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Adjuvant effect of Garlic extracts (*Allium sativum* L.) on the production of γ globulin in mice immunized with ovalbumin

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The antigens used in vaccines are usually attenuated or inactivated microorganisms, toxoids or purified particles. The purified particles have a better biosecurity but their capacity to generate an immune response is low, therefore vaccines include adjuvants that seek to improve immunogenicity. Unfortunately, adjuvants have side effects so only aluminum salts are currently used as adjuvants. So that this work evaluated an adjuvant of garlic extracts, a plant with immunomodulatory properties, in mice immunized with ovalbumin. To formulate the adjuvant, biotoxicity and cytotoxicity assays with a model of *Artemia salina* and haemolytic activity were considered respectively. A qualitative phytochemical analysis and quantification of phenolic compounds were carried out and in the immunization scheme 100 µg of antigen with adjuvant were administered at day 1, 50 and 100 µg of antigen on days 14 and 28 respectively. The sacrifice of the animals was done on day 30. Leukocytes and γ globulins were quantified at the beginning and at the end of the experiment. A lethal dose 50% of 1430 µg/mL was calculated for the Garlic extracts, a haemolytic activity of 2.66% and 7.53% was observed ($p<0.05$) for the concentrations of 10 and 100 µg/mL. And it was only possible to identify the presence of tannins in the aqueous extract of Garlic. With the results obtained, significant differences were observed in leukocyte counts and concentration of γ globulins at the end of the immunization scheme ($p<0.05$). Concluding that the results with the adjuvant of Garlic at 10 µg/mL concentration were comparable to those found with the adjuvant of aluminum salts.

Keywords: Adjuvant, *Allium sativum L*, Garlic, Mouse, γ globulin

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An immune-adjuvant or simply adjuvant is a substance that when administered in a person or an animal simultaneously with an antigen, i.e., another substance capable of generating a response of the immune system, facilitate a more effective immune response[1,2]. Incorporating adjuvants in immunization schemes or vaccines (biological preparations that provide acquired immunity), allows an antigen and time economy, as well as a greater production of specific γ globulins or antibodies[3].

The purpose of incorporating adjuvants in human or veterinary vaccines relays on their capacity for enhancing the production of antibodies in the immunized subjects[4,5]. The applications of immunization schemes, with regard to antibody production, can be of two Types: 1) In research, antibodies are used to identify or isolate molecules, or 2) In medicine, antibodies are used to prevent infectious diseases or hypersensitivity reactions[6].

The mechanisms of action of the adjuvants depend on intrinsic characteristics capable of potentiating the immune response for example physicochemical factors such as hydrophilicity, lipophilicity, amphipathicity, etc. or for example biological origin if they are bacterial or cellular products such as toxoids or cytokines, respectively[1,7]. According to the above

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intrinsic characteristics, adjuvants can act on three levels: 1) Immunological stimulation dependent on the antigenic presentation, where a lipophilic adjuvant with deposition effect, guarantees a slow and prolonged release of antigen, in addition it recruits antigen-presenting cells at the site of inoculation[8]; 2) Stimulation of receptors and co-receptors since the immunological response requires in addition to the antigenic presentation and stimulation of receptors such as those of the major histocompatibility complex (MHC), B (BCR) or T cell receptors (TCR), co-stimulation of co-receptors such as CD40-CD40L or CD28-CD80/86[9,10]. Where the physicochemical properties of the adjuvant can lead to a cross-linking of receptors resulting from molecular interactions such as electrostatic charges or hydrogen bridges, to mention a few and 3) Polarization of immune response to a pattern Th1, Th2, Th17, etc., whose principle of action would be determined by the cytokines profile that are released by the cells involved in the immune response. This polarization depends on the type of antigen the route of inoculation and mainly the adjuvant used. The latter could be a bacterial or cellular product capable of polarizing the type of response[11].

The vaccines classifies mainly into 4 types depending on the antigen used in their formulation: 1) attenuated microorganisms, 2) inactivated microorganisms, 3) toxoids and 4) purified or recombinant microorganism particles. In the same order from highest to lowest, its ability to produce an immune response in a healthy person is represented as well as the pathogenic potential[2]. The potential risk when using vaccines with attenuated microorganisms in people with a weakened immune system is the plausible activation of the disease or a failure in the process of inactivation of the pathogenic microorganism will lead to catastrophic results. Vaccines currently used consist of sub units, purified or recombinant parts of microorganisms with an important negative impact on its immunogenicity[12]. For these reasons, the relevance of studying adjuvants relays on vaccine enhancement of effectiveness[13] and the importance is that unfortunately adjuvants are not free of undesirable effects therefore the adjuvants currently used in the formulation of vaccines are a few and consist mainly of aluminum salts[1,2,4,12].

Medicinal plants have shown to have immunomodulatory activity in *in vitro* studies or animal models[14]. To mention some of the medicinal plants with immunomodulatory activity, is *Uncaria tomentosa* commonly known as cat's claw, *Allium sativum* commonly known as garlic, *Croton lechleri* commonly known as dragon's blood and *Moringa oleifera* commonly known as moringa[15-18].

Garlic is a plant belonging to the *Liliaceae* family, originally from Central Asia, it is a plant used since ancient times for its culinary applications, as well as for its many medicinal properties. Garlic has immunomodulatory, antioxidant, lipid-lowering, antiatherogenic, antithrombotic, hypotensive, antimicrobial, antifungal and anticarcinogenic activity according to recent publications[16].

In vitro and *in vivo* studies have demonstrated the ability of garlic both to stimulate the proliferation of lymphocytes and phagocytosis of macrophages and the release of interferon-gamma. Likewise, it has been attributed to increase the activity of natural killer cells. Therefore, it can be assumed that the components of this plant increase the immunogenicity of an antigen[19-21].

In summary, the problem of the antigens used in vaccines or immunization schemes is the low immunogenicity of the antigen when it comes to highly purified molecules. And the aim of this work to approach a solution was to evaluate an adjuvant based on Garlic extracts on the production of γ globulin in Swiss mice immunized with ovalbumin.

Materials and methods

Biological material

All protocols used in this study were approved by the Ethics Committee of the Faculty of Medicine, Universidad Autónoma de Coahuila Unidad Torreón (reference number CB061017).

Twenty-five Swiss male mice with an age of 12 weeks old, weighing 15 to 20 g were used. The animals were divided in 5 groups of 5 mice each. The animals were housed in plastic boxes using sawdust as bedding with stainless steel grill covers. Water and food were offered *ad libitum*. The environmental parameters were monitored by means of a temperature and relative humidity meter. The photoperiod was 12 h of light and 12 h of dark.

The blood samples taken from the tail of the animal were less than 10% of the volume of circulating blood of mouse of approximately an amount of 150 μL, first the tail was heated with water at 40°C for 5-10 min, then cut perpendicularly with a sterile blade of scalpel, approximately 1-2 mm from the tail end, a gentle pressure was applied proximally to the cut and the blood was collected in BD microtainer tubes with

EDTA® (Catalog No. 365974), and BD microtainer tubes yellow with coagulation activator and SST® gel (Catalog No. 365967). Bleeding was carried out for a maximum time of 5 min at constant flowing; the final sample was taken from cardiac puncture prior to sacrifice.

The samples of garlic (*A. sativum L.*) were obtained from a local market in the city of Torreón, Mexico. An amount of 100 g was washed with water and macerated in a mortar with porcelain pistil. The extract was prepared with the macerated infusion of distilled water at 60°C in a 1:9 ratio for 30 min. Subsequently the supernatant was filtered on Whatman No. 40 filter paper and the filtered solution was evaporated at 40°C for 72 h in a hot air oven. The extract was stored until use at a temperature of -20°C.

General procedures

Phytochemical tests were qualitative tests 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. For the following tests, a standard solution of 10,000 μg/mL concentration was used.

Alcaloids (Dragendorff and Mayer' reagents)

This test based on the presence of nitrogen in the alkaloids which reacted with reagents containing bismuth or mercury and formed insoluble iodides or soluble oxyio duromercuriamonic compounds; respectively, both of color bright yellow.

For this test, 1 mL of the solution to be evaluated was placed in three test tubes, 1 drop of concentrated hydrochloric acid was added to each tube, the sample was then heated gently and the test tubes were left to cool. Then one drop of the Dragendorff reagent [Bi $(NO_3)_3$: $5H_2O$ 8%; HNO_3 20%; KI 1.6M], Wagner and Mayer [KI 5%; HgCl 0.05M] was added. The presence of a bright yellow color indicated the presence of alkaloids. An atropine solution was used as a positive control (Sigma Aldrich St. Louis, MO Catalog No. A0132) and distilled water as a negative control.

Aldehydes (Tollens' Reagent)

This test involved the oxidation of the aldehydes to the corresponding carboxylic acid, using a 5% ammoniacal silver nitrate solution. The positive test consisted of the formation of a silver mirror or a black silver precipitate. For this test, 5 drops of 5% silver nitrate, 1 drop of 2N sodium hydroxide and 3 drops of 10% ammonium hydroxide were placed in a test tube. Until this moment the solution was transparent, then 1 mL of the standard solution to be evaluated was added. A glucose solution was used as a positive control (Sigma Aldrich St. Louis, MO Catalog No. D9434) and distilled water as a negative control.

Carbohydrates (Brady' Reagent)

This reaction based on Brady's Reagent (with the reactant 2,4-dinitrophenylhydrazine) that quickly formed 2,4 dinitrophenylhydrazones with aldehydes and ketones, yellow or red pigments.

For this test 1 mL of the standard solution was placed in a test tube and 8 drops of 2,4-dinitrophenylhydrazine 20% were added in concentrated sulfuric acid, plus 3 drops of 70% ethanol. The formation of a red or yellow color indicated the presence of aldehydes or ketones. A glucose solution and fructose solution were used as a positive control and distilled water as a negative control.

Sterols (Liebermann-Burchard's reagent)

This test based on the reaction that occurred between the steroid rings with acetic anhydride with the formation of green or blue pigments. For this test, 1 mL of the standard solution to be evaluated was placed in a water bath of 50°C until the solvent evaporated completely, the sample was solubilized in 2 mL of acetic anhydride and cooled in ice, then 3 drops of concentrated sulfuric acid were added. The formation of a green or blue color indicated the presence of the steroid ring. Vitamin D was used as a positive control and distilled water as a negative control.

Flavonoids

This test based on the formation of pigments by the reaction that occurred between the gamma benzopirone ring with hydrochloric acid and Magnesium. For this test, 1 mL of the standard solution to be evaluated was placed in a test tube, a piece of Magnesium metal (10 mg) and 2 drops of concentrated hydrochloric acid were added. The formation of a red or blue color indicated the presence of flavonoids. A catechin solution was used as a positive control (Sigma Aldrich St. Louis, MO Catalog No. C1251) and distilled water as a negative control.

Proteins (Biuret's Reagent)

This test based on the ability of copper (II) sulfate in an alkaline medium to react with the peptide bonds and form a complex of violet color.

For this test, 1 mL of the standard solution to be evaluated was placed in a test tube and 1 mL of copper sulfate - sodium potassium tartrate was added ($CuSO_4$: $5H_2O$ [6 mM] $NaKC_4H_4O_6$: $4H_2O$ [0.02M], 3% NaOH). The color change of the Biuret reagent from Blue to Violet indicated the presence of proteins. A bovine albumin solution was used as a positive control (Sigma Aldrich St. Louis, MO Catalog No. A2153) and distilled water as a negative control.

Tannins

This test based on the tannins property to form precipitates of urea-soluble proteins which form pigments with ferric chloride.

For this test, 1 mL of the standard solution to be evaluated was placed in a test tube, 1 mL of a solution of gelatin 1% in physiological saline solution was added, and afterwards the sample was centrifuged at 3500 rpm for 5 min. The precipitate was resuspended in 1 mL of 10M urea, and then 3 drops of 5% ferric trichloride were added. The formation of a blue color indicated the presence of tannins. A tannic acid solution was used as a positive control (Sigma Aldrich St. Louis, MO Catalog No. 403040) and distilled water as a negative control.

Terpenoids

For this test, 1 mL of the standard solution to be evaluated was placed in a water bath at 50°C until the solvent evaporated in its entirety, 1 mL of a solution of glacial acetic acid and sulfuric acid [1:1] was added, then 1 mL of chloroform was added. The formation of a red or blue color indicated the presence of terpenoids. An ursolic acid solution in DMSO was used as a positive control (Sigma Aldrich St. Louis, MO Catalog No. U6753) and distilled water as negative control.

Quantification of total phenolic compounds (Folin Ciocalteu method)

This test required the use of a spectrophotometer. A sample of 150 µL of standard solution was mixed with 150 µL of Folin Ciocalteu 0.2 N reagent (Sigma AldrichSt. Louis, MO Catalog No. F9252). The mixture allowed to incubate at room temperature and left in the dark for 5 min, and the reaction was stopped with 300 µL of 0.35 M sodium hydroxide solution. The absorbance was measured at a wavelength of 760 nm. This result was extrapolated in a standard curve with concentrations of 0, 2, 4, 8, 10, 15, 20, 30 and 50 µg/mL of gallic acid (Sigma Aldrich St. Louis, MO Catalog No. G7384).

Biotoxicity assay with the Artemia salina model

To evaluate the biotoxicity of the extract the *in vivo* biotoxicity assay with the *Artemia salina* model was used. The *Artemia salina* was cultivated by placing 0.01 g of *Artemia salina* eggs in artificial seawater (40 g of sea salt in one liter of distilled water) with 0.06 g of yeast extract. This mixture was placed in an Artemia chamber at a temperature of 28°C for 48 h for the eggs to hatch. Once the eggs of *Artemia salina* hatched, a standard curve of the extract to be evaluated at a concentration of 0, 1, 10, 100, 250, 500, 1000, 5000 µg/mL was prepared in enough seawater for 10 mL. A sample of *Artemia salina* (N=10) was added in triplicate in test tubes for each of the concentrations under study. Potassium dichromate was used at 1000 µg/mL in seawater 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% (LD_{50}) was estimated by Probit regression.

Citotoxicity assay with the haemolytic activity model

An aliquot of 50 µL of mouse's blood with EDTA anticoagulant was washed 3 times with 950 µL of 0.89% NaCl sterile saline solution with centrifugation at 3500 rpm for 5 min. After the third wash, the cell pellet was re-suspended in a final volume of 50 µL of saline solution. Next, 950 µL of the extract to be evaluated was added in the saline solution at concentrations lower than the lethal dose 50% estimated in the biotoxicity assay with the *Artemia salina* model. The samples were incubated for 30 min at 37°C, then the sample was centrifuged again at 3500 rpm for 5 min, and the free hemoglobin was measured to the supernatant with a spectrophotometer at 412 nm. Saline solution and distilled water were used as minimum and maximum haemolytic controls, respectively.

Manual count of leukocytes

First, blood was taken with EDTA anticoagulant with the Thoma pipet up to the 0.5 mark avoiding the formation of bubbles. Subsequently, Turk's solution (2% glacial acetic acid and 0.1% gentian violet) was taken up to the 11 mark (which allows a 1:20 dilution). The sample was shaken vigorously for 3 min. The first 3 drops of the dilution were removed, an aliquot was placed in the Neubauer chamber (0.100 mm) and it left to rest for 3 min so that the leukocytes settle. To make the leukocyte count, the sample was observed at 40 x. The total of cells was

represented as the average in 4 quadrants that was multiplied by the factor obtained between the dilution of the blood and the depth of the chamber whose units were cells per μL.

Immunization scheme

The immunization scheme was developed according to Table 1.

The concentration of the adjuvant was prepared according to the results of the LD_{50} in the biotoxicity assay with the *Artemia salina* model and the results of the citotoxicity assay with the haemolytic activity model.

Purification of γ globulins

A half volume of supersaturated solution of ammonium sulfate was added slowly and with constant agitation to a volume of serum, contained in a conical tube, for 15 min, followed by centrifugation at 3500 rpm for another 15 min at room temperature, the sediment was resuspended in saline solution pH 7.8 until the original volume of the serum was reached. This treatment was repeated three times, in the fourth repetition the sediment was re-suspended in Borate buffered saline (95 parts of 0.89% saline solution and 5 parts of borate buffer [0.1M] and dialyzed into cellulose acetate membrane (12,000 kDa Sigma-Aldrich St. Louis, MO Catalog No. D9777-100FT) for 5 days with two daily changes against borate buffered saline at 4°C. A solution of 10% BaCl was used as dialysis control.

Protein quantification by Bradford method

The Bradford reagent was prepared with 5 mg of Coomassie blue G-250 (Sigma-Aldrich St. Louis, MO Catalog No. 27815) which was mixed with 2.5 mL of ethanol and 5 mL of 88% phosphoric acid which was gauged to 50 mL. For protein quantification, 1 mL of the Bradford reagent was mixed with 100 μL of the sample and the absorbance was read at a wave length of 595 nm in a spectrophotometer (Spectronic 20 Genesys; Sigma-Aldrich St. Louis, MO Catalog No. Z376027). The results were extrapolated from a calibration curve with bovine albumin at concentrations of 0.0, 2.0, 4.0, 6.0, 8.0 and 10.0 g/L.

Statistical analysis

The variables of the phytochemical tests were nominal. The rest of the variables were continuous and they were described with means and standard deviation. Student's t-test was used for difference of means in paired samples; linear regression and Probit regression were calculated with IBM SPSS 21 and GraphPad Prism 6 software.

Results and discussion

Phytochemical tests of the aqueous extract of Garlic

The phytochemical tests of the aqueous extract of Garlic showed only the presence of tannins as seen in Table 2.

Biotoxicity and citotoxicity assays with the Artemia salina and haemolytic activity models

Based on the results of the biotoxicity assay with the *Artemia salina* model, a LD_{50} of 1430 μg/mL (95% IC 1096-2245 μg/mL) was calculated. Therefore, we proceeded to evaluate the haemolytic activity of garlic extracts at concentrations lower than the LD_{50} that were 10, 100, 500 and 1000 μg/mL with the results obtained in Fig. 1. Only concentrations of

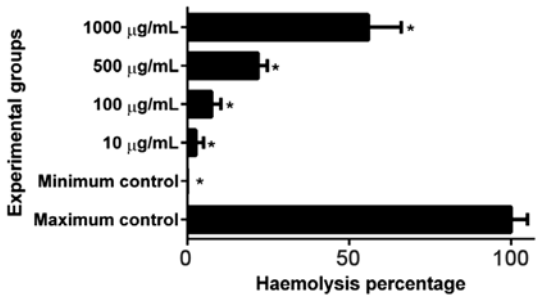

Fig. 1 — Haemolytic proportions with Garlic extracts. * Significant *p value*<0.05 compared with controls

Table 1 — Immunization scheme used.

Day	Activity	Content
1	Subcutaneous administration	Sterile adjuvant*, 100 μg of antigen in 500 μL of solution.
14	Subcutaneous administration	50 μg of antigen in 500 μL of solution.
28	Intraperitoneal administration**	100 μg of antigen in 500 μL of solution.
30	Sample taking and sacrifice	

*Sterilized with a syringe filter of 0.2 μm.** The route of administration in this application changed with the intention that the antigen reached the circulation faster.

Table 2 — Phytochemical tests of the aqueous extract of Garlic.

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

+ Positive. – Negative.

10 and 100 µg/mL showed haemolytic activity of less than 10%, which were 2.66% and 7.53% ($p<0.05$); respectively. In the case of the concentration at 500 µg/mL, a haemolytic activity of 21.85% ($p<0.05$) was shown and the concentration at 1000 µg/mL showed a haemolytic activity of 55.92% ($p<0.05$).

Manual count of leukocytes and γ globulins

According to the results obtained in the LD_{50} of the biotoxicity assay and the citotoxicity assay, only the aqueous extracts of Garlic were tested as adjuvant at concentrations of 10 and 100 µg/mL; an aluminum hydroxide adjuvant of 20 mg/mL was included. A group only immunized with the antigen in saline solution (Ovalbumin. Sigma Aldrich St. Louis, MO Catalog No. A5253) and a group only with saline solution were used as controls. The results of the leukocyte counts at the beginning and at the end of the immunization scheme are shown in Table 3.

After the immunization scheme, an increase in γ globulin concentrations was observed between the beginning and the end of the immunization scheme, although only the results of the negative control (which included only saline solution) were not significant as shown in the Table 4.

The statistical analysis of the results was carried out with the Student's t-test for the difference of means in paired groups. The results obtained between the beginning and the end of the experiments were contrasted for each of the experimental groups so each experimental animal had its own control to avoid bias due to biological variability. With these results, it could be corroborated that there were no significant differences in the case of the negative control group to which only saline solution was administered; the rest of the experimental groups showed significant differences both for leukocyte counts and for the concentration of γ globulins. In the case of leukocyte counts, the differences observed at the beginning and at the end of the immunization scheme are probably a consequence of the last administration of antigen on day 28, which would lead to cellular activation or recruitment of immune cells after the administration of a foreign substance such as the antigen. In the case of the concentration of γ globulins after an immunization scheme, a stimulation of the adaptive immune response would be expected, as it is possible to observe in the results found. It should be noted that the best results were obtained with the Garlic adjuvant at 10 µg/mL concentration (Table 4). Unfortunately, with the garlic adjuvant, a response with linear tendency was not observed because the best results were obtained at the lowest concentration under study. However, considering the results of the biotoxicity and cytotoxicity tests, it can be speculated that the effect could be masked by a cytotoxic activity at higher concentrations. Although other explanations are possible, *e.g.,* in a biochemical context, because phytochemical tests only showed the presence of tannins, *i.e.,* phenolic compounds capable of binding to proteins, an explanation for the non-linear results found could be that the amount of reactive chemical functional groups of both the tannins (hydroxyl groups) and the protein antigen (carboxyl groups of the amino acids) will limit the conjugation of both substances (chemical union of a hydroxyl group with a carboxyl group, this type of bonds may holds the biopolymers together)[22]. The immunogenic effect of such union could be explained as a consequence of the incorporation of rigid structures (as phenolic compounds) to the chemical structure of the antigen[23].

Table 3 — Leukocyte counts (cells/µL) between experimental groups at the beginning and at the end of the immunization scheme (30 days).

Group	N	Beginning $\bar{x}$ (S)	End $\bar{x}$ (S)	p-value*
Saline solution control	5	9660 (1760)	10871 (2208)	0.546
Antigen control in saline solution	5	9385 (1736)	13052 (1297)	0.038
Antigen control with aluminum adjuvant	5	8966 (1185)	12181 (2217)	0.022
Antigen with garlic adjuvant at 10 µg/mL	5	9631 (1515)	12638 (2521)	0.011
Antigen with garlic adjuvant at 100 µg/mL	5	9260 (1548)	12276 (2523)	0.078

* Student's t-test.

Table 4 — Concentration of γ globulin g/L in experimental groups after the immunization scheme.

Group	N	Beginning $\bar{x}$(S)	End $\bar{x}$ (S)	Difference	*p*-value*
Saline solution control	5	3.16 (0.28)	3.31 (0.36)	0.14	0.185
Antigen control in saline solution	5	3.24 (0.66)	4.42 (0.54)	1.18	0.001
Antigen control with aluminum adjuvant	5	3.13 (0.33)	4.33 (0.61)	1.20	0.003
Antigen with garlic adjuvant at 10 µg/mL	5	3.38 (0.39)	4.71 (0.31)	1.33	<0.001
Antigen with garlic adjuvant at 100 µg/mL	5	3.28 (0.73)	3.98 (0.45)	0.70	0.047

* Student's t-test.

Because tannins are generally attributed abrasive properties, the latter could be buffered to a limited extent by a protein antigen[24].

Another explanation of the non-linear response could be a consequence of the presence of phytochemical remnants in the extract since the phytochemical tests were qualitative but not quantitative. And the presence of lectins in Garlic extracts has already been reported; lectins are proteins that bind specifically to glycosidic residues such as those present in cell receptors[25-28]. The lectins of plants such as L-type lectins and galectins have shown to be very resistant to extraction methods and have even reported activity at nanomolar concentrations[29]. A non-linear response may be observed with lectins since they are specific at low concentrations but become nonspecific at high concentrations[29].

Conclusion

When evaluating the immunological adjuvant effect of *A. sativum L.* in mice in this work, it can be concluded that significant differences were observed in the leukocyte counts and concentration of γ globulins at the end of the immunization scheme using an adjuvant based on aqueous extracts of Garlic, with the best results at a 10 μg/mL concentration.

Conflict of interest/competing interests

The authors declare no conflict of interest.

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Authors' contributions

PED and SGLB conceived the hypothesis. PED, CMI and RGMA designed the study. EVD, DSCR, BHC, RBFC and LGS conducted the experiments. LGS, THM and DGD analyzed the data. CMI, SGLB, RGMA and PED wrote the manuscript. DGD checked and made necessary corrections. The authors thanks to Lizully Azeneth Alvarado-Ortiz and Paulina Lizeth Rivas-Herrera from the Centro Universitario Angloamericano for the English-translation of this work. All authors read and approved the final version of the manuscript.

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