Inhibición del crecimiento de Giardia Lamblia por acción del extracto acuoso y metanólico de semillas de Cucurbita Pepo

Giardia lamblia growth inhibition by action of the aqueous and methanolic extract of cucurbita pepo seed.

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Resumen

Giardia lamblia es el protozoario parásito causante de la giardiasis, la cual se caracteriza por molestia abdominal, pérdida de peso y desnutrición. La droga de elección para su tratamiento es el metronidazol sin embargo, presenta diversos efectos secundarios adversos en el paciente. Por otra parte, en la herbolaria se conoce a la semilla de *Cucurbita pepo* por sus propiedades desparasitante, principalmente sobre *Entamoeba histolytica* y *Taenia solium*. Por lo anterior se planteó la hipótesis de que las semillas de *C. pepo* contienen principios activos capaces de inhibir el crecimiento de *G. lamblia* bajo condiciones axénicas *in vitro*. El objetivo de esta investigación fue evaluar la actividad biológica del extracto acuoso y metanólico de las semillas de *C. pepo* sobre el crecimiento de *G. lamblia.* Tanto el extracto metanólico como el acuoso se identificaron siete grupos funcionales y la actividad antioxidante no fue significativa. En el extracto metanólico se observó mayor efecto giardicida seguido del acuoso, esta diferencia puede deberse a las saponinas que se encuentran sólo en el

extracto metanólico. Los resultados sugieren que las semillas contienen principios activos que pueden emplearse para la investigación de nuevos tratamientos para la giardiasis.

Palabras clave: Giardia lamblia, Cucurbita pepo, saponinas.

Abstract

Giardia lamblia is the protozoan parasite that causes giardiasis, which is characterized by abdominal discomfort, weight loss and malnutrition. The drug of choice for treatment is metronidazole however, presents several adverse side effects in the patient. Moreover, in the herbalist is known seed Cucurbita pepo by desparasitante properties, mainly Entamoeba histolytica and Taenia solium. Therefore it was hypothesized that the seeds of C. pepo contain active substances which inhibit the growth of G. lamblia axenic in vitro conditions. The objective of this study was to evaluate the biological activity of aqueous and methanolic extract of the seeds of C. pepo on the growth of G. lamblia. Both aqueous methanol extract as identified seven functional groups and antioxidant activity was not significant. The methanol extract showed higher aqueous followed giardicida effect, this difference may be due to the saponins found only in the methanol extract. The results suggest that the seeds contain active ingredients that can be used for research into new treatments for giardiasis.

Key words: Giardia lamblia, Cucurbita pepo, saponins.

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Introduction

Giardiasis

Giardiasis is a diarrheal disease caused by the protozoan *Giardia lamblia*, also know as *Giardia duodenalis* o *Giardia intestinalis*; a flagellate that resides in the small intestine of humans and other mammals. This protozoan presents two stages in its life cycle; the pyriform trophozoite and the cyst, which is the infective form. Infection in the host begins when the cyst is ingested through contaminated food and water or through fecal-oral contact. The cyst is relatively inert, allowing prolonged survival in a variety of environmental conditions. After exposure to the acidic environment of the stomach, the cysts excyst and the trophozoite is released into the proximal small intestine. The trophozoite is the vegetative form and replicates in the small intestine, causing symptoms of diarrhea and malabsorption, the incubation period is estimated to be 12 to 19 days. After exposure to bile fluid, trophozoites form cysts in the jejunum, which are passed in the feces, allowing *Giardia lamblia* complete its transmission cycle by infecting a new host (Adam, 2001), (Fig. 1).

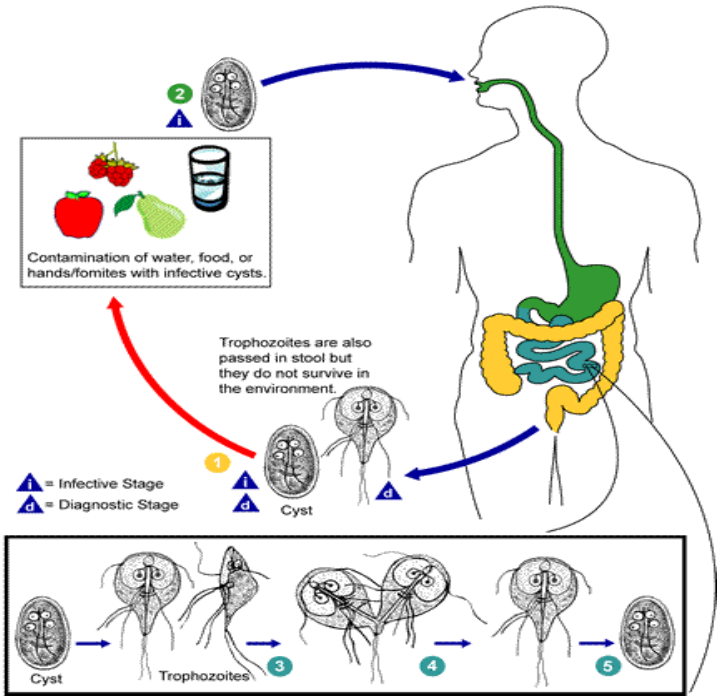

Fig. 1. Life cycle of Giardia lamblia. G. lamblia infection begins by ingesting mature cysts (2), excystation occurs in the duodenum, two trophozoites are released (3) which multiply by binary fission (4). As they migrate to the final part of the intestine, encystment occurs (5), the infective cysts are eliminated in the feces (1) (image taken from www.cdc.com).

> Because the transmission of G. lamblia requires the ingestion of parasite cysts, the level of environmental health is inversely proportional to the prevalence of the disease; thus, the highest prevalences are observed in developing countries (Vázquez and Campos; 2009). The high figures in the frequency of infection are due to the contamination of water or food with fecal matter, mainly affecting children and adults (Tay et al., 1996; Botero and Restrepo 1998; and Vázquez and Velasco; 1987).

The World Health Organization (WHO) has reported that there are 280 million people with symptomatic giardiasis and that 500,000 people are infected annually in America, Asia and Africa. In developed countries the prevalence is 2 to 5% and in developing countries it is between 20 and 69%. In Mexico, a frequency of 7.4 to 68.5% was reported (Tay et al; 1994 and Cifuentes et al., 2004), presenting the highest prevalence in preschoolers and schoolchildren (Vázquez and Campos; 2009). In our country, the morbidity rate of giardiasis in the years from 1995 to 2000 was less than two cases per 1,000 inhabitants in children between 1 and 4 years of age (Ximénez, 2002). 60% of children infected with *G. lamblia* They develop symptoms associated with bloating, epigastric pain, dehydration, abdominal pain, and chronic diarrhea with thick or steatorrheic stools containing large amounts of mucus and fat. High parasite loads have been shown to interfere with fat absorption mechanisms as well as the absorption of lactose, glucose, xylose, vitamin A and B12 (Cordingley and Crawford, 1986; Gillon 1985 and Beaver *et al.,* 2003).

Chronic giardiasis can last several months and is devastating in children, because abdominal pain is exacerbated during food intake and children stop eating, in addition to bloating, abdominal distention, fetid flatulence, malaise, asthenia, adynamia, weight loss, short stature and cognitive deficit. The stools are soft, steatorrheic and foul-smelling. It can alternate with periods of constipation or bowel movements of normal consistency. In this phase, patients can develop malabsorption of vitamins A and B12,

micronutrients such as iron and zinc, proteins, lipids and carbohydrates, especially lactose, sucrose, maltose and isomaltose (Becerril, 2008).

For the treatment of infections caused by *G. lamblia* the drugs of choice are metronidazole and other nitroimidazole derivatives such as tinidazole; furazolidone, quinacrine, albendazole and paramomycin. All these therapeutic agents produce side effects, such as nausea, metallic taste, yellowing of the skin (quinacrine), increased liver enzymes (albendazole) and nausea after taking them together with alcohol, an effect observed with metronidazole (Zaat et al; 2006; Huang and White; 2006). However, studies in mice have shown that metronidazole is both mutagenic and carcinogenic (Legator, et al., 1975). On the other hand, an in vitro study with mouse leukocytes showed that tinidazole at concentrations of 100, 250, and 500 µg/mL increases the percentage of damaged cells as the dose increases; in vivo it was observed that a dose of 100 mg/kg of weight is capable of damaging the DNA of mouse leukocytes (Rodríguez *et al,* 2001).

Natural alternatives are currently being sought to treat giardiasis using active ingredients isolated from different plants popularly used in traditional herbal medicine that do not cause side effects to the body. Traditionally, the seeds of Cucurbita pepo have been extensively studied from the pharmacological point of view, proving their anthelmintic and vermifuge activity, mainly due to the presence of cucurbitacins that accumulate in high concentrations in this part of the plant. (Osuna *et al*., 2005).

Cucurbita pepo

It is an annual herbaceous plant belonging to the Cucurbitaceae family, it has long stems stretching up to 10 m long, with very large leaves divided into 5 lobes and covered by rigid hairs. The fruits are very voluminous, contain flattened and whitish seeds. It is native to tropical countries, it is currently cultivated throughout the world for its edible fruits (Bravo, 2003).

> Pumpkin seeds contain fatty oil, proteins and carbohydrates, they are also rich in sterols, including lutein, carotenes and beta carotenes, and are also a source of vitamin A. The presence of small amounts (0.4 0.6%) of the amino acid has also been reported. cyclic cucurbitin (3-aminoi-3carboxy-pyrrolidine), to which it owes its vermifuge properties. Its oil content is high (45-50%), predominantly linoleic and oleic acids (Gopal et al., 2009), the use of dry or fresh pumpkin seeds without the shell is recommended for the treatment of benign prostatic hypertrophy, preventing prostate enlargement from occurring (Bravo, 2003).

Aqueous and methanolic extracts made from the seed have shown anthelmintic activity. Cucurbita pepo also has cytotoxic, antitumor and stomachic properties; as well as laxative, toxic and diuretic effects. In the stomach, cucurbitacins stimulate gastric secretion, due to the powerful bitter flavonoids it contains. It causes paralysis and expulsion of intestinal parasites, it has been scientifically proven that it has substances that destroy parasites of the digestive tract without affecting the mucosa or causing other undesirable effects (Osuna et al; 2005).

Since giardiasis is a disease that affects the world and the treatments currently used have undesirable side effects, there is a need to seek effective and economical alternatives to treat this disease. Compounds isolated from plants such as Cucurbita pepo, a plant native to Mexico widely used for many years, may be an option for the isolation and identification of giardicide compounds that act on the trophozoite stage of *Giardia lamblia.*

HYPOTHESIS

The aqueous and methanolic extract of the Cucurbita pepo seed contain active principles that can possibly inhibit the growth of Giardia lamblia under axenic conditions in vitro..

OBJECTIVES

To evaluate the biological activity of the aqueous and methanolic extract of the seeds of C. pepo on the growth of Giardia lamblia under axenic conditions in vitro.

METHODOLOGY

Giardia lamblia

1 Giardia lamblia maintenance: 16 x 150mm borosilicate tubes containing 10 mL of TYI-S-33 medium (Diamond, 1978) added with 1.0 mL of serum and 0.1 mL of penicillin-streptomycin solution (1000x) were used. 2x104 cells/mL were inoculated into each tube and incubated at 37°C for 72 h.

2 Growth kinetics of Giardia lamblia: The tube containing the inoculum was cooled at 0-4°C for 15 min. Subsequently, the number of cells/mL was determined. Eighteen tubes containing 10 mL of TYI-S-33 medium with 1.0 mL of serum and 0.1 mL of antibiotic were placed; subsequently they were inoculated with 2x104 cells/mL, incubated at 37°C/6 days. Every 24 h, triplicate counts were performed through a Neubawer chamber to determine the number of cells, thus obtaining the maximum cell yield.

Cucurbita pepo

1 Obtaining seeds: The seeds were extracted from the fruit of C. pepo.

2 Processing of C. pepo seeds

Pumpkin seeds were processed according to the following steps:

Drying: the seeds extracted from the inside of the pumpkin were left to dry in the sun

for two days at room temperature

Peeled: the shell of the seed was removed to obtain only the nugget

Macerated: the seeds were macerated in a porcelain mortar

Extraction: obtaining extracts from C. pepo seeds was carried out

as follows:

a) Hexanic extract: 400 mL of hexane and 209.33 g of the macerated sample of C. pepo seeds were added to an Erlenmeyer flask. The flask was covered from light and kept in this way in the shaker for 7 days. The phases were extracted with the help of a micropipette, placed in beakers and allowed to evaporate to dryness in an oven at 45°C. The extract obtained was covered with aluminum foil and kept refrigerated at 4°C until use.

b) Methanolic extract: from the sediment (macerated seeds) that remained in the hexanic extract, 400 mL of methanol were added, covered from light and kept in a shaker for 7 days. The phases obtained were separated with a pipette and placed in beakers to evaporate to dryness in an oven at 45°C. The extract obtained was transferred to conical tubes, covered from light and kept at 4°C until use.

c) Aqueous extract: 150 g of whole nugget were weighed in an Erlenmeyer flask, ground in a blender with 400 mL of warm purified water. Then it was passed through a gauze in a funnel to filter it, the filtrate obtained was recovered in a beaker; subsequently, the solid retained in the gauze was transferred back to the blender and ground with 300 mL of warm purified water, filtered and recovered again in a beaker, allowed to evaporate to dryness in an oven at 45°C . The extract obtained was transferred to a conical tube which was covered with light and kept refrigerated at 4°C until use.

Bioassays

1 Preparation of stock solutions. 4g of each extract were weighed, dissolved in 10 mL of TYI-S-33 medium, from this solution the aliquots were taken for the doses to be evaluated.

2 Chemical screening. The aqueous, hexanic and methanolic extract of C. pepo was subjected to phytochemical tests (Domínguez, X.A, 1973).

3 Evaluation of the biological activity of the aqueous and methanolic extract of Cucurbita pepo seed on the in vitro axenic growth of G. lamblia.

Twenty-two 13 x 100 mm borosilicate tubes were placed with 5 mL of TYI-S-33 medium added with 0.5 mL of serum and 0.05 mL of the penicillin-streptomycin solution mixture and 0.005 mL of bovine bile. the concentrations of each extract

were added: aqueous (0.1, 1.0, 5 mg/mL) and methanolic (5.16, 51.6, 516, 2460 µg/mL). Each tube was inoculated with 2x104 cells/mL and incubated at 37°C. At 72 h, counts were made in triplicate using a Neubauer chamber and the results obtained were subsequently analyzed.

4 Determination of the median lethal dose (LD50): The LD50 corresponds to the concentration required to reduce population growth by 50% at the dose analyzed. To determine the corresponding LD50 value of each chemical agent, B-Probit tables and the Microsoft Excel 2007 program were used.

5 Determination of antioxidant activity by the DPPH technique: a DPPH solution was prepared, for which 3.9 mg were weighed and calibrated with 100 mL of methanol. Stock solutions of the aqueous extract and the methanolic extract were prepared. 10 mg of each extract were weighed and dissolved in 10 mL of ethanol, from this solution the following concentrations were prepared: 25, 50, 100, 200 and 300 µg/mL. They were performed in duplicate, the DPPH reagent was added and they were left to stand for 30 minutes in the dark, immediately read at 517 nm in a Spectronic®Genesys 5 spectrophotometer.

Statistic analysis: Statistical analysis will be performed using ANOVA P>0.05 and the Dunnet-T Test (2-side) with the statistical package SSPS for Windows version 2007.

RESULTADOS

Aqueous and methanolic extract of C. pepo seed

1 Identification of functional groups. The results of the phytochemical tests for the extracts: aqueous and methanolic are shown in Table I. It is observed that all the extracts present alkaloids, carbohydrates, sterols, triterpenes, flavonoids, carbonyl groups and unsaturations. Aromaticity was detected in the aqueous and methanolic extract. Saponins were not detected in any extract.

Table I. Color tests for C. pepo extracts

Determinación de:	**Prueba colorida de:**	**Extracto de semilla**	
		Acuoso	**Metanólico**
Alcaloides	Dragendorff	+	+
Aromaticidad	Ácido sulfúrico-formaldehído	+	**+**
Carbohidratos	Molish	+	+
	Cumarinas	-	-
	Lactonas	-	-
Esteroles y Triterpenos	Liebermann-Burchard	**+**	-
	Salkowski	+	+
Flavonoides	H_2SO_4	+	+
	Leucoantocianinas	-	-
Grupo Carbonilo	2-4-Dinitrofenilhidracina	+	+
Insaturaciones	$KMnO_4$	+	+
Saponinas	Agitación	-	-
	Bicarbonato de sodio	-	-
	Salkowski	-	**+**
Sesquiterpenlactonas	Baljet	-	-
Oxhidrilos fenólicos	$FeCl_3$	-	-

2 Antioxidant activity: The results obtained from the antioxidant activity of the methanolic and aqueous extracts are shown in Table II. Vitamin C, whose EC50 was 15,070 µg/mL, was used as a positive control. The results indicate that the extracts do not have significant antioxidant activity.

Table II. Antioxidant activity of the aqueous and methanolic extract of *C. pepo*

Extracto de semilla de *C. pepo*	**CE_{50} (µg/mL)**	**Actividad**
Acuoso	>300	No antioxidante
Metanólico	>300	No antioxidante
Vitamina C (control)	15.070	Antioxidante

Giardia lamblia

1 Growth kinetics: In the growth kinetics of G. lamblia, no adaptation phase is observed, which is an indication of the good physiological state of the cell culture, a logarithmic growth phase is observed from its beginning until day three, reaching a maximum yield of 3,087,500 cells/mL on the fourth day. Subsequently, a moderate decrease in cell yield is observed, which continued for nine days, this kinetics is the result of three independent events in triplicate (Fig. 2).

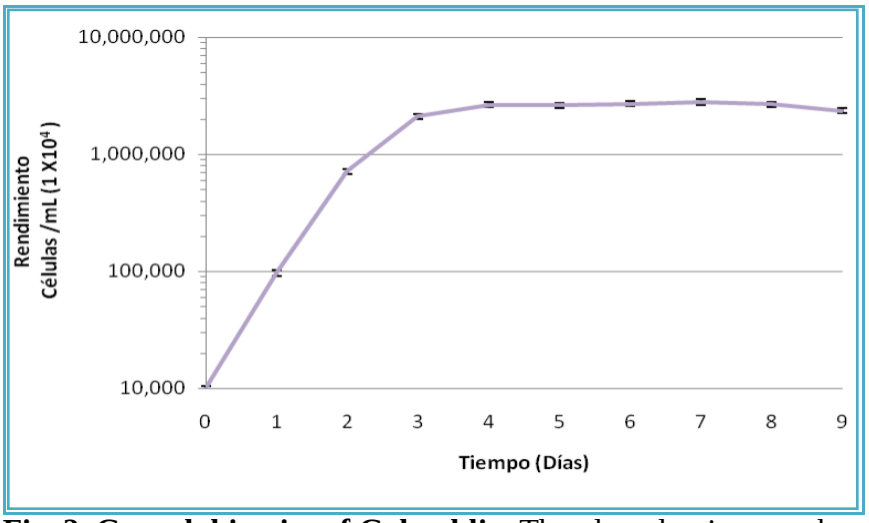

Fig. 2. Growth kinetics of G. lamblia. The plotted points are the result of three independent events in triplicate, plotting as mean standard deviation (+SD).

bioassay

1 Biological activity of the aqueous and methanolic extract of C. pepo on the in vitro axenic culture of G. lamblia: When evaluating the giardicidal activity of the aqueous and methanolic extract of C. pepo seed, it was observed that both extracts show inhibition, the aqueous extract it inhibited 78.6% at the concentration of 1.0 mg/mL (Fig. 3). On the other hand, the methanolic extract at a dose of 0.516 mg/mL inhibited 100% the culture of Giardia lamblia, which shows that it has greater giardicide activity (Fig. 4).

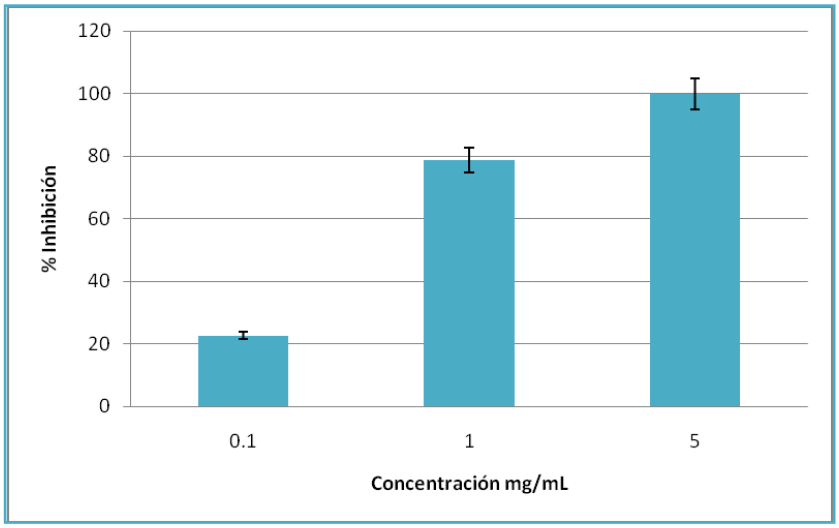

Fig.3 Biological activity of the aqueous extract of the seed of C. pepo on the in vitro axenic culture of G. lamblia.

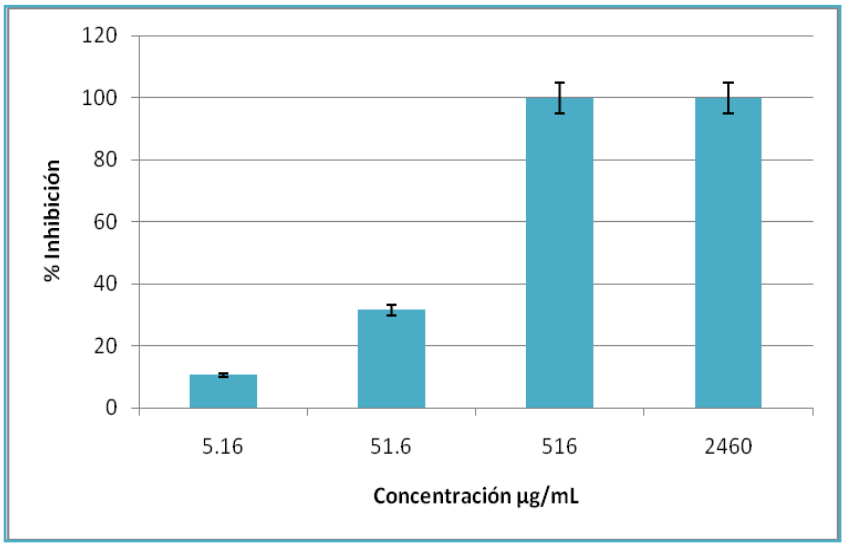

Fig. 4 Biological activity of the methanolic extract of the seed of C. pepo on the in vitro axenic culture of G. lamblia.

2 PROBIT analysis: Figures 5 and 6 show the median lethal dose (LD50) calculated for each of the extracts evaluated on the growth of G. lamblia, at 0.2563 mg/mL of the aqueous extract of C. pepo seed is inhibited 50% of the population of G. lamblia and the methanolic extract required 0.0435 mg/mL, this dose being lower when compared to that required with the aqueous extract.

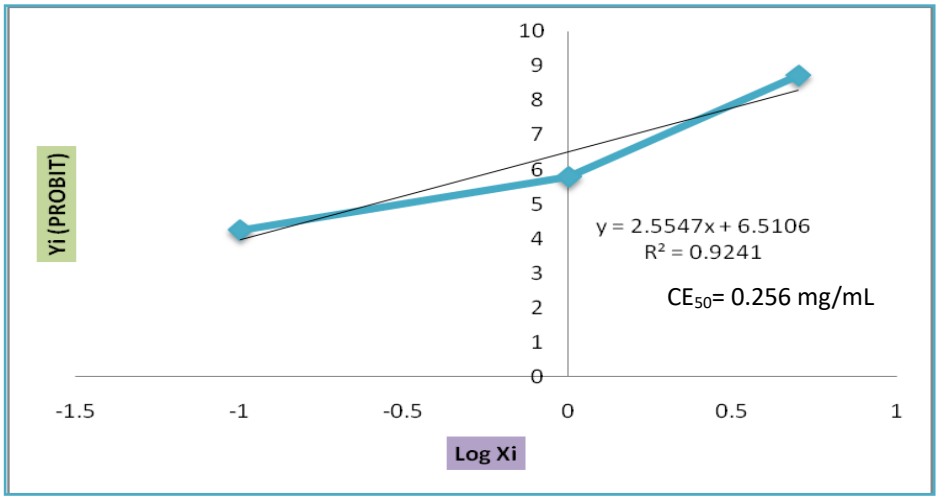

Fig.5 Dispersion diagram of the variables Yi PROBIT and Log Xi of the bioassay of the aqueous extract of C. pepo seed on the culture of G. lamblia. An R2 of 0.924, a high reliability value, was obtained and, according to the equation, the PROBIT value was calculated.

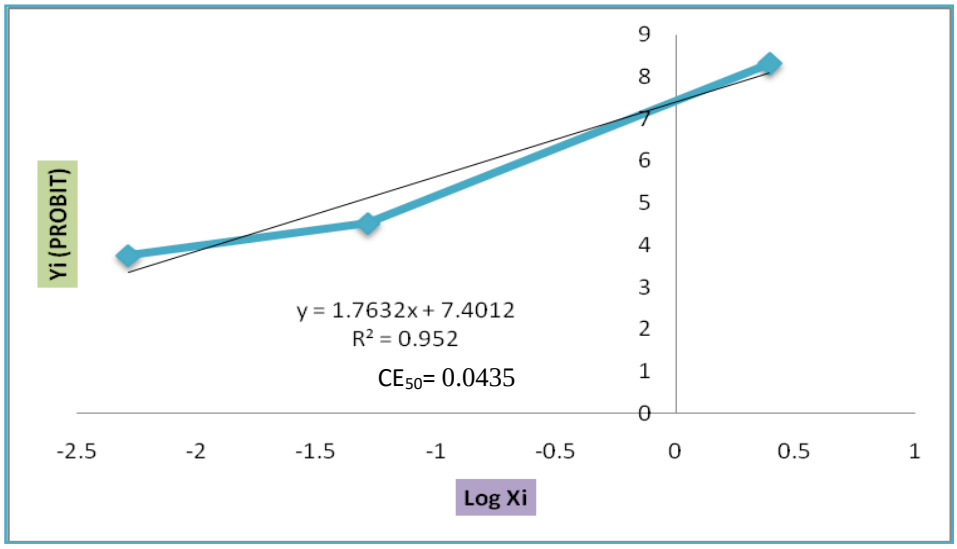

Fig. 6 Dispersion diagram of the Yi PROBIT and Log Xi variables of the bioassay of the methanolic extract of C. pepo on the G. lamblia culture. From the concentrations evaluated, an R2 of 0.952 was obtained, a high reliability value, and the PROBIT value was calculated according to the equation obtained.

The evaluated extracts showed giardicide activity, the aqueous extract presented a 78.6% inhibition in the axenic growth of G. lamblia at a concentration of 1.0 mg/mL, the methanolic extract presented 100% inhibition at a concentration of 0.516 mg/mL , showing that the methanolic extract has greater giardicide activity. This may be due to

the saponins present in the methanolic extract since, when counting G. lamblia, the morphology was altered, some cells presented lysis, it also has alkaloids that could enhance its effect, in other plants alkaloids such as emetine have amebicidal effects (http://www.bvsde.paho.org/texcom/manualesMEC/fitoterapia/cap4.pdf), although the identity of the alkaloid is unknown, this may support the results obtained, although further studies are recommended to determine the molecule responsible for producing the giardicide effect.

The mean inhibitory concentration (IC50) exhibited by the aqueous extract of the C. pepo seed against the in vitro axenic culture of Giardia lamblia was 0.29 mg/mL and the IC50 of the methanolic extract was 0.055 mg/mL, the IC50 of the metronidazole on Giardia lamblia is 0.000221 mg/mL (Cedillo and Muñoz, 1992).

Although the properties of Cucurbita pepo seeds are known, such as: antidiabetic, antihypertensive, anthelminic, antiamibian, antitumor, antibacterial, as well as anti-inflammatory and analgesic action; To date, there are not many studies that demonstrate its biological activity against protozoa such as Giardia lamblia.

The extracts can be used in the future for the development of research in the field of pharmacology for the development of drugs that inhibit the growth and encystment of G. lamblia, offering an alternative option of natural origin for the treatment of giardiasis without presenting side effects. undesirable secondaries. These preliminary results suggest the presence of an active ingredient with a marked inhibitory effect on the growth of Giardia lamblia in the aqueous and methanolic extract of Cucurbita pepo.

conclusion

The aqueous and methanolic extract of Cucurbita pepo seed inhibit the growth of Gardia lamblia under axenic conditions in vitro.

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