Research Article

Cytokine Profiles of the Pro-Inflammatory and Anti-Inflammatory Response to Bacterial Antigens when Combined with Vaccine Adjuvant

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Abstract | This study aim to investiage a natural alternative (alcoholic extract of propolis and nigella oil) to current vaccine adjuvants (alum) using inactivated antigens (sonicated and whole-killed bacteria) of E. coli. We also aimed to reveal tthe nature of host innate and adaptive immune responses by measuring the extent of their effects on the level of pro-inflammatory (interferon γ and interleukins (IL)-12) and anti-inflammatory cytokines (IL-4 and IL-10) and innate immune responses (total white blood count, phagocytosis and respiratory burst). Our results demonstrated that all vaccines have induced both innate and adaptive immune responses. However, the adjuvant with alum hydroxide mixed with sonicated antigens and killed bacteria induced higher pro-inflammatory cytokine effects (IFN- γ $P < 0.05$, $P < 0.01$) and anti-inflammatory cytokines (IL-4 $P < 0.0001$, $P < 0.001$), respectively. Interestingly, all immunized groups switched immune responses toward Th2 and reduced pro-inflammatory cytokines to produce homeostasis and reduce damage to body tissue. In conclusion, the use of natural vaccine adjuvants and alum led to a type 2 immune response and diverted immunity towards Th2. Importantly, all adjuvant types increased the level of innate immune responses. However, alum performed better in induction of adaptive immunity than the rest of adjuvents used.

Keywords | Alcoholic extract of propolis, *Nigella sativa*, Interferon, Interleukin, Iraq

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INTRODUCTION

Vaccines are significant medical achievements, preventing diseases by inducing the immune response. Purified antigen-based vaccines require adjuvants to improve their strength, quality, and duration (Martión et al., 2019). Adjuvants are substances that are injected with an antigen to boost the antigen-stimulating humoral and/or cell-mediated immune response. Adjuvants can modify the immune system reaction to the antigen and often allow for the use of lower antigen doses. There have been almost a hundred adjuvant preparations described in previous years (Vogel and Powell, 1995). Aluminum-based adjuvant will remain a crucial part of current and upcoming approved vaccines, particularly significant combination vaccines (Laera et al., 2023). However, it has side effects that increase the risk of autoimmunity, chronic brain inflammation, and related neurological problems; These can have serious and far-reaching negative effects on health (Tomljenovic and Shaw, 2011), so it is necessary to find a new adjuvant that can replace the alum-type adjuvant (Sivakumar et al., 2011), so drug research now focuses more on natural medicine than on synthetic drugs (Fan et al., 2015).

Propolis, a natural adjuvant with immunomodulatory properties, is being evaluated for its potential as a novel medication due to its faster, more effective, and less toxic response (El Ashry and Ahmad, 2012; Woods et al., 2017). Furthermore, *Nigella sativa*, a medicinal plant with non-specific immunostimulant effects and an induced immune response, is recommended due to its proper action and the absence of side effects (Alishahi et al., 2012; Mady et al., 2013). The mediators' cytokines are frequently measured to evaluate immune responses to vaccines since activated T cells release these immune mediators (Lin et al., 2014); the cytokines play a crucial role in regulating the body's immune response to infection (Abduljabbar and Ibrahim, 2022).

Cytokines are classified into different groups; Th1 cytokines, usually termed pro-inflammatory, are involved in the regulation of cell-mediated immune responses (Poncin et al., 2008), which are induced by immune cells, such as macrophages, T cells, and other cells that promote inflammation and immunity. Pro-inflammatory cytokines include interleukin-1 (IL-1), IL-12, interferon-gamma (IFN-γ), tumor necrosis factor alpha (TNF) and colony-stimulating factor of granulocyte macrophages (G-MCSF). Pro-inflammatory cytokines are involved in the upregulation of immune responses, including macrophage activation, induction of apoptosis, and recruitment of additional immune cells (AL-Sadoon et al., 2018), and cause tissue injury (Hashim et al., 2021). On the contrary, Th2 produces cytokines such as (IL-4, IL-5, IL-6, and IL-10) (AL-Sadoon et al., 2018), anti-inflammatory cytokines, including IL-4 and IL-10, are secreted by immune cells such as regulatory T cells and macrophages to suppress inflammation and immunity (Cicchese et al., 2018). Therefore, our objective was to examine innate and adaptive immune responses and detect the interface between pro- and anti-inflammatory cytokines by vaccination of rats with different types of adjuvant (classical and natural vaccine adjuvant) and then find out which cellular and humoral immune responses in the different types of adjuvants with inactivated antigens to detect the appropriate vaccine adjuvant to use safely.

MATERIALS AND METHODS

Laboratory animals

The experiments in this study used 42 female Wistar albino rats, weighing between (220-260) grams. The College of Veterinary Medicine of Basrah University provided all the animals. Before being used in lab tests, the rats were housed in plastic cages for two weeks with unrestricted access to food and water. Throughout the experiments, they were kept in regulated settings (temperature 24-26°C, appropriate humidity, and a 12-hour light-dark cycle). All animal handling was done following the approval of the Ethics Committee requirements of the College of Veterinary Medicine (Ref. No. 3/2023).

Bacterial isolate

An isolate of local Shiga-producing *E. coli* (STEC) was obtained from cattle in the Basrah governorate and submitted by the Department of Microbiology of the University of Basrah, College of Veterinary Medicine (Farhan and AL-Iedani, 2019).

Preparation of dead bacteria (whole cell vaccine)

The stock *E. coli* was grown for 24 hours at 37 ° C while shaken in brain-heart infusion broth. After incubation, cells were exposed to 3.7% formalin whole overnight. Four washings with phosphate buffer saline (PBS) were performed on inactivated bacteria (Sunwoo et al., 2006) and then, comparing with the 0.5 McFarland Standard Solution, adjusted to 2×10^9 CFU/ml. Until used, the preparation was stored at 4 ° C and then streaked onto blood agar and McConkey agar plates for 24 to 48 hours to verify sterility (Manzoor et al., 2017).

Preparation of crude antigens using sonication

For cell lysis, to avoid considerable heating of the sample during sonication, the sample vial was kept in an ice water bath containing an *E. coli* solution in sterile phosphate buffer saline at a concentration of 2×10^9 CFU/ml (Shrestha et al., 2012), followed by ten cycles of a 60-second pulse with 90-second intervals at a frequency of 20 kHz (Shrestha et al., 2012).

Preparation of alum hydroxide gel

The solution of sodium hydroxide (NaOH) was prepared by dissolving 40 g of sodium hydroxide: Sodium Hydroxide with 1000 ml of distilled water and mixed with 1L of 10% solution of potassium aluminium sulfate (Alum) K2SO4. $Al_2(SO_4)_3$. 24H2O. Both solutions were mixed, leading to the formation of 500 ml of white gelatinous precipitate of Alum Hydroxide gel, and stored at 4 ° C. After overnight storage, the supernatant on the gel was discarded and the gel was mixed with the same amount of agitated distilled water and placed in the refrigerator for 20 minutes. The gel was then taken out, and the supernatant was discarded. This procedure was repeated until the supernatant was free of sulfate ions. The sulfur ions in the supernatant were verified by taking 5 ml of the supernatant in a test tube and mixed with 1-2 drops of 1% silver nitrate ($AgNO_3$). The supernatant was observed for any change in color or precipitate formation. Upon a cloudy color change or the formation of a white precipitate in the supernatant, the gel was mixed again with distilled water, repeating the procedure of washing sulfate ions. Washing of the gel was carried out until the gel was free of sulphate ions. The pH

of the gel was determined and maintained at 7.0 and autoclaved (Manzoor et al., 2017). The whole killed bacteria and crud antigen suspension 2×10^9 CFU/ml in PBS mixed with alum as an adjuvant in a ratio of 1:1 (volume/volume) (Arshadi et al., 2020).

Preparation of propolis vaccine adjuvant

In this study, Iraqi propolis was prepared as a natural adjuvant, and the alcoholic extract of propolis was prepared as described by (Alishahi et al., 2019). Briefly, 15 g of propolis collected from Basrah city were suspended in 60 ml of 95% ethanol shaking at 25 ° C for 1 day in a water bath. Subsequently, the suspension was stored in the dark using closed containers at 4 ° C to prevent excessive oxidation until processing (Ghazi and Al-Bayati, 2019). Propolis extract was filtered through gauze layers many times, then sterilized through 0.4 µm membranes and used as a stock solution. Before use, the propolis extract was suspended in PBS at a concentration of 30 mg/ml and mixed with killed *E. coli* and sonicated antigen in a concentration of 2×10^9 CFU / ml in a 1:1 ratio (Alishahi et al., 2019).

Preparation of Nigella sativa Adjuvant Emulsion

The emulsion was prepared by mixing the oil phase of *Nigella sativa* with the aqueous phase of the prepared antigen as follows:

Preparation of the oil phase of Nigella sativa: The oil was obtained by cold pressing *Nigella sativa* seeds by the procedure described by (Kiralan et al., 2014). Subsequently, it was combined with span 40 (an emulsifier) from Alpha Chemicka, India, and thoroughly mixed in a ratio of 9:1, or nine parts oil to one part of span 40, after that was sterilized by filter syringe filtration of 0.45µL. The mixture of oil and span 40 was kept at room temperature in sterile containers until used (Mady et al., 2013).

Preparation of the aqueous phase of the vaccine*:* The aqueous phase was prepared by mixing 96% inactivated *E. coli* or crude antigen solution with a span of 4% 40 (Madbouly and Tamam, 2000).

Preparation of *E. coli* antigens and Nigella sativa adjuvant: The stable emulsion of vaccines was prepared by thorough mixing of the prepared aqueous phase and the oil phase in a 1:4 ratio where one part of the aqueous phase was mixed with 4 parts of the oil phase with continuous mixing until the production of stable emulsion (Tamam et al., 2015).

Vaccine dose and route of injection

The total dose used in this study was 0.5 ml per rat, injected subcutaneously [vaccine suspension of Alum hydroxide, propolis and *Nigella sativa* vaccine adjuvant] (Al-Hariri and Abualait, 2020).

Immunization

Seven groups were used, each group was composed of 6 rats that were subcutaneously immunized using standard hygiene precautions [sterile needles after having disinfected the animal skin with 70% ethanol] (Lindblad, 2008), each rat was injected into the dorsal region in four sites, three sits 0.1 ml and the fourth 0.2 ml (IACUC, 2022). The first group of animals was subcutaneously injected with normal saline as control (CO), the second group G2 was immunized with Alum hydroxide adjuvant and crude antigens of bacteria (ALSO), G3 was immunized with Alum hydroxide adjuvant and whole dead bacteria (ALKi), G4 was immunized with propolis adjuvant and crude antigens of bacteria (ProSO), G5 was immunized with propolis adjuvant and whole dead bacteria (ProKi), G6 was immunized with *N. sativa* oil adjuvant and crude antigens of bacteria (NeSO) and G7 was immunized with *N. sativa* oil adjuvant with whole dead bacteria (NeKi). Regarding the booster dose, all groups were injected subcutaneously using the same vaccine adjuvant after 14 days after the first injection.

Blood samples and plasma collection

Blood samples were collected after the second injection, using tubes with heparin to plasma collection tubes, and inverted eight times, followed by centrifugation at 300 RPM for 10 min at 20 ° C. The tubes were stored in a frozen state until analysis (Sotelo-Orozco et al., 2021).

Measurement of IL-4, IL-10, IFN-γ and IL-12 concentrations

Cytokine concentrations (IL-4, IL10, IFN-γ, and IL-12) were measured in the plasma of rats using ELISA kits purchased from (Bioassay Technology Laboratory/ China), these kits used the quantitative sandwich enzyme immunoassay according to the manufacturer's protocol, quantification of cytokine proteins was determined by comparing samples with the standard curve generated from the respective kits.

Data Analysis

The statistical analysis of the results was determined on the basis of the differences that exist between the means of the seven groups. The analysis was performed using GraphPad Prism version 8 (Gharban et al., 2023).

RESULTS

The result of total white blood cell count shows an increase in all vaccinated groups compared to the control group, the alum hydroxide vaccine adjuvant groups had the highest increase in WBC count than others (Table 1, Figure 1).

Table 1: Total leukocyte count, phagocytosis rate, and respiratory burst activity. in various immunization groups of rats using different adjuvant vaccines

Group	Total white blood cell count	Phagocytosis rate	Respiratory burst activity
G1	6.805×10^3 ±0.7068	35.08 □1.195	28.63 ±1.250
G2	8.438×10^3± 0.09161 ****	55.35±0.8928 ****	40.61±1.229 ****
G3	8.213×10^3±0.1458 ****	57.88±0.913 ****	44.64±1.862 ****
G4	7.275×10^3±0.3327 ns	43.93±1.200 ****	35.54±1.573 ****
G5	7.213×10^3 ±0.21 ns	54.4±0.8709 ****	33.61±0.9031 ****
G6	7.363×10^3± 0.2264 *	41.35±1.047 ****	30.58 ±1.276 ns
G7	7.656×10^3±0.362 ***	53.74±1.043 ****	34.15±1.327 ****

All data are presented as mean ± standard deviation of the total count of white blood cells, phagocytosis rate, and respiratory burst activity

****= P < 0.0001, ***= P < 0.001, ** =P < 0.0 1, *= P < 0.05, ns = not significant

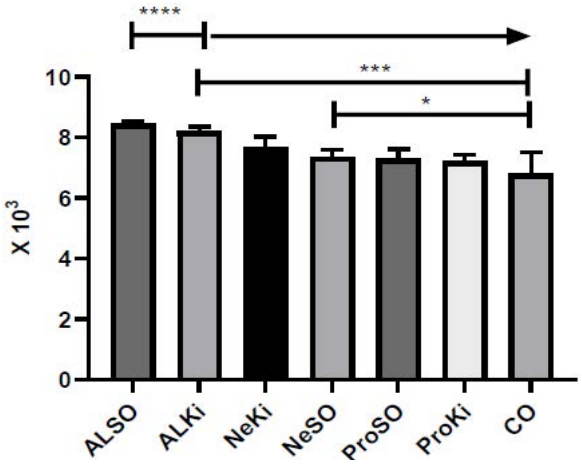

Figure 1: Total leukocyte counts in the adjuvant groups of the alum hydroxide vaccine and the natural adjuvant vaccine groups (propolis and *Nigella sativa* oil) compared to the control group

Note: G1=CO, G2=ALSO, G3= ALKi, G4=ProSO, G5=ProKi, G6=NeSO, G7-NeKi

The phagocytosis rate significantly increased in all vaccinated groups compared to the control, and the killed vaccine adjuvant also had a higher rate of phagocytosis than the sonicated vaccine in all vaccinated groups, as shown in (Table 1, Figure, 2).

The results show that the respiratory burst significantly increased in all vaccinated groups, except G6 with a nonsignificant increase, as shown in (Table 1, Figure 3).

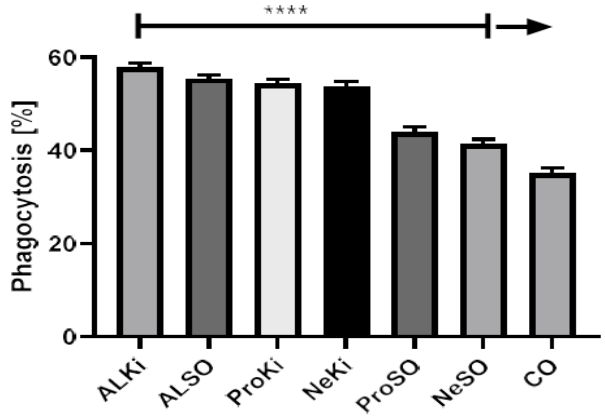

Figure 2: Phagecytosis rates in adjuvant groups of alum hydroxide vaccine and natural adjuvant vaccine groups (propolis and *Nigella sativa* oil) compared to control group

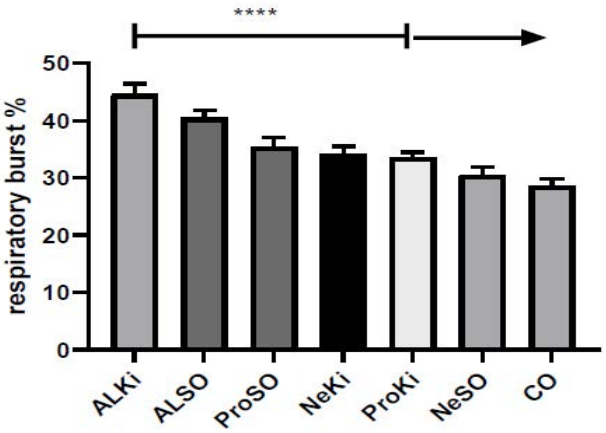

Figure 3: The respiratory burst rates in the adjuvant groups of the alum hydroxide vaccine and the natural adjuvant vaccine groups (propolis and *Nigella sativa* oil) compared to control group.

Table 2: IL-12, IFN-γ, IL-4 and IL-10 concentration in all vaccinated groups and control group

No. of groups	IL-12	IFN- γ	IL-4	IL-10
G1	45.516±3.4653	29.959±2.8743	32.178±4.4258	113.49± 5.4498
G2	45.69±15.6252 ns	50.3788±15.506 *	73.666±7.72153 ****	141.5±33.4016 ns
G3	52.3563±11.5144 ns	55.8688±13.8594 **	63.52±23.0646 ***	143.065±46.5382 ns
G4	34.195±3.72467 ns	39.05±9.83707 ns	51.5675±10.8001 ns	163.6±19.6311 ns
G5	28.7175±5.36644 *	49.6225±8.07731 *	55.335±11.0836 *	245.25±38.2601 ****
G6	40.3488±4.30855 ns	32.985±4.95629 ns	49.3013±4.79651 ns	155.9±18.0216 ns
G7	50.41±7.48833 ns	40.07±7.14681 ns	62.09±11.0305 **	134.19±33.872 ns

All data are presented as mean ± standard deviation of interleukin concentrations.
****= P < 0.0001, ***= P < 0.001, ** =P < 0.0 1, *= P < 0.05, ns = not significant

The results show that the pro-inflammatory cytokines IL-12 did not increase significantly in G3, G7 and G2, respectively, and did not decrease significantly in G6 and G4, however, the decreases were significant in G5 (Table 2, Figure 4-A). The IFN- concentration of IFN-γ was increased in all groups compared to a control group and was statistically significant in G2, G3 and G5 as shown in (Table 2, Figure 4-B).

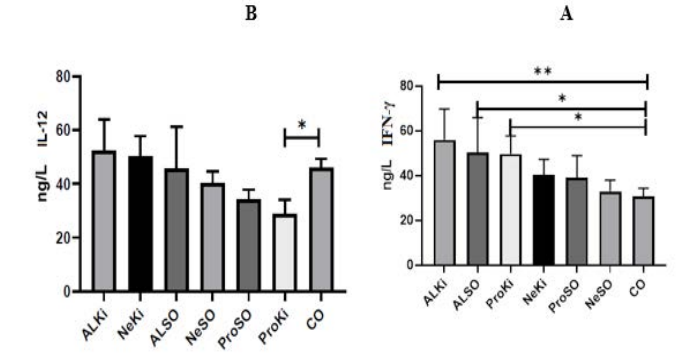

Figure 4: Concentration of pro-inflammatory cytokines (A- IL-12, B- IFN-γ) in different groups compared to the control group.

The results show that anti-inflammatory cytokines in IL-4 increased significantly in G2, G3, G7, and G5, respectively, and a nonsignificant increase in G4 and G6, while the result of IL-10 reveals a highly significant increase in G5 and a nonsignificant increase in other groups, this result illustrated in (Table 2, Figure 5).

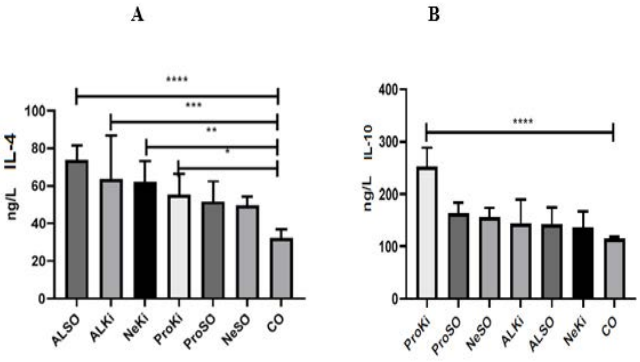

Figure 5: Concentration of anti-inflammatory cytokines (A- IL- 4, B-IL-10) in different groups compared to the control group.

The results of the ratio of IFN-γ to IL-4 indicated that all groups tend to humoral immune responses (Table 3). According to Mozaffari et al. (2019), who stated that the ratio of IFN-γ to IL-4 was found to be a supplementary signal for the type of response. The Th1 response was determined if the ratio was greater than one and a Th2 response if it was less than one. All vaccine adjuvant groups oriented immunity toward Th2 immune responses.

Table 3: The ratio of IFN-γ to IL-4 of the control group and different vaccine adjuvant groups

Groups	IFN-γ /IL-4 ratio
G1	0.967±0.1370
G2	0.6809±0.1858
G3	0.8675 ±0.59
G4	0.794±0.271
G5	0.946±0.30243
G6	0.6747±0.1209
G7	0.6586±0.1464

DISCUSSION

Immunization is widely recognized as one of the most significant public health achievements of the twentieth century (Abdalsaid et al., 2017). Vaccination works by manipulating the immune system of the body, preparing it for the rapid elimination of infectious agents and/or their toxic products (O'Hagan and Valiante, 2003). Innate immune responses accomplish rapid identification, elimination of foreign objects, and triggering an inflammatory response by identifying PAMP present in numerous bacterial infections (Al-Aalim et al., 2021a).

Adjuvants are administered in conjunction with vaccines to enhance the immune response to the target antigen by stimulating the innate immune system. This, in turn, stimulates resident immune cells, which generate cytokines and chemokines, leading to cell recruitment and recognition of APCs. Subsequently, these APCs initiate the presentation of antigens in the lymph nodes, increasing adaptive immune responses (Pulendran et al., 2021).

An essential component of the blood system, white blood cells (WBCs) are considered one of the important arms of the innate immune system, which is in charge of responding to and defending the body against infectious diseases by transporting certain cells, known as neutrophils, to infection sites to combat invasive infections (Al-Aalim et al., 2021b).

The results of this study showed an increase in total white blood cells in all groups, G2 and G3 have increased significantly, then G7 this is due to increased IFN- production of IFN-γ in these groups, a similar result of Resend et al. (2017) who showed that the IFN-γ is responsible for inducing leukocyte recruitment to the infection site. Although G5 did not significantly increase in WBC count despite a significant increase in IFN-γ, this study suggested that the lower WBC count may be due to the increase in IL-10 in this group, this study is in agreement with Iyer and Cheng (2012) who illustrate that IL-10 is a potent anti-inflammatory cytokine that plays a central role in limiting the host's immune response to infection, this group also had a significant increase in IL-4, and this interleukin responsible limits the movement and recruitment of neutrophils, and these cells are the first innate immune cells migrate to the site of action, quickly executing effector functions such as secretion of cytokines and chemokines, attracting additional innate and adaptive immune cells (Woytschak et al., 2016).

One of the primary innate immune functions is phagocytosis, which involves the removal of pathogens by phagocytic cells such as neutrophils and macrophages. It is one of the most important host defense mechanisms (Kartikasari et al., 2022).

When comparing adjuvant groups with control group, the phagocytosis rate increased significantly in all immunization groups; however, the increase in alum groups agrees with the findings of Coffman et al. (2010), who hypothesize that alum may activate the NLRP3 inflammasome and result in the creation of mature IL-1β, this result is in line with the increase in phagocytosis observed in alum groups; phagocytosis of alum crystals appears to be a part of this mechanism.

Propolis improves phagocytosis, as cited by Cuesta et al. (2005) who found that propolis adjuvant increased leucocyte phagocytosis. Also, Nigella sativa oil has increased phagocytosis, this result is similar to that of Mady et al. (2013).

In this study, there is an association between IFN-γ and-phagocytosis, due to an increase in this interleukin in all groups, and this is in agreement with Spellberg and Edwards (2001) who illustrated the proinflammatory IFN-γ stimulates phagocytosis and intracellular killing of microbes.

A respiratory burst (RB) is a surge in reactive oxygen species (ROS) that occurs during the phagocytosis of microorganisms (Victor et al., 2004). Cavinato et al. (2020) stated that phagocytic cells use reactive oxygen species (ROS), which are small molecules generated from oxygen, to control infections. Furthermore, RB is necessary for innate immunity for phagocytic cells to eliminate pathogens (Victor et al., 2004).

All groups in this study showed a highly significant increase in respiratory burst activity (RB), except G6, the increase was not significant, while the groups that received the alum hydroxide adjuvant increased in this activity, as Martinon et al. (2009) noted that ROS are essential secondary messengers that induce activation of the NLRP3 / NALP3 inflammasome and are generated by NLRP3/ NALP3 activators. Furthermore, this increase in alum vaccine may be due to an increase in IFN-γ concentration in this group, which was higher than in other groups. This is similar to what Spellberg and Edwards (2001) recorded.

The increase in RB activity in the propolis adjuvant groups was notable. This finding is consistent with that of Magnavacca et al. (2022), who showed that propolis extract acted on oxidative and inflammatory processes to provide a protective impact. In addition, the Nigella sativa oil vaccine adjuvant induces RB as noted by Abdevand et al. (2021). The nonsignificant increase in G6 may be due to a nonsig-

nificant increase in IFN-Y, as noted by Spellberg and Edwards (2001), that interleukin increased the effect of R. B. The immune system activates pro-inflammatory cytokines in response to antigens, but excess inflammation can cause harm. Anti-inflammatory mechanisms exist to limit damage and restore tissue homeostasis (Iyer and Cheng, 2012). Several factors induce polarization toward TH1 (pro-inflammatory) or TH2 (anti-inflammatory). Important of these factors are the local cytokine milieu; the dose and route of antigen administration; the type of antigen-presenting cell that stimulates the T cell; (IL-12) is mainly produced by antigen-presenting cells and stimulates T and NK cells to produce interferon-γ (IFN-γ), which in turn increases the synthesis of IL-12 in monocytes and polymorphonuclear cells. IL-12 is a key component in the start of Th1 responses against certain infections (Kriegel et al., 2006).

In this study, the results showed that IL-12 was not significantly increased in G3, G7, and G2, respectively, and this increase may be due to the dominant effect of IL-10 as mentioned by Ma et al. (2015), who noted that LPS induces IL-12. However, this study reveals a nonsignificant decrease in G6 and G4 and a low significant decrease in G5 because Nigella sativa oil and propolis adjuvants induce a high concentration of IL-4 productivity and thus the induction of IL-10. These results are in agreement with Majdalawieh and Fayyad (2015) and Mojarab et al. (2019). Induction of a high concentration of IL-4 is perhaps related to the anti-inflammatory characteristics of some compounds in propolis, which agrees (Wolska et al., 2019), also in nigella oil this result agrees with others (Uçan et al., 2020). The lower induction in TH1 activity recorded in this study is similar to the result observed by Mitchell et al. (2017), who illustrated that Th2 cytokines inhibit the Th1 phenotype. In this study, all groups show a nonsignificant increase in IL-12 because all groups have an increase in IL-4 and a highly significant increase in IL-10 results like this reported by Poncin et al. (2008), who illustrated that anti-inflammatory cytokines such as IL-4 and IL-10 are involved in the control of antibody production and make suppression of actions of immune responses resulting from Th1. Mountford et al. (1999) show that IL-4 predominates over IL-12 during the priming step; therefore, Th2 cells are developed.

IFN-γ is secreted mainly by natural killer cells (NK) and activated T cells; it can mediate antiviral and antibacterial immunity, improve antigen presentation, activate the innate immune system, coordinate interaction between lymphocytes and endothelium, regulate Th1 / Th2 balance and control apoptosis and cell proliferation (Tau and Rothman, 1999).

The results of IFN-γ revealed an increase in all groups, the increase is significant in three groups (G3, G2, G5), increases in G3 and G2 may be due to an increase in IL-12 in these groups, Kriegel et al. (2006) show that IL-12 induces IFN-γ production while G5 shows a significant increase, and other groups have a non-significant increase in IFN-γ, despite a decrease in IL-12, another study suggested that the results of IFN-γ increase in IFN- may be due to induction by IL-18 or IL-1B cytokines (Tominaga et al., 2000).

Interferon-γ production is inhibited by IL-4 and IL-10 (Gattoni et al., 2006), but this result is not very clear in this study, Cicchese et al. (2018) recorded a similar result who suggested that the balance in downstream activation and inhibition is not necessarily quantitative, but rather a qualitative harmonization of pro- and anti-inflammatory cytokines.

Th2 cells, stimulated by the cytokines IL-4, IL-10, and IL-13, promote B cell proliferation, antibody production, and class switching (Spellberg and Edwards, 2001).

Anti-inflammatory cytokine results show that IL-10 increased in all groups, a significant increase in G5, and a nonsignificant increase in all other groups may be due to IL-4 induced, as noted by (Mitchell et al., 2017), who illustrated that IL-4 triggers IL-10 production by CD4+ T cells, regulating pro-inflammatory and anti-inflammatory cytokines.

The three types of adjuvants make an orientation in immune responses toward humoral; this result agrees with Mozaffari et al. (2019), an additional signal for the type of response was mentioned to be the ratio of IFN-γ to IL-4. The vaccine adjuvant groups are all oriented toward Th2 immune responses, with a Th1 response identified if the ratio was greater than one and a Th2 response if it was less than one; this result is illustrated in Table (3).

Regarding the comparison between the types of soluble antigen and the whole bacteria, the results were fluctuating and were not clear considering the direction of immunity and cytokine stimulation. This study suggests that the reason is the interaction of the type of antigen with the type of adjuvant, where the type of interaction that occurs with each type of adjuvant leads to a difference in response between the types of vaccines, and this study agrees with Fox et al. (2013).

Therefore, maintaining a balance between pro-inflammatory and anti-inflammatory cytokines is crucial to prevent the breakdown of body tissue and lead to antibody production depending on the nature of the antigen and other

factors.

CONCLUSIONS

The adjuvant of the alum hydroxide vaccine induces a higher production of pro-inflammatory and anti-inflammatory cytokines compared to natural adjuvants. Additionally, it is more inducible of TH2 and the production of antibody responses. However, natural adjuvants induce less innate and adaptive immunity than those of alum; this means that they may be less harmful than alum vaccine adjuvant, so they may be the best alternative for alum and have fewer side effects.

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CONFLICT OF INTEREST

There are no disclosed conflicts of interest for the authors.

NOVELTY STATEMENT

This study is significant because it shows that using nigella oil and an alcoholic propolis extract to boost the immune system in rats can boost type 2 immune responses, which are higher in these animals than in control groups.

AUTHORS CONTRIBUTION

The authors attest to having sole responsibility for the following: planning and designing the study, gathering data, analyzing and interpreting the findings, and preparing the manuscript.

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