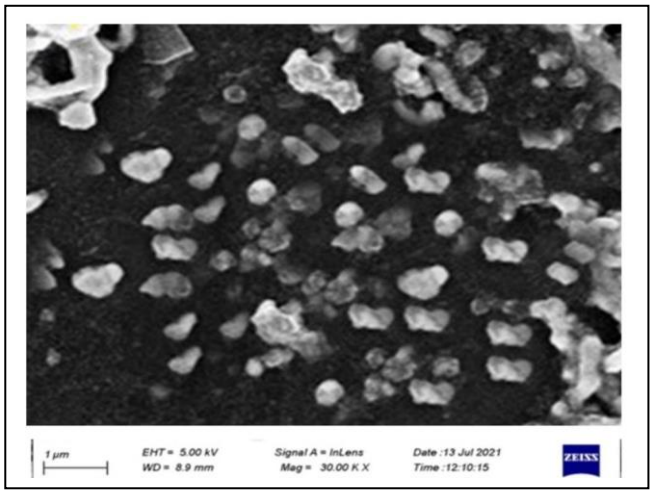

Figure 2. Scanning Electron Microscope of propolis AgNPs

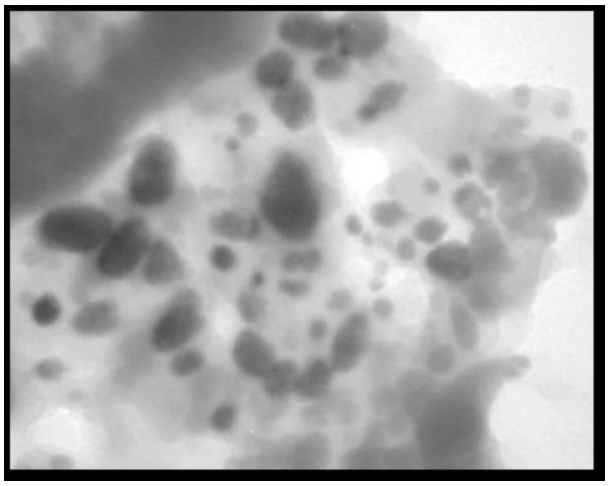

Figure 3. Transmission Electron Microscopy of propolis AgNPs

3.1.2. Delayed-Type Hypersensitivity Skin Test

The cell-mediated immunity of the immunized and control rats was assessed by a DTH skin test. Only the immunized rat groups were positive for the skin reaction, compared to the control group. The results of the DTH skin test have shown increases in the thickness of the footpad skin of all immunized groups (WKCSA), compared to the control group (PBS). The result obtained from the WKCSA group (first group) showed an increase in thickness at 24 and 48 h (6.48±0.12 mm and 8.25±0.13 mm, respectively), followed by a decrease at 72 h (5.57±0.14), with a significant difference ($P<0.05$).

Results of induration of the second group showed a significant ($P<0.05$) increase in the thickness of the skin at 24 and 48 h (7.41±0.14 and 8.57±0.15, respectively), followed by a decrease at 72 h (6.32 ± 0.13) with a significant difference ($P<0.05$). Findings of the third group (the group that was immunized with WKCSAg+10% propolis nanoparticles) showed an increase in the thickness of the skin at 24 and 48 h (6.31±0.07 and 8.27±0.12, respectively), followed by a decrease at 72 h (5.57±0.14) with a significant difference ($P<0.05$). Results of the fourth group, which was immunized with 30% propolis nanoparticles, revealed a less thickness in the footpad at 24 and 48 h (4.67±0.13 and 5.52±0.18, respectively), followed by a

larger decrease at 72 h (4.46±0.12) with a significant difference (P<0.05).

These results indicated that the group that received the antigen combined with propolis nanoparticles had the most DTH reaction, as shown in the second and third groups. The thickness increase in the group that received 30% propolis nanoparticles indicated that the nanoparticles increased the cellular immune response in the immunized animals (Table 1).

These findings agree with those of a study conducted by Al-Tae (19), who discovered that only the immunized group in a study on rats inoculated with Pseudomonas aeruginosa antigen were positive for a skin test. The DTH response, which depends on T helper 1 (Th1)-driven responses as well as cell recruitment and chemotaxis to a local region, has been utilized as an indicator of a cell-mediated immune state. Therefore, interruption of either Th1-driven, antigen-dependent T cell growth or migration of sensitized T cells to a local location may impact the DTH functional response.

3.2. Interleukin 4 Concentration

According to table 2, the concentrations of IL-4 in the first group that was immunized with WKCSAg CF, reached 21.26±0.28 pg at day 21 post-immunization, then increased to reach 21.70±0.29; 24.00±0.46; 25.69±0.33, and 27.36±0.43 pg on days 28, 35, 49, 53 post-immunization, respectively. Results of this group were significantly different from those of the control group on days 21, 28, 35, 49, and 53 (P<0.1).

In the second group, the concentrations of rat IL-4 were 39.44±0.50, 41.38±1.46, 45.57±2.49, 43.44±1.01, and 42.02±0.90 on days 21, 28, 35, 49 and 53 post-immunization, respectively. The rats in this group showed the highest value on day 35 (45.57±2.49 pg) with significant differences (P<0.05), compared to the control group on the same day. In the third group of rats, the IL-4 concentrations were 21.88±0.43, 23.86±0.70, 26.05±0.89, 29.52±1.34, and 36.84±0.61 on days 21, 28, 35, 49, and 53 respectively. The fourth group showed lower IL-4 concentrations, including 11.77±0.49, 14.20±0.35, 9.68±0.65, 7.86±0.58, and 5.30±0.37 on days 21, 28, 35, 49 and 53, respectively.

All these results were compared with those of the fifth (control) group (PBS), which showed the lowest concentrations of IL-4, and the levels remained low till the end of the experiment. Concentrations of IL-4 in this group were 4.66±0.74, 4.52±0.41, 4.48±0.41, 4.38±0.50 and 4.32±0.57 pg on days 20, 28, 35, 49, and 53 post-immunization, respectively. The levels of rat IL-4 in the control group were lower than those of the experimental groups; however, the differences were not significant.

We agreed with Al-Samarraae (20); they claimed that Salmonella typhimurium (KWCSA-ST) killed full sonicated antigen can raise IL4 concentration.

Our findings concurred with those of Al-Samarraae (20), who prepared antigens from sonicated Salmonella typhimurium (KWCSA-ST) and Lactobacillus acidophilus (KWCSA-LBA), and used these antigens to evaluate (IL-4) showing there was an increase in the concentration of IL-4 significant differences (P<0.05) in the first group. They also concurred with those of Mohammed and Al-Samarraae (6), who reported a significant increase in IL. There is an agreement with Galdiero et al. (1998), who stated that mice treated with dead vaccinations or pure bacterial components have an IL-4-dominated immune response. Additionally, Marinaro et al. (1996) demonstrated that mice have more IL-4, IL-5, and IL6-secreting cells in Payer's patches after oral infection with attenuated Salmonella.

3.3. Immunoglobulin Concentration

The IgG concentration in the first group (immunized with KWCSAg-CF (1,000 µg/ml S/C) were 20.05±0.64, 20.88±0.73, 24.74±1.14, 30.36±2.24, and 39.74±1.30 at days 21, 28, 35, 49, and 53 post-immunization, respectively, with significant differences (P<0.05). However, in the second group (immunized with KWCSAg-CF (1,000 µg/ml S/C)+30% propolis nanoparticles) IgG concentrations were 23.38±1.09, 25.36±0.81, 30.64±1.09, 37.98±0.45, and 46.11±0.81 at days 21, 28, 35, 49, and 53 post-immunization, respectively, with a significant difference (P<0.05).

In the third group (immunized with KWCSAg-CF (1,000 µg/ml S/C)+10% propolis nanoparticles), IgG concentrations were 21.30±1.19, 22.01±1.31, 26.85±0.81, 34.82±1.58, and 43.75±1.08, respectively, at days 21, 28, 35, 49 and 53 post-immunization, with significant differences (P<0.05). In the fourth group (immunized with 30% propolis nanoparticles) IgG concentrations were 15.73±0.43, 20.03±1.16, 20.20±0.83, 17.84±0.79 and 14.53±1.36 at days 21, 28, 35, 49, and 53 post-immunization, respectively, with significant differences (P<0.05). Moreover, in the fifth (control) group, IgG concentrations were 5.99±0.15, 6.22±0.20, 5.72±0.20, 5.75±0.34, and 5.61±0.21, at days 21, 28, 35, 49, and 53 post-immunization, respectively, with significant differences (P<0.05). There were significant differences (P<0.05) among the second, third, fourth, and fifth groups, compared to the fifth (control) group (Table 3).

Table 1. The DTH-skin test of immunized rats by *Citrobactor Freundii* antigen

Hours / Groups	24hr	48hr	72hr
KWCSAg-CF	6.48±0.12B b	8.25±0.13A b	5.57±0.14C b
KWCSAg-CF+30 mg /ml propolis nanoparticles	7.41±0.14B a	8.57±0.15An a	6.32±0.13C a
KWCSAg-CF+10 mg/ml propolis nanoparticles	6.31±0.07B b	8.27±0.12A b	5.57±0.14C b
30 mg /ml Propolis	4.67±0.13B c	5.52±0.18A c	4.46±0.12B c
Control	3.84±0.05A d	3.89±0.06A d	3.94±0.05A d
LSD	0.35		

Means with a different small letter in the same column are significantly different (P<0.05)
Means with a different capital letter in the same row are significantly different (P<0.05)

Table 2. IL4 concentration of rats immunized by Citrobactor Freundii antigen in different periods of days

Days / Groups	21	28	35	49	53
KWCSAg-CF	21.26±0.28D b	21.70±0.29CD b	24.00±0.46BC b	25.69±0.33AB c	27.36±0.43A c
KWCSAg-CF+30mg/ml propolis AgNPs	39.44±0.50C a	41.38±1.46BC a	45.57±2.49A a	43.44±1.01AB a	42.02±0.90B a
KWCSAg-CF+10mg/ml propolis AgNPs	21.88±0.43D b	23.86±0.70D b	26.05±0.89C b	29.52±1.34B b	36.84±0.61A b
30mg/ml propolis AgNPs	11.77±0.49AB c	14.20±0.35A c	9.68±0.65BC c	7.86±0.58C d	5.30±0.37D d
Control	4.66±0.74A d	4.52±0.41A d	4.48±0.41A d	4.38±0.50A e	4.32±0.57A d
LSD	2.45				

Means with a different small letter in the same column are significantly different (P<0.05)
Means with a different capital letter in the same row are significantly different (P<0.05)

Table 3. IgG concentration in the immunized groups with different antigens by ELISA test

Days / Groups	21	28	35	49	53
KWCSAg-CF	20.05±0.64D b	20.88±0.73D b	24.74±1.14C b	30.36±2.24B c	39.74±1.30A b
KWCSAg-CF+30mg/ml propolis AgNPs	23.38±1.09D a	25.36±0.81D a	30.64±1.09C a	37.98±0.45B a	46.11±0.81A a
KWCSAg-CF+10mg/ml propolis AgNPs	21.30±1.19D ab	22.01±1.31D b	26.85±0.81C b	34.82±1.58B b	43.75±1.08A a
30mg/ml propolis AgNPs	15.73±0.43BC c	20.03±1.16A b	20.20±0.83A c	17.84±0.79AB d	14.53±1.36C c
Control	5.99±0.15A d	6.22±0.20A c	5.72±0.20A d	5.75±0.34A e	5.61±0.21A d
LSD	2.83				

Means with a different small letter in the same column are significantly different (P<0.05)
Means with a different capital letter in the same row are significantly different (P<0.05)

These results agreed with those of a study carried out by Mohammed and Al-Samarraae (6), who discovered that IgG concentration increased considerably ($P<0.05$) until day 60 after being inoculated with various *C. freundii* antigens, including the whole cell sonicated antigen, LPS, and DNA antigens. According to the present research, the usage of nanoparticles to promote immunity may increase the efficiency of vaccines. In the second and third groups, propolis AgNps administered with bacteria antigen showed the greatest IgG concentration (21). The AgNPs have been demonstrated to increase ovalbumin-specific IgG antibody production in the serum of mice that received ovalbumin parenterally (22) and to play the role of an adjuvant for a rabies vaccine intraperitoneally administered to mice (23).

Authors' Contribution

Study concept and design: R. J. S.
Acquisition of data: I. A. A. A.
Analysis and interpretation of data: I. A. A. A.
Drafting of the manuscript: I. A. A. A.
Critical revision of the manuscript for important intellectual content: R. J. S.
Statistical analysis: R. J. S.
Administrative, technical, and material support: R. J. S.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article according to the ethics committee of the University of Baghdad, Baghdad, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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