RESEARCH ARTICLE

IMMUNOLOGIC ADJUVANT ACTIVITY OF NEEM LEAF EXTRACT ON ENHANCING HUMORAL IMMUNE RESPONSE

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Abstract: The immune response is divided into the innate and the adaptive immunity, both include humoral and cellular components. The immune responsiveness leading to a specific immunity,*e.g.*, humoral immune response, depends on the antigen that the body sees as harmful or foreign. Unfortunately, not all the antigens are able to stimulate the immune system. So that, immunologic adjuvants are used to reinforce the immune response against a weak immunogenic antigen. Thus, the aim of this work was to evaluate the immunologic adjuvant activity of Neemaqueous leaf extract over humoral immune response of *Wistar* rats immunized with bovine serum albumin (BSA).Phytochemical compositionof the extract was determined and cytotoxicity tests were performed on the extract to stablish the concentrations to be tested. Later,25 rats were divided into 5 groups of 5 animals each and immunized in a 30-day immunization scheme with bovine serum albumin as an antigen and several adjuvants.The groups were: 1) PBS, 2) BSA, 3) Aluminum adjuvanted BSA, 4) Neem adjuvanted BSA, and 5) Freund's complete adjuvanted BSA. At the end of the immunization the antibodies were purified, quantified and isotyped. Finally, the specificity of the antibodies was evaluated by immunoprecipitation against BSA. Neem extract showed no cytotoxicity (LD50 > 400 µg/ml), in the phytochemical profile carbohydrates, flavonoids and tannins were identified. No significant differences ($p > 0.05$) were found among antibody isotypes and concentration in the study groups. However, significant differences were found in the specificity of antibodies ($p < 0.05$) when compared Freund's complete adjuvant against other groups but Neem.

Keywords: Immunologic adjuvant, Neem, *Azadirachta indica*

INTRODUCTION

The immune system is confirmed by cells, molecules, organs and tissues that protects the body from endogenous or exogenous foreign substances, which may be harmful, such as malignant cells, microorganisms or toxins (Parkin & Cohen, 2001; Satler, 2017; Beutler, 2004). Besides,the immune system involves the cooperation and interaction of different types of cells such as macrophages, neutrophils, lymphocytes, among others; and soluble or humoral proteins such as those of the complement system and antibodies. In this way, the immune system includes the cell-mediated immunity and the humoral immunity, respectively (Prager *et al.*, 2017; Fischinger *et al.*, 2019).

The immune response generated by the cellular and protein components of the immune system is fundamental when a foreign substance is in contact with the body and it should be neutralized or eliminated in order to avoiding any kind of damage (Huang, 2021). The immune response is divided into: 1) Innate, that is also defined as natural and non-specific immunity; and 2) Adaptive, that is also defined as adjustable and specific. The innate immune response is ready to procced even before the foreign substance be in contact with the organism, its mechanism of action involves generic recognition such as patter recognition; for instance, the pattern-

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recognition receptor known as TLR-4 binds to bacterial lipopolysaccharides which triggers a series of reactions to eliminate the pathogen (Thompson et al., 2011; Park *et al.,* 2013). So that, innate immunity occurs quickly within minutes or hours. On the other hand, the adaptive immune response requires that the foreign substance be in contact with the organism before proceeding, its mechanism of action involves the development of specialized recognition such as that provided by the antibodies (Goulet et al., 2020). Consequently, adaptive immunity takes much longer to become effective, typically a few days.

The B and T lymphocytes are the cells of the adaptive immunity, whose effector mechanism are: 1) humoral, as the case of the immunoglobulins or antibodies produced by the B lymphocytes; or 2) cellular, as the case of the cytotoxic CD8+ T lymphocytes (Germain, 2002; Raposo*et al.*, 1996).

The humoral immune response, in the case of immunoglobulin synthesis, begins with the uptake of a foreign substance by a professional antigen presenting cell (APC) (Zanoni & Granucci, 2010); then, the APC processes the up taken antigen which implies molecular degradation, for instance from a big complex protein of thousands of amino acids into a small simple peptide of a few amino acids (Riese& Chapman, 2000; McCarthy *et al*., 2015). Then the peptide couple to a molecule known as MHC-II (major histocompatibility complex II), and this MHC-II-Peptide is transported towards the cellular membrane where it will be bind with a specific T helper lymphocyte through the T-Cell receptor (TCR) leading to the lymphocyte sensitization (Roche *et al.*, 2015; Harton*et al*., 2016; Szeto *et al*., 2020). Later, the sensitized T lymphocyte will activate a specific B lymphocyte with a MHC-II-peptide that matches with the TCR of the sensitized T lymphocyte. Once the B lymphocyte is activated, it will go through several cellular transitions to become anantibody-producing plasma cell (Fillatreau, 2019).

In this context, the humoral immune response is lead by the antigen itself, but unfortunately not all the antigens are able to stimulate the immune system (Duquesnoy, 2014). The antigen capacity of stimulating an immune response, known as immunogenicity, depends mainly in psyco-chemical characteristics for instance being a particulate or soluble molecule, a small o big molecule, a complex o simple chemical structure, and so on (Irais*et al.*, 2020; Reyna-Margarita *et al*., 2019). For these reasons, when antigens are used as a vaccine formulation that pretends to generate immunity as a pre-exposition prophylaxis, this formulation includes adjuvants which are diverse kind of molecules that improves the antigen immunogenicity with the aim of enhancing an immune response such as the humoral immunity.Thus, the aim of this work was to evaluate the immunologic adjuvant activity of Neem aqueous leaf extract over humoral immune response of *Wistar* rats immunized with bovine serum albumin.

MATERIALS AND METHODS

Neem leaf extract

A sample of 800 g of fresh Neem leaves was collected during the months of December and January in the city of Lerdo (Durango, Mexico) from a local vivarium. After being collected, the leaves were rinsed several times with tap water, separated and allowed to dry at room temperature.

Once dried, it was grinded with a manual mill, and mixed in a 1:10 solution with hot distilled water at 60° C for 1 h, afterwards, it was filtered with a Watman filter number 40, and allowed to dry in a hot air oven at a temperature of 40° C for a period of 7 days.

Biotoxicity and cytotoxicity assays

The *Artemia salina* model was used to evaluate the biotoxicity. First, the *Artemia salina* was cultivated by placing 10mg of *Artemia salina* eggs in 250 mL of artificial seawater (450 mM NaCl, 10 mM KCl, 9 mM $CaCl_2$, 30 mM $MgCl_2*6H_2O$, 16 mM MgSO4*7H2O) supplemented with 60mg of yeast extract. Then the eggs were incubated at 28 °C for 48 h. Once the eggs of *Artemia salina* hatched, a standard curve (0, 1, 10, 100, 250, 500, 1 000, 5 000 µg/mL) of the extract was prepared up to 10 mL with artificial seawater. A sample of *Artemia salina* (N=10) was added in triplicate into test tubes for each of the concentrations under study. Potassium dichromate was used at 1 000 µg/mL as a positive control. During this assay, the samples were incubated at 28 °C for 24 h; subsequently, live and dead *Artemia salina* larvae were quantified, and the lethal dose 50% (LD50) was estimated by regression.On the other hand, the cytotoxicity assay was done as follows. First, a two-fold dilution (0.00, 3.125, 6.25, 25.00, 100.00, 400.00µg/mL) of the extract was done with RPMI-1640 supplemented medium into a ninety-six-well tissue culture plate. Then, the plates were seeded with $1.5x10^6$ cells, of thecell line J774A.1 (ATCC), and the cultures were incubated at 37°C, 5% CO2 and 95% humidity for 72h. Later, 10 µL of resazurin dye solution were added to each well and incubated for another 2 h. Finally, samples were measured spectrophotometrically by monitoring the decrease in absorbance, in a Magellan TECAN Microplate reader, at a wavelength of 535 and 595 nm.

Phytochemical tests

These tests were qualitative to identify the main chemical groups of organic compounds present in plant extracts. The principle of these tests based on chemical reactions between the functional chemical groups of organic compounds present in plant extracts and chemical reagents that led to the formation of precipitates or colored substances. The following determinations were done according to the

methodology published elsewhere (Barboza-Herrera et al., 2021): 1) Alkaloids (Dragendorff and Mayer's method); 2) Aldehydes (Tollens' method); 3) Carbohydrates (Brady'smethod); 4) Flavonoids (Magnesium method); 5) Sterols (Liebermann-Burchard's method); 6) Tannins (Indirect precipitation method); and 7)Terpenoids (Chloroformmethod).

Handling of animals

All protocols used in this study were approved by the Ethics Committee of Faculty of Medicine, Universidad Autonoma de Coahuila Unidad Torreon (reference number CB031118).The blood samples were collected, by cardiac puncture, in BD microtainer tubes with SST® coagulation activator polymer gel (Catalog No. 365967).The animals were sacrificed by cervical dislocation.To evaluate the humoral immune responseinduced with the neem-based adjuvant, twenty-fivespecific-pathogen-free (SPF) *Wistar* rats with an age of 6 weeks and a weight of 200-250 g were used. The animals were randomly divided into five groups, of fiverats each one. The groups included the following treatments: 1) PBS, 2) PBS+BSA (100 µg), 3) NEEM+BSA (10 µg/mL and 100 µg, respectively), 4) AL+BSA (500 µg of aluminum hydroxide and 100 µg of BSA), and 5) FCA+BSA (The emulsion of Freund's Complete Adjuvant 1:1 and 100 µg of BSA).

Immunization scheme

The first day, the animals were primed intradermally (*i.d.*), at three different sites (thigh pocket, base of tail, and mediastinum), with a final volume of 500 µL of the treatment according to the group to which the animal belonged. The rats received a booster dose on day 14^{th} (a half dose of the treatment in a final volume of 500 µL phosphate buffered saline (PBS); and finally, the rats received a second booster dose intraperitoneally (*i.p.)* on day 28^{th} (a complete dose of 500 µL of the treatment according to the group to which the animal belonged). Blood samples were collected on day 30^{th} for analysis of humoral immunity and the animals were sacrificed by cervical dislocation. This design with three applications was selected based on what was reported by other authors for other conventional immunologic adjuvants (Gupta, 1998; Maruyama *et al.*, 2002; Lindblad & Schonberg, 2010).

Immunoglobulin purification and quantification

Immunoglobulinwas precipitated two times with ammonium sulfate saturated solution [80% w/v (NH4)2SO4, pH 7.8] in a proportion 2:1, samples were centrifuged at 10,000 revolution per minute (rpm) for 5 min. A third precipitation was done and the pellet was diluted with Borate buffered saline (BBS, 100 mM H3BO3, 25mM Na2B4O7, 75mM NaCl, pH 8.2), then the immunoglobulin precipitate was dialyzed for 3 days at 4 °C in cellulose membrane of 14,000 Da molecular weight cut-off (Cat. D9402. Sigma-Aldrich St. Louis, MO, USA) with BBS. The dialysate solution was changed every 12 h, and a solution of Barium chloride (10% BaCl2) was used as a dialysis control. Immunoglobulin quantification was done by Biuret method. Briefly, a two-fold standard curve of BSA (0.00, 0.11, 0.22, 0.43, 0.87, 1.75, 3.50 and 7.00 g/dL in BBS) was prepared. A sample of 25 µL mixed with 1 mL of Biuret reagent (6 mM CuSO4•5H2O, 22 mM NaKC4H4O6•4H2O, 0.75 mM NaOH) by triplicate and the optic density (OD) was measured spectrophotometrically at 540 nm. The immunoglobulin concentration was calculated by interpolation of the OD against the BSA standard curve.

Immunoglobulin isotyping

Immunoglobulin samples were isotyped by ELISA (Rat Ig isotyping Ready-SET-go! Cat. 88-50640, Thermo Fisher Scientific Waltham, MA, USA) according to the specifications of the manufacturer to determine Ig G1, Ig G2a, Ig G2b, Ig G2c, Ig A and Ig M.The blocking buffer used in the ELISA test was 1% Ovalbumin (OVA) and the OD was measured at 450 nm in an ELISA reader.

Immunoglobulin precipitation against BSA

The humoral-specific immune response was measured as anti- BSA antibody titers by means of the antigen-antibody precipitation inhibition. First, a two-fold dilution, 60 µL (adjusted to 0.100 g/dL) from 1:2 until 1:1024, was done with the dialyzed immunoglobulin precipitates and PBS into 96- well microtiter plates. Then 20 µL of each dilution was slowly mixed with 20 µL of antigen solution (0.5 µg/µL w/v BSA/PBS) into a capillary glass tube. In addition, the capillary was incubated at room temperature for 24 h. Each immunoglobulin precipitate was analyzed by triplicate. The highest dilution that prevented antigen-antibody precipitation was considered as the Log2 titer.

Statistical analysis

The results were expressed as mean ± standard errors (SE) and examined for their statistical significance with ANOVA and *post-hoc* minimum significant difference (MSD). *p value < 0.05 and **< 0.01 were considered to be significant. The statistical package Graph Pad Prism Version 9.3.1was used.

RESULTS AND DISCUSSION

According to the results of the biotoxicity test with the*Artemia salina* model, a lethal dose 50(LD_{50}) was calculated on 1077 µg/mL;and, this concentration permitted the researchers to classify it as not toxic, considering the toxicity cut-off point of200 µg/mL where lower concentrations are considered toxic. Besides, these datawas corroborated with the results of the cytotoxicity assay with the macrophage cell line J774A.1 where none of the neem extract concentrations tested had an effect on cell viability; thus, concluding that the LD50 was higher than 400.00 µg/mL.

The qualitative phytochemical tests of the aqueous extract of Neem leaf allowed identifying the presence of carbohydrates, flavonoids, and tannins as shown in Table 1.

Table 1. Phytochemical tests of the Neem extract.

Phytochemical test	Result
Alkaloids	-
Aldehydes	-
Carbohydrates	+
Sterols	-
Flavonoids	+
Tannins	+
Terpenoids	-

+ positive, - negative.

The analysis of humoral immunity comprised immunoglobulin quantification, isotyping and anti-BSA antibody titers. The immunoglobulin concentrations among the groups were: PBS (0.116 ± 0.03), PBS+BSA (0.134 ± 0.06), NEEM+BSA (0.203 ± 0.05), AL+BSA (0.151 ±0.02), and FCA+BSA (0.157 ± 0.02). ANOVA analysis showed no significant differences among the groups' means ($p=0.706$) as shown in Figure 1. Immunoglobulin isotypes are shown in the radial plot of Figure 2; where, ANOVA analysis showed no significant differences among the antigen (BSA) immunized groups PBS+BSA, NEEM+BSA and FCA+BSA, which indicated that none of the adjuvants seems to polarize the immune response towards an specific type of response. In the case of the PBS control the differences against previous groups are attributed to the antigen since this group was not immunized with BSA. Even though, the immunoglobulin concentration did not show differences among the groups it was important to evaluate the specific anti-BSA antibodies among the groups, as can be seen in Figure 3, ANOVA analysis showed significant differences ($p<0.01$) when comparing the PBS group against all other groups. A similar pattern was observed when comparing the FCA+BSA group against all other groups ($p<0.01$) but the NEEM+BSA group ($p>0.05$).

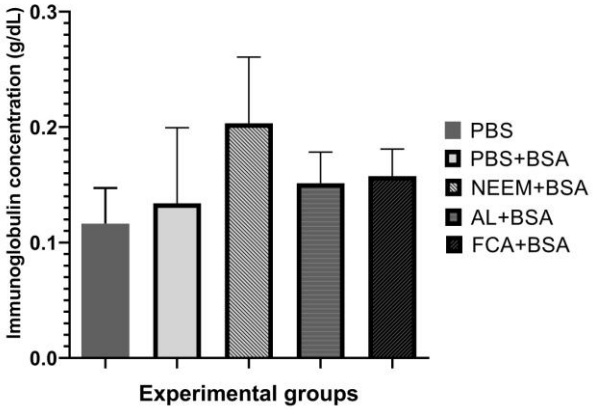

Figure 1. Immunoglobulin concentration (g/dL) among the experimental groups.

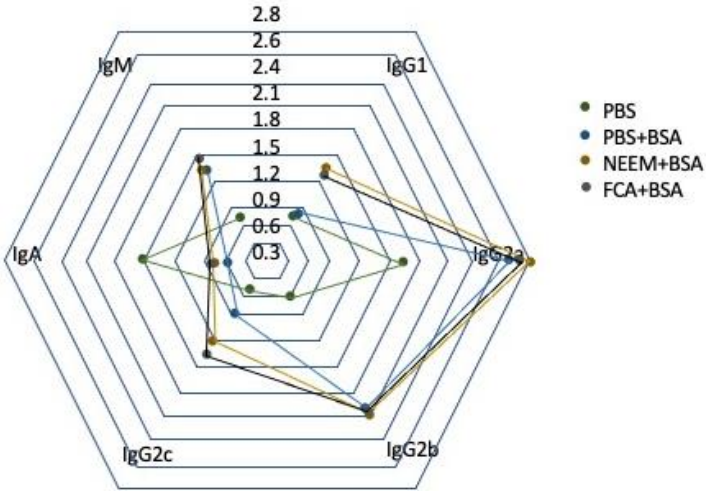

Figure 2. Radial Plot of immunoglobulin isotypes (as means of OD 450 nm) among the experimental groups. The ±SE are not shown to permit visual comparison.

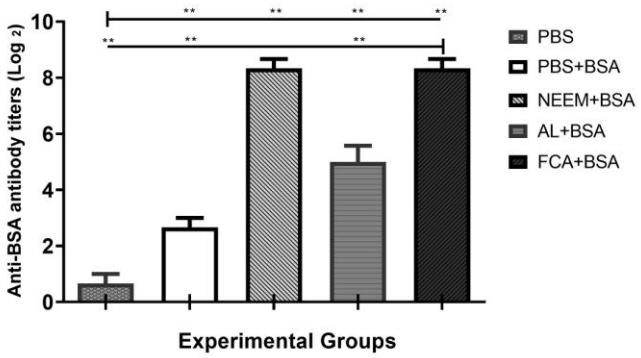

Figure 3. Anti-BSA antibody titers (Log2) among the experimental groups. *p-value <0.05 and **p-value <0.01 were considered to be significant.

CONCLUSION

The Neem based adjuvant showed to be safe based by the biotoxicity and cytotoxicity assays; besides, this adjuvant induced specific anti-BSA antibody titers comparable to those induced byFreund's complete adjuvant and similar results were found when comparing the immunoglobulin isotypes induced. Since this novel adjuvant did not show the high toxicity exhibited by the FCA the authors propose it as a promising vaccine adjuvant candidate.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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