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Research Article

Activity of *Lepidium meyenii* Walp (purple maca) in immunosuppressed *Oryctolagus cuniculus* (albino rabbits)

Angel T. Alvarado[1], Carlos Navarro[2], Mario Pineda[2], Laura Villanueva[2], Ana María Muñoz[3], María R. Bendezú[4], Haydee Chávez[4], Jorge A. García[4]

1 *International Research Network in Pharmacology and Precision Medicine, Human Medicine, San Ignacio de Loyola University, Lima, Peru*
2 *Peruvian Association of Immunogenomics and Personalized Medicine, Lima, Peru*
3 *Research Unit in Nutrition, Functional Foods for Health and Nutraceuticals, San Ignacio de Loyola University, Lima, Peru*
4 *Faculty of Pharmacy and Biochemistry, San Luis Gonzaga National University of Ica, Ica, Peru*

Corresponding author: Angel T. Alvarado (eaa.alvarado@hotmail.com)

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Abstract

Various properties are attributed to "maca", including immunomodulatory properties due to its secondary metabolites such as macamides, glucosinolates, isothiocyanates and flavonoids. Immunosuppression, hemolytic anemia, and thymic involution were induced with cyclophosphamide. Three concentrations of doses of dehydrated hydroalcoholic extract of purple maca (EHADM) were used for 30 days, the analysis of variance and Duncan's multiple comparisons test the results are statistically significant ($p<.05$) which shows immunostimulatory activity in the marrow bone (monocytes, lymphocytes and white blood cells) and antianemic (hematocrit 31%) compared to the negative control group (G-1). At 84 mg/kg and at 167 mg/kg, it shows immunomodulatory activity on the humoral response in 66.70% of the experimental animals (G-3 and G-4). It is concluded that the dehydrated hydroalcoholic extract of purple maca presents immunostimulating and immunomodulatory activity on the humoral response in 66.7% of the *Oryctolagus cuniculus* induced to immunodeficiency with cyclophosphamide.

Keywords

Ecotype, immunosuppression, macamides, purple maca

Introduction

Lepidium meyenii Walp (maca), belongs to the genus Lepidium and family Brassicaceae (Granados et al. 2020) native to the highlands of the central Andes of Peru that grows at high altitudes (3500–4700) and severe environmental conditions (Chen et al. 2017; Granado et al. 2020); being cultivated in recent years in Tibet, Yunnan and Xinjiang of China (Chen et al. 2017). The hypocotyls of maca are roots of different phenotypic colors such as yellow, white, purple and black, which do not affect the composition of the primary metabolites such as fatty acids (2.2%: linolenic, oleic and palmitic), carbohydrates (59%), proteins (10%: alanine, arginine,

aspartate, phenylamine, glycine, glutamate, isoleucine, lysine, leucine, serine, methionine and others) and fibers (8.5%), which are the primary nutritional source (Dini et al. 1994; Clément et al. 2010; Meissner et al. 2016); however, the phenotype influences the composition of secondary metabolites (McCollom et al. 2005; Zhao et al. 2012; Esparza et al. 2015; Meissner et al. 2016; Granados et al. 2020). Among the secondary metabolites, glucosinolates have been determined: isothiocyanates, p-methoxybenzylglucosinolate, m-methoxybenzylglucosinolate, 5-methyl-sulfinylpentyl (glucoalisin), p-hydroxybenzyl glucosinolate (glucosinalbin), glucoscobenantyl-4-enenzine glucosinolate (glucosinalbin), indolyl-3-methylglucosinolate (glucobrassicin) and 4-methoxyindolyl-3-methyl glucosinolate (4-methoxy-glucobrassicin) (Fahey et al. 2001; Dini et al. 2002; Lock and Rojas 2002); among the polyunsaturated fatty acids are macanes (0.15%) and macamides (0.84%) (Ganzera et al. 2002).

The reported macamides (N-benzylated alkamides) are acyclic keto acid, N-benzyloctadecanamide, N-benzyl-16-hydroxy-9-oxo10E, 12E, 14E-octadecatrienamide, N-benz1-9,16-dioxo-10E, 12E, 14E-octadecatrienamide, N-benzylhexadecanamide, N-benzyl-5-oxo-6E, 8E-octadecadienamide, 5-oxo-6E, 8E-octadecadienoic acid and macaridine (1,2-dehydro-N-hydroxypyridine) (Zheng et al. 2000; Lock and Rojas 2002; Zhao et al. 2005; Almukadi et al. 2013).

Hypocotyls have been studied according to the ecotype, reporting that red maca has an inhibitory effect on benign prostatic hyperplasia (BPH), black maca has aphrodisiac properties (Alzamora et al. 2007); in other studies, it is mentioned that macamides are responsible for antifatigue (Yang et al. 2016), antiosteoporotic (Zhang et al. 2006), analgesic, antidepressant and anxiolytic (Almukadi et al. 2013; Hajdu et al. 2014), and neuroprotective (Zhou et al. 2017). In traditional medicine it has been used for centuries to increase sexual activity and fertility (Gonzales et al. 2006; Uchiyama et al. 2014; Chen et al. 2017).

After conducting a review in the PubMed-NCBI database on the studies of the immunomodulatory activity of maca, it was found that the yellow ecotype has antitumor and immunomodulatory activity attributed to the stimulation of macrophages, increasing phagocytosis and production nitric oxide (NO), and other cells of the immune system (Alzamora et al. 2007). In another study it has been reported that the immunomodulatory activity of purple maca is due to alkaloids, macamides, glucosinolates and isothiocyanates (Alvarez and Alzamora-Gonzales 2013). This has prompted a preclinical phase 0 study of *in vivo* experimental pharmacology with purple maca ecotypes.

Our objective was to study the immunomodulatory activity of *Lepidium meyenii* Walp (purple maca) in *Oryctolagus cuniculus* (albino rabbits) induced to immunosuppression with cyclophosphamide.

Materials and methods

Chemicals

Sheep red blood cells (GRC) at 10% obtained from the National Institute of Public Health of Peru (INS), Sandoz cyclophosphamide 500 mg powder for solution for injection, dehydrated hydroalcoholic extract of purple maca (EHADM).

Type of study

A preclinical experimental study (phase 0) was carried out with a double blind and explanatory type.

Study period

The study was carried out from May 2019 to August 30, 2021.

Sample and study population

Convenience and intentional sampling of 18 albino rabbits (*Oryctolagus cuniculus*) between males (n = 10) and females (n = 8) of the New Zealand breed, with an average age of 3 months and body weight of 1900 ± 100 g.

The albino rabbits (*Oryctolagus cuniculus*) were acquired from the Bioterium of the National Center for Biological Products (CNPB) of the National Institute of Health. The 18 rabbits were kept under standardized laboratory conditions (12 h light/dark cycle, 22 °C) and in cages designed for this species. They were provided adequate food for their species and drinking water ad libitum. The animals were acclimatized for two weeks, after that time the experiment began.

Plant materials

The *Lepidium meyenii* Walp "ecotype purple maca" is a herbaceous plant, whose fresh hypocotyls were collected in the month of May 2019 in the Pazos district (Geographically it is located between 12°15'32"S, 75°04'13"W; 3840 masl), Tayacaja province, Huancavelica department, central Peru. The plant was identified and classified, according to the Cronquist Classification System (1988). A specimen of the plant is deposited in the Herbarium of the Natural History Museum of the National University of San Marcos, Lima, Peru, certificate N° 307-USM-2019.

Preparation of dehydrated hydroalcoholic extract of maca

37 kg of fresh hypocotyls of *Lepidium meyenii* Walp (purple maca) were collected, then all foreign substance was separated from the hypocotyl, it was washed until it is totally clean, then they were dried in the environment for a month, in its place of origin. , obtaining 30 kg of dried maca.

Then, the dry hypocotyls were pulverized, allowing them to macerate for 7 days in 70% ethanol; after that time, the mash was filtered, the hydroalcoholic phase was concentrated and evaporated in the forced recirculation evaporator equipment; The concentrate obtained was atomized at 70 °C obtaining a light beige fine powder. It was packed in a sachet to avoid humidity and stored in a dry environment until the day of the phase 0 experimental pharmacology experiment. The yield was 36.66% (11 kg/30kg) and with a shelf life of 2 years.

Design of the preclinical phase experiment

After acclimatization (15 days), the preclinical phase (phase 0) was carried out, for this, the albino rabbits were weighed and six groups were formed (n = 3 rabbits), following the methodology of Álvarez (2008):

Day 1 to 30, dehydrated hydroalcoholic extract of purple maca (EHADM) was administered according to doses to three groups (G-3, G-4 and G-5).

On day 22, the humoral immune response was evaluated by administering 10% sheep red blood cells (GRC) according to dose to five groups (G-2, G-3, G-4, G-5 and G-6).

Day 24 immunodeficiency is induced in four groups of albino rabbits by administering cyclophosphamide (G-3, G-4, G-5 and G-6).

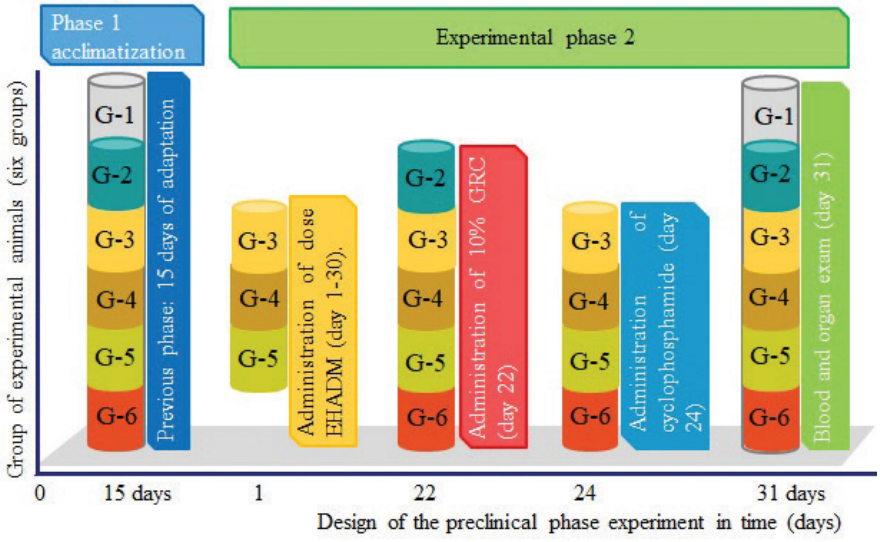
Day 31 blood test (complete blood count) and study of lymphoid tissues (spleen and thymus cellularity), for this, the rabbits were sacrificed fasting, as recommended by the Ethics Committee. After extracting the organs, they are cut and imprints are made on object slides of each of the organs, they are left to dry in the environment and then Wright staining is carried out and then the reading is made, in each group, taking in consideration of the different cell types that arise. Scheme 1 summarizes the experimental design.

Table 1. Administration of doses of dehydrated hydroalcoholic extract of purple maca, red blood cells of sheep, cyclophosphamide and physiological saline to *Oryctolagus cuniculus*.

Group	Placebo/drugs/biological product / EHADM	Animal treatment
G-1	Physiological saline	The negative control group was administered physiological saline (2 mL/kg of body weight) by means of a gastric gold tube.
G-3	EHADM-1	EHADM-1 was administered at a daily dose of 89 mg/kg of body weight by means of a gastric gold tube; administration time 30 days.
G-4	EHADM-2	EHADM-2 was administered at a daily dose of 167 mg/kg of body weight by means of a gastric gold tube; administration time 30 days.
G-5	EHADM-3	EHADM-3 was administered at a daily dose of 339 mg/kg of body weight by means of a gastric gold tube; administration time 30 days.
G-2 to G-6	GRC 10%	10% GRC was administered at a dose of 0.4 mL/kg body weight, intraperitoneally (IP).
G-3 to G-5	Cyclophosphamide	Cyclophosphamide was administered at a dose of 100 mg/kg body weight intramuscularly (IM).
G-6	Cyclophosphamide	The positive control group was administered cyclophosphamide at a dose of 100 mg/kg of body weight via IM.

The administration of placebo (saline solution), EHADM, 10% GRC and cyclophosphamide was carried out according to the design of the preclinical phase experiment, the same as described in Table 1.

During the experiment, severe pain and distress in the animals was avoided. At the end of the experimental study, all the animals were sacrificed with an intravenous overdose of sodium pentobarbital (120 mg/kg) (jugular vein). After the extraction of the lymphoid organs, all the dead animals were discarded according to the NTS N° 144-MINSA/2018/ DIGESA, Technical Health Standard "Comprehensive Management and Solid Waste Management in Health Establishments Support Medical Services and Research Centers " (Chávez et al. 2021).



Scheme 1. Summary of the design of the preclinical phase experiment.

Statistic analysis

The experimental data were entered into a database in the Microsoft Excel program. Analysis of variance (ANOVA) and Duncan's multiple comparisons test were applied to the results with a p value <.05; and the values were reported as average.

Ethical aspects

The study was carried out in strict compliance with the ethical standards of experimental procedures and animal care. The research protocol and consent for handling animals was approved by the Ethics Committee of the Faculty of Pharmacy and Biochemistry, National University of San Marcos, by official letter No. 0197/FFB-UDI-2019.

Results

Figure 1 shows the values of monocytopenia, lymphopenia and leukopenia induced by cyclophosphamide and the immunomodulatory effect of *Lepidium meyenii* Walp (purple maca) in the samples of agranulocyte white blood cells (monocyte and lymphocyte) obtained from *Oryctolagus cuniculus*. By analysis of variance (ANOVA) and Duncan's multiple comparisons test, the administered doses of EHADM for 30 days and after having induced immunosuppression with the alkylating agent are significant (p<.05), except in G-3 lymphocytes and G-5 compared to G-1 (φ not significant).

Table 2 Shows the ANOVA and Duncan's multiple comparisons test, it is observed that the means of agranulocytes (G-3, G-4 and G-5 with G-1) are greater than the critical values to compare two means with corresponding distance in range p (Rp), so the difference is significant (ω), except in two groups of lymphocytes.

Table 2. Results of the analysis of variance (ANOVA) and Duncan's multiple comparison test between the treatment groups with the control group.

Groups compared	Monocytes		Lymphocytes		White blood cells		ANOVA and Duncan
							p<.05
	Dm	Rp	Dm	Rp	Dm	Rp	
G-3 and G-1	0.17	0.13	0,17	0.54^{φ}	0.40	0.32	
G-4 and G-1	0.40	0.13	0.90	0.54	1.59	0.32	
G-5 and G-1	0.50	0.13	0.50	0.54^{φ}	1.67	0.32	
G-5 and G-6	0.57	0.13	0.67	0.52	1.30	0.31	

Dm: difference of means; Rp: distance in range p; ^{φ}Not significant.

After treatment with EHADM for 30 days, the percentage of hematocrit (Fig. 2A) and hemoglobin (g/L) (Fig. 2B) were evaluated in a blood sample obtained from *Oryctolagus cuniculus*, after the induction of hemolytic anemia immune system generated by cyclophosphamide. The ANOVA showed that at the proposed doses of EHADM the percentage of hematocrit is significant (p <.05) and for the values of g/L of hemoglobin the results were not statistically significant (p>.05). Duncan's analysis shows that the differences in the hematocrit means (G-3, G-4 and G-5 with G-1) are less than the corresponding Rp, so the difference is not significant (φ).

The hemagglutination of the rabbit serum was evaluated, after sensitization with GRC, observing serum anti-GRC antibodies (Ig M) in mesh form (positive reaction) in 66.70% in two groups of experimental animals (G-3 and G-4). In the control group (G-1) the presence of a button was observed (negative reaction).

Discussion

The present study shows that cyclophosphamide caused monocytopenia, lymphopenia, a decrease in the number

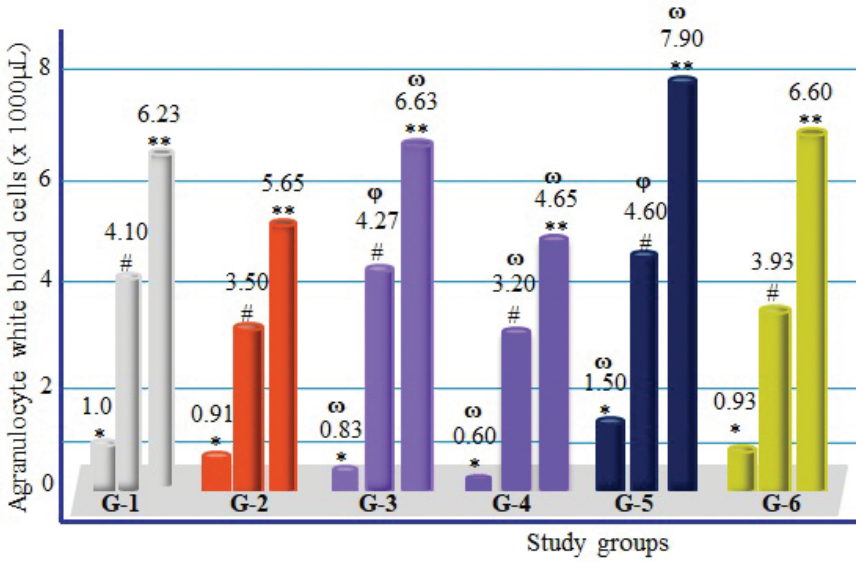


Figure 1. Immunomodulatory effect of *Lepidium meyenii* Walp (purple maca) on white blood cells agranulocytes of *Oryctolagus cuniculus*. *Monocyte; #lymphocyte; **white blood cell; Duncan's multiple comparisons: ω significant and φ not significant.

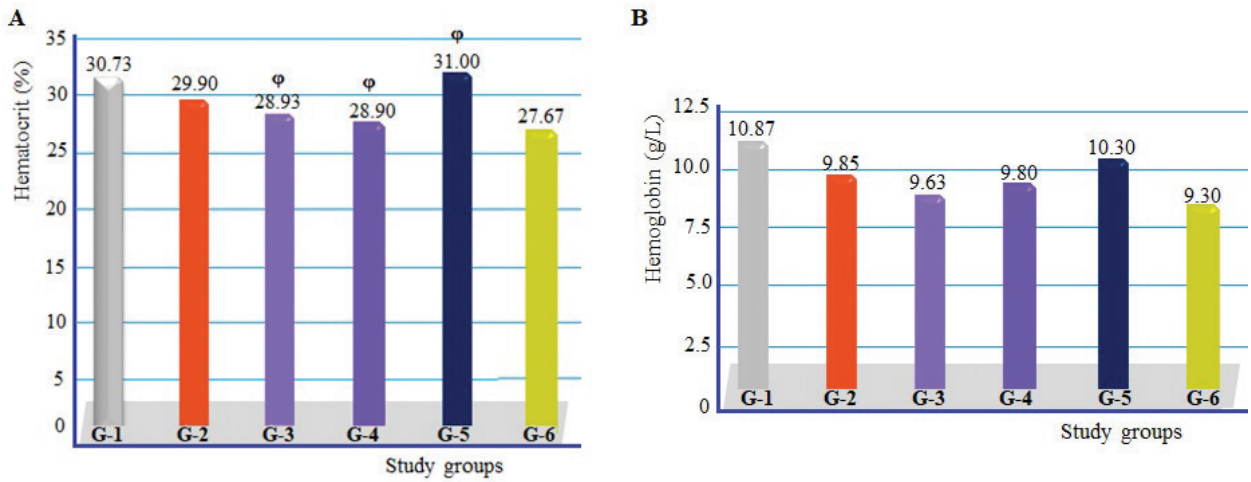


Figure 2. Antianemic effect of Lepidium meyenii Walp (purple maca) determined by hematocrit percentage and hemoglobin weight (g/ L).

Table 3. Percentage of antibodies in *Oryctolagus cuniculus* after sensitization with 10% sheep red blood cells (GRC).

Study group	Antibodies		
	Identification criteria	Result (negative or positive)	Percentage (%)
G-1	Button	Negativo	0
G-2	Mesh	Positive (n=1/3)	33.40
G-3	Mesh	Positive (n = 2/3)	66.70
G-4	Mesh	Positive (n = 2/3)	66.70
G-5	Mesh	Positive (n = 1/3)	33.40
G-6	Mesh	Positive (n = 1/3)	33.40

of white blood cells, a decrease in hematocrit and hemoglobin; involution of the thymus. After administering dehydrated hydroalcoholic extract of purple maca (EHADM) for 30 days, the immunostimulating and immunomodulatory effect was evaluated. At three EHADM dose concentrations, immunostimulatory activity is evidenced in the bone marrow, increasing the population of monocytes (cells of innate immunity), lymphocytes and total white blood cells; and an increase in hematocrit values, with respect to the control group (G-1). In previous studies, Alvarez (2008) demonstrated that the methanolic extract of purple maca induces a percentage increase in leukocytes in mice compared to the control group; Alvarez and Alzamora-Gonzales (2013) reported that yellow maca administered at a dose of 200 mg/kg in immunosuppressed mice favors the production of interleukin mRNA (IL-3 and IL-7 responsible for the stimulation of lymphocytes) and the granulocyte and monocyte colony stimulating factor (GM-SCF) in bone marrow, which supports what was reported in the present study. Lobatón (2003) reported an antianemic effect of maca in 50 people with iron deficiency anemia, as iron (Fe) increased significantly.

Immunomodulatory activity on the humoral response was observed in the present study at two dose concentrations of EHADM (89 mg/kg G-3 and 167 mg/kg G-4), after sensitizing with 10% GRC and inducing immunodeficiency with cyclophosphamide to experimental animals. In an experimental study carried out in mice immunosuppressed with cyclophosphamide, by Alvarez (2008) it is concluded that the administration of the purple maca extract increases the weight and cellularity of the thymus; while Torres (2008) observed that the yellow maca ecotype in immunosuppressed mice induces a high concentration of antibodies against GRC, this is possible, since the yellow ecotype stimulates type 2 helper T lymphocytes (Th2 or CD4+) produce IL-4, IL-5, IL-10, and IL-13, which are responsible for generating antibodies, as indicated by Alzamora et al. (2007). Quispe (2010) promotes the consumption of 3 g/day of the spray of the yellow ecotype of *Lepidium peruvianum* Chacón, to increase the production of surface antibodies to the hepatitis B virus (anti-HBs), the same that is generated between 1.2 and 6 months after starting the consumption of maca; while the count of Th2 lymphocytes (CD4 + involved in humoral immunity) and CD8+ cytotoxic lymphocytes (involved in cellular immunity) increased 2 months after the start of treatment compared to the control group.

Zhang et al. (2016), characterized a new polysaccharide of 11.3 kDa (MC-1: made up of arabinose, mannose, glucose and galactose) from *Lepidium meyenii* Walp, evaluating its activity in murine macrophages (Raw 264.7) and its immunomodulatory effect, concluding that MC- 1 stimulates macrophages (phagocytic and pinocytic capacity) and induces the expression of NO, IL-6 and TNF-α. Huayhua (2018) indicates that the methanolic extract of the purple ecotype of *Lepidium meyenii* Walp (maca) has an activating and immunomodulatory effect on peripheral blood mononuclear cells (PBMC) including T, B, NK lymphocytes, dendritic cells and monocytes, increasing the nitric oxide (NO) production and mRNA expression for TNF-α.

Through in vitro methods, the antioxidant activity of the aqueous extract of maca has been demonstrated, being capable of sequestering DPPH (1,1-diphenyl-2-picryl-hydrazyl) radicals at a concentration of 0.61 mg/mL, and at 0.43 mg/mL sequestering peroxynitrile radicals, the latter being a compound that occurs in chronic inflammations

and oxidizes DNA (Sandoval et al. 2002); In a study carried out on macrophages RAW 264.7 it has been shown that at a concentration of 1 mg/mL of aqueous extract of maca it inhibits apoptosis induced by peroxynitrile and maintains the synthesis of intracellular ATP under conditions of oxidative stress caused by hydrogen peroxide (H_2O_2) (Lock and Rojas 2002); However, when making a comparison of the catechin content in maca, it has been shown that it is low (2.5 mg/g) compared to green tea (145 mg/g), so that the antioxidant activity in vitro is higher in tea (Lock and Rojas 2002); while the anthocyanin content in purple corn is 0.86–2.87 mg/g, with a higher antioxidant activity (Ramos et al. 2012). Tang et al. 2017 reported that maca polysaccharides at high doses of 100 mg/kg bw/day of mice could significantly improve the activities of glutathione peroxidase and creatine kinase ($p < 0.05$), decrease the activity of lactate dehydrogenase ($p < 0.01$).

Liu (2019) postulates that purple maca contains thymic phytohormones that increase the size and cellular activity of the thymus. Alvarez and Alzamora-Gonzales (2013), have reported that the immunomodulatory activity of purple maca is due to alkaloids, macamides, glucosinolates and isothiocyanates, which stimulate the production of IFN-γ by human T lymphocytes cultured in vitro. Recently Batiha et al. (2020), have described that the anthocyanins and flavonoids (quercetin and catechins) in maca are responsible for the antioxidant and anti-inflammatory activity.

The main limitations of the study lie in the small size of the sample (n = 18), not having extracted the main secondary metabolites of maca and not having individually tested the immunological activity of glucosinolates, macaenes, macamides, flavonoids and anthocyanins. However, the main strength of this preclinical study (phase 0) is to generate scientific evidence of the dehydrated hydroalcoholic extract of purple maca in the immune system, which will allow the initiation of phase 1 clinical studies. In this sense, our research team does not promote the consumption of maca as a treatment, but as a functional food that could help maintain immune capacity.

Conclusions

In conclusion, our results suggest that the dehydrated hydroalcoholic extract of purple maca at the concentrations studied shows significant immunostimulatory and immunomodulatory activity on the humoral response in 66.7% of Oryctolagus cuniculus induced to immunodeficiency with cyclophosphamide.

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