**ISOLATION OF A BIOACTIVE PROTEIC FRACTION OF *Solanum marginatum***

**AISLAMIENTO DE UNA FRACCIÓN PROTEICA BIOACTIVA DE *Solanum marginatum***

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**RESUMEN:**

*Solanum marginatum* pertenece a la familia de las solanáceas, se utiliza comúnmente en la medicina tradicional mexicana. Sin embargo, sus propiedades biológicas no han sido evaluadas; por lo que en este estudio hemos evaluado algunas actividades biológicas de una fracción proteica aislada de *S. marginatum*. Para lograr esto, la fracción proteica fue extraída de las hojas de *S. marginatum* por precipitación con sulfato de amonio seguido de cromatografía líquida rápida de proteínas (PF-FPLC), se cuantificó el contenido de proteína por el método de ácido bicinconínico (BCA), se realizó un ensayo de actividad enzimática (método de Kunitz), y se identificaron sub-fracciones de proteína por electroforesis (SDS-PAGE). También, se midió la capacidad para capturar radicales (métodos DPPH y ABTS. El efecto tóxico se evaluó con el ensayo de A*. salina* mientras que la viabilidad celular en células mononucleares de sangre periférica (PBMC). El aislamiento de PF-FPLC se llevó a cabo a Rt = 2,13 min. Así mismo, se separaron e identificaron siete sub-fracciones con pesos moleculares que oscilaban entre 18-112 kDa, mientras que las condiciones óptimas de actividad enzimática fueron 37ºC, pH 7 y 120 min. Los métodos ABTS y DPPH demostraron una capacidad antioxidante de 20 y 62%, respectivamente. Por otra parte, las proteínas PF-FPLC carecieron de efectos tóxicos en el ensayo de *A. salina* y presentaron un leve efecto antimicrobiano (inhibición ≤ 35%), así como efecto en la viabilidad de PBMC.

**Palabras clave:** antimicrobiano, antioxidante, actividad enzimática, efecto proliferativo*, Solanum marginatum*.

**SUMMARY:**

*Solanum marginatum* belong to the family of Solanaceae, is widely used in traditional Mexican medicine. However, their properties have not been evaluated; so that in this study we tested some biological activities of a protein fraction of *S. marginatum*. For this, proteins were extracted from *S. marginatum* leaves, a protein fraction isolated by FPLC, protein content quantified by BCA method, an enzyme activity assay performed by Kunitz method, protein sub-fractions were identified by SDS-PAGE. The ability to capture radicals was determined by the DPPH and ABTS methods. The toxic effect was assessed with the *A. salina* assay while cell viability in peripheral blood mononuclear cells (PBMC) was tested by MTT method. Isolation of PF-FPLC was carried out at Rt = 2.13 min, further, separated and identified seven subfractions with molecular weights ranging from 18-112 kDa, while the optimal conditions of enzymatic activity were 37 ° C, pH 7 and 120 min. The ABTS and DPPH methods showed a radical capture percentage of 20 and 62 % respectively.PF-FPLC proteins had no toxic effect in *A. salina*, however, displayed a low antimicrobial effect (inhibition ≤ 35%) as well as an effect on the cellular viability of PBMC.

**Keywords:** antimicrobial, antioxidant, enzymatic activity, proliferative effect, *Solanum marginatum.*

## INTRODUCTION

The plants of the Solanaceae family are cultivated in Mexico for the nutritional and medicinal properties; which are conferred by the compounds they contain (Wang *et al*., 2011, Amagase and Farnsworthm, 2011, Canales *et al*., 2005). Some plants of this group have anti-inflammatory, antioxidant and antithrombotic actions.On the one hand, the medicinal properties are due to compounds derived from secondary plant metabolism such as phenylpropanoids, flavonoids, alkaloids, acetogenins, polyketides, terpenes, steroids, and carotenoids. On the other hand, they are due to compounds derived from primary metabolism such as peptides and bioactive proteins(Nirmal, *et al*., 2012, De Lucca, 2000). Since some vegetal bioactive proteins have been isolated and characterized, glucanases, chitinases, several families of peptides rich in basic-cysteine and aspartic proteinases with antimicrobial effect are some examples (Guevara *et al*., 2002). Solanum exhibits the ability to synthesize proteins with different biological activities (Vega et al., 2006, Duarte et al., 2007, Verpoorte 1998, Shamsi *et al*., 2016). Some bioactive proteins isolated from Solanum are dioscorine capable of inducing the expression of cytokines (Fu *et al*., 2006). Caspase and aspartic protease isolated from *Solanum tuberosum* with antimicrobial effect (Mendieta *et al*., 2006; Guevara et al., 2002) and lunar isolated from *Solanum nigrum* effective against cancer (Jeong *et al*., 2010). *S. marginatum* is native to Africa, in Mexico, it is known as Sosa (Rzewski and Rzedowski, 2005). The aqueous extracts of its leaves are used to treat fungal infections of the skin (Villaseñor and Espinosa, 1998). Additionally, in traditional Mexican medicine, it is used as an antimicrobial and anticarcinogenic remedy. However, the mechanisms and compounds associated with traditional use have not been reported (Villaseñor, 2016; Sánchez *et al*., 2011). For this reason, this research focused on isolating and evaluating some of the biological activities a protein fraction of *S. marginatum*.

**METHODOLOGY**

**Protein extraction, isolation, and quantification**

**Protein extraction**

*Solanum marginatum* leaves were collected at Tenosique Tabasco Mexico, cutted, lyophilized and crushed in a food processor. Then, 10 g of crushed leaves were extracted with a sodium acetate buffer 50 mM and NaCl 5% (Ahmed *et al*., 2009) under constant stirring. Subsequently, the proteins were separated by precipitation with ammonium sulfate (80% saturation index) for 24 hours at 4 ° C.

The separation was performed by centrifugation for 30 min at 10,000 rpm and 4 ° C. Finally, the pellet was resuspended in 50 mM sodium acetate buffer.

**Proteic fraction isolation**

The proteic fraction was desalting out by fast protein liquid chromatography (PF-FPLC). In this technique, a gel filtration column Sephadex G-25 was used (Llorente *et al*., 2014).

The conditions used were a flow of 1 mL/min, an elution volume of 10 ml, a pressure of 1 mPa, and an injection volume of 500μL. FP-FPLC was collected in 1 ml aliquots which were lyophilized and stored at -20 ° C until use in assays subsequent.

**Protein quantification**

Protein content in a 96 well plate by the bicinchoninic acid method (BCA) was measured. For this purpose, a calibration curve of bovine serum albumin (0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 ppm) was done. In the assay 50 μL of the extract and 200 μL of BCA were mixed, and then this mixture was incubated at 37 ° C for 45 minutes and was read at 550 nm.

The protein content in the crude extract as well as in PF-FPLC was measured (Guevara *et al*., 1999; Zheng *et al*., 2016).

**Enzyme activity assay**

The enzymatic activity was evaluated by the Kunitz method (Mazorra-Manzano et al., 2013). For this, a mixture of FP-FPLC with 1% casein for 30 minutes at 37 °C was incubated. This reaction was stopped using the addition of trichloroacetic acid at 10%. The resulting solution at 4000 rpm for 10 min centrifuged and the protein content of the supernatant was detected. Then, absorbance was measured at 280 nm using a microplate reader (GEN 5, Biotek, Intruments, Inc). The specific activity measured at different pH (1, 5.2, 6, 7 and 10), temperature (8, 25, 30, 40, 50, 60, 70 and 80 ° C) and reaction time (0 to 180 minutes). Additionally the protein yield percent of every one purification step was calculated.

**Protein separation by SDS-PAGE**

The PF-FPLC was separated by the technique of polyacrylamide gel electrophoresis (SDS-PAGE), under reducing conditions (Menegassi *et al*., 2008; Konozy *et al*., 2013).

The electrophoretic separation was carried out on a Bio-Rad Miniprotean III system, applying 100 V for 120 minutes. Protein fragments were revealed with Coomassie R-250 blue staining and a commercial marker was used for comparison.

**DPPH assay**

The DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay adapted to a 96-well microplate. This performed by mixing 100 μL of each sample with 100 μL of 0.1 mM DPPH. Then, the mixture was incubated for 30 minutes in the dark at room temperature. Glutathione (GSH) solution (1,2,3,4 and 5 ppm) was used as a positive control for the sequencing effect of the protein extract and the effect was measured at 517 nm employing a microplate reader (TECAN, InfiniteM200PRO).

**ABTS assay**

The ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) assay adapted to a 96-well microplate. This assay performed by means mixing 100 l of 7 mM ABTS with 100 μL of potassium persulfate 2.4 mM. The mixture was incubated at 23°C in darkness for 16 h. This solution diluted with PBS (pH 7.2) (Tironi and Añón, 2010). Additionally, a PF-FPLC solution at 10 ppm and GSH curve of 1, 2, 3, 4, and 5 ppm were tested. The readings at 514 nm in a microplate reader were realized.

**Antibacterial effect evaluation**

The antibacterial effect of PF-FPLC was carried out by microdilution method in a 96-well plate. *Escherichia coli* (ATCC 11229), *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 15442) and *Salmonella choleraesuis* (ATCC 1070) strains were used. For each strain, a bacterial suspension at 1.5 × 108 CFU / mL was prepared using the McFarland scale of 0.5 (Kim et al., 2009). Concentrations of 0.01, 0.02, 0.04, 0.08, 0.16, 0.33, 0.63, 1.25, 2.50, 5.00, 10.00 μg/mL from PF-FPLC at 37 ° C for 24 h were tested. The absorbance at 625 nm in a Bioteck 800 XL microplate reader was read. Then, the bacterial inhibition percentage (%) was determined (Tavares *et al*., 2008; Kaewpiboon *et al*., 2012).

**Toxicity evaluation**

The *A. salina* assay was performed to evaluate the toxicity of proteins isolated from PF-FPLC and was carried out in a 96-well plate. *A. salina* cysts were hatched in artificial sea water at 37 g/L at 25 °C with aeration and constant light source for 24 h (Déciga-Campos *et al*., 2007). Concentrations of 0.01, 0.02, 0.04, 0.08, 0.16, 0.33, 0.63, 1.25, 2.50, 5.00, 10.00 μg/mL from PF-FPLC for 24 h at 25 °C were tested. A concentration-response curve with K2Cr2O7 at 5.00, 10.00, 15.00, 20.00 and 25.00 g/mL, as well as a negative control with PBS pH 7 (viability control) was performed. LC50 with the percent of mortality M (%) by a linear regression analysis was determined (Jegathambigai *et al*., 2014).

**Isolation of Peripheral Blood Mononuclear Cells (PBMCs)**

Peripheral blood from healthy volunteers was obtained. The peripheral blood mononuclear cells were separated by Ficoll-Histopaque-1077 density gradient method (Liu *et al*. 2007). The mononuclear cells were washed three times with PBS, and then centrifuged at 1250 rpm at 4 for 5 min, finally, the cells were resuspended in RPMI 1640 supplemented with 10% fetal bovine serum (GIBCO, Grand Island, N.Y. USA) and 0.04% ceftriaxone. After, 1x106 PBMC/mL were plated in a 96-well plate, subsequently, PF-FPLC concentrations of 0.01, 0.02, 0.04, 0.08, 0.16, 0.33, 0.63, 1.25, 2.50, 5.00, and 10.00 μg/mL were added. Finally, the plates were incubated for 24 hours at 37 ° C in a humidified atmosphere of 5% CO2, and the cell viability was measured by MTT method. The reading was carried out on a microplate reader (Bioteck 800 XL) at 450 nm (Wang *et al*., 2016).

**Statistical analysis**

The results of the statistical analysis present as the mean and standard deviation (n=3). The data obtained were analyzed by one-way ANOVA and a Tukey test (\*p ≤ 0.05). The data to evaluate the specific activity of the extract against FPF-PF were analyzed by the "t-student" test. Both tests were performed using the statistical program GraphPad Prism 5 ©. Statistical significance was accepted when p values were ≤0.05.

**RESULTS**

**Protein extraction, isolation, and quantification**

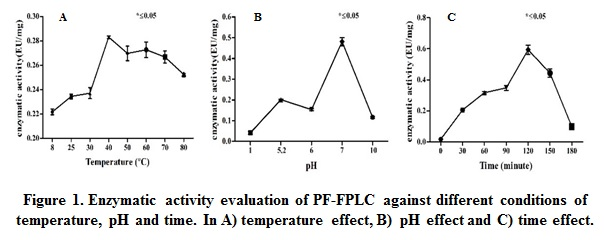
The extraction procedure of *S. marginatum* showed a protein content of 315.8 ± 0.08 ppm, a yield of 71.05 % and a purification factor of 1.407.

The PF-FPLC isolation by FPLC showed a signal with a retention time Rt of 2.13 min with a resolution of 2.81. Additionally, the equation allowed detecting protein content in the PF-FPLC of 9,259 ppm.

**Enzyme activity assay**

Assays to evaluate enzyme activity showed that in purification steps, the specific activity was decreased while total activity was increased. In the precipitated extract, a TA of 0.02 as well as a specific activity of 6.00 x 105 TA/mg was found. Additionally, in PF-FPLC values of 0.13 and 1.78 x 10-3 TA/mg of total activity and specific activity respectively were found.

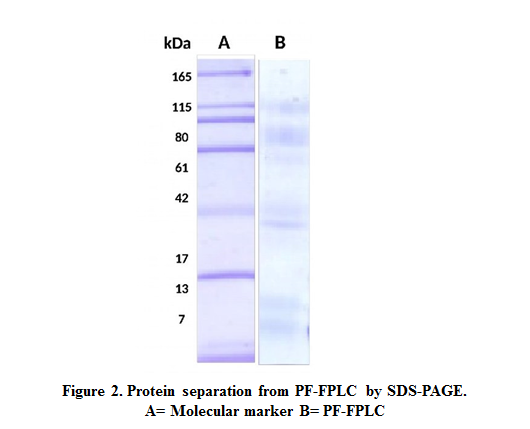
Furthermore, in enzyme activity assays the optimum conditions were determined to be 37 ° C, pH 7 and 120 min, results are presented in Figure 1.



The yield of PF-FPLC was 71.05 % and a purification factor of 0.152; also, a decrease in protein content was observed at each stage of purification.

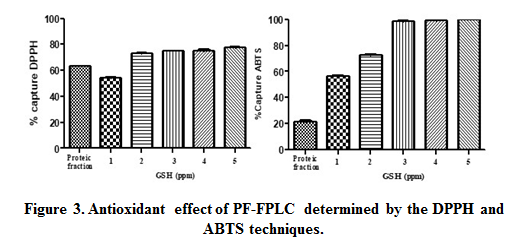
**Protein separation by SDS-PAGE**

The protein separation from PF-FPLC using SDS-PAGE showed seven bands. Although only seven bands were observed, there is a possibility that each band contains a group of proteins with different biochemical and biological properties. The results are presented in Figure 2.



**Antioxidant effect evaluation**

In PF-FPLC an antioxidant effect of 20.03 ± 0.09% and 62.08 ± 0.08% was found by the ABTS and DPPH methods, respectively. The results are shown in Figure 3.



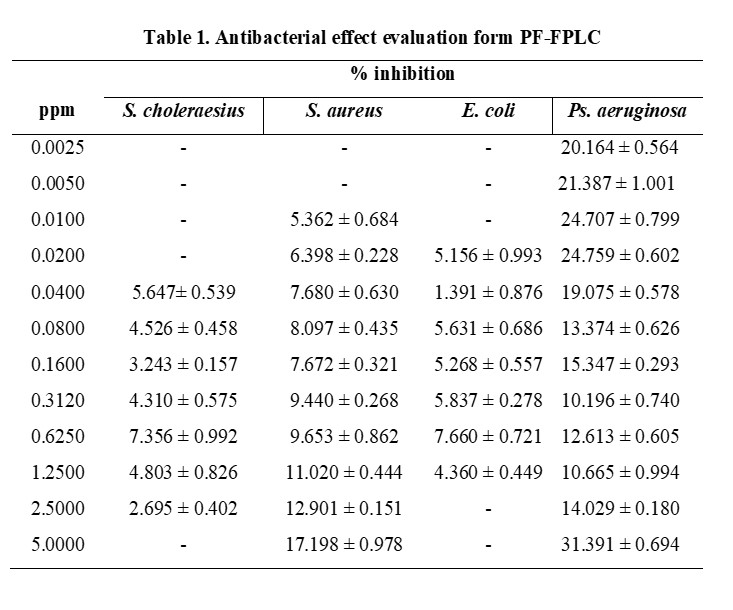
In PF-FPLC an antiradical effect of less than 25% was found; while the GSH used as a positive control (1, 2, 3, 4 and 5 ppm) showed an effect of 98%.

**Toxicity evaluation**

*A. salina* assay showed that PF-FPLC had no toxic effect at the concentrations tested. The positive control showed an LC50 of 13.90 ± 0.51 ppm.

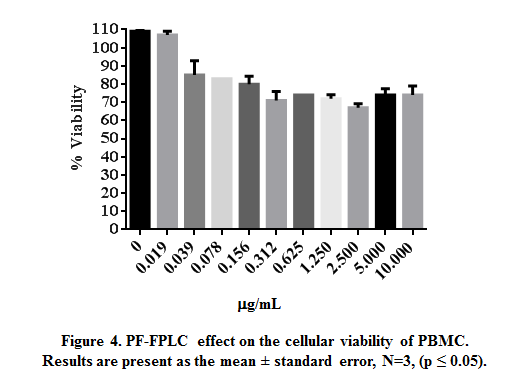
**Antibacterial effect evaluation**

The PF-FPLC showed an antimicrobial effect on *S. aureus* and *Ps. aeruginosa* with a percent inhibition value 30 % to 5 ppm. This fraction showed an effect in *E. coli* and *S. choleraesuis* with a percentage of bacterial inhibition ≤ 8%, however, hormesis phenomenon between 0.312 and 0.635 ppm was observed. The concentrations tested on *S. aureus* and *E. coli* showed a significant difference (\*p ≤ 0.05) by the Tukey test. The results showed that there is not a significant antimicrobial effect because the percentage of inhibition on *S. choleraesius,* *S. aureus* and *E. coli* was ≤ 20% and in *Ps. aeruginosa* had no effect. The results are shown in Table 1.



**Isolation of Peripheral Blood Mononuclear Cells (PBMCs)**

The MTT assay was used to determine the cellular viability of PBMC after treatment with PF-FPLC (Figure 4). The results indicate that the cell viability was not modified reduced by FP-FPLC at 0.019 g/mL. In contrast, at 0.039 to10 g/mL, the cell viability was reduced significantly

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**DISCUSSION**

The protein content on *Solanum marginatum fruits* is higher than reported for other Solanaceae plants (Mohamed Ahmed *et al*., 2009). The resolution ensured the effectiveness of chromatographic separation since it showed a value ≥1.5 (Harris, 2002).

Assays to evaluate enzyme activity showed that in purification steps specific activity was decreased while total activity was increased; because some lower molecular weight proteic compounds lost during desalate (García *et al*., 2013). According to specific activity results, in *S. marginatum*, were found values below the values reported for the cysteine proteases (15 and 16 EU/mg) of the crude extract of the fruit of *Solanum granulosum* (Vallés *et al*., 2011). On the other hand, assays of PF-FPLC enzymatic activity at optimal conditions showed that this fraction contains more than one type of protein and belongs to the group of proteases because they have activity at neutral, acidic and basic pH (Ahmed *et al*., 2009; Mohamed-Ahmed *et al*., 2009). However, when specific activity values compared, increased activity at neutral pH was observed by the ability of each protease to work at specific pH. Some examples are cysteine protease isolated from *S. granulosum* having activity at pH 6, whereas proteases isolated from *Solanum dubium* act at pH 11, 60 ° C and 25 h (Mohamed-Ahmed *et al*., 2009). Therefore, the enzyme activity of each protein depends on its catalytic mechanism (van der Hoorn, 2008). The percent yield determination showed that there is a decrease in protein content at each stage of purification.

The protein separation by SDS-PAGE assay showed that PF-FPLC contains proteins similar to the serin protease, identified in most plants (Kumari *et al*., 2012); because their molecular masses are of 19-110 kDa (Antão and Malcata, 2005). Additionally, there is a possibility that PF-FPLC contains some reductases; such as s-nitrosoglutathione reductase isolated from *Solanum lycopersicum*, having a molecular weight of 45 kDa (Kubienová *et al*., 2013); as well as some oxidoreductases, since in *Solanum tuberosum* some have been identified that have molecular weights that oscillate of 47-68 kDa (Batista *et al*., 2014). Additionally, there is a possibility that the proteins contained in PF-FPLC could be similar to those *Solanum dubium* since it contains proteins with molecular weights of 10-95 kDa (Mohamed *et al.,* 2009); as well as aspartic protease of *S. tuberosum* since it has a molecular weight of 40 kDa.

The antioxidant effect of PF-FPLC was below GSH, this behavior reported with other proteins (Townsend *et al*., 2003; Chen *et al*., 2012; Chang-Bum *et al*., 2014). This antiradical effect presented because the amino acids residues are capable of promoting radicals elimination (Sarmadi and Ismail, 2010). However, the antiradical effect shown by the PF-FPLC is higher than that reported in another's species of solanum genus. Some examples are patatin of *Solanum tuberosum* that has an antiradical effect of 50% to 582 ppm, (Liu *et al*., 2003), as well as proteins isolated from *S. tuberosum* and *Solanum betaceum*, which have an antiradical effect of 50% using 55 and 73 ppm respectively (Ordóñez *et al*., 2011). These results suggest that PF-FPLC purification could increase its enzymatic activity and its biological effect. The antioxidant effect could occur by cysteine content in the amino acid sequence because it is known that this amino acid is a precursor of glutathione and has a powerful antioxidant effect (Carrasco and Guerra, 2010). Furthermore, the antioxidant effect of a protein compound is greater when it has residues of 5 to 20 amino acids, a molecular mass ˂ 5 kDa, 41% hydrophobic amino acids and 12% aromatic amino acids (Sarmadi and Ismail, 2010).

*A. salina* assay suggest that the proteins contained in PF-FPLC have a selective effect since also the antibacterial and lymphoproliferative effects in peripheral blood mononuclear cells were observed. Briefly, it should be noted that the antibacterial effect exhibited by PF-FPLC is related to molecular weight, amino acid residues (AA) and lysine or arginine content since these amino acids have antimicrobial effects. This effect is due to the structural conformation of the amino acids which includes properties such as net charge, amphipathicity, and hydrophobicity (Yeaman and Yount, 2003). Therefore, the PF-FPLC proteins might be able to destabilize bacterial membranes and kill some pathogens (Zhao *et al*., 2012), and the effect is similar to *S. tuberosum* aspartyl protease since present an effect on *Bacillus cereus*, *E. coli*, and *S. aureus* of 0.24-4.24 M (Mendieta *et al*., 2006).

The reduction PBMC treated with different concentrations of PF-FPLC occurs by an interaction between cellular sensitivity and protein chemical structure. However, the reduction in viability caused by PF-FPLC is acceptable since there are compounds which present a viability reduction in small concentrations; paclitaxel is an example (Kasemwattanaroj *et al*., 2013). The PF-FPLC proteins showed an effect on the decreased viability of PBMC directly proportional to test concentrations. There are insufficient data on the mechanism of *S. marginatum* proteins action on normal blood mononuclear cells. Nevertheless, could be related to the ability of cells to adapt to the effects of proteins involved in chemotactic effects (Alfaro Leon *et al*., 2005), such as serine proteases capable of producing cytokines (IL-4, IL-10 and TGF -β) (de Matos Guedes *et al*., 2010).

Additionally, some compounds with antioxidant effect diminish PBMC viability since they give a rise oxidative change, induce apoptosis, and cause changes in the granularity and size of these cells. Therefore, the PF-FPLC antioxidant effect could be implicated in the viability decrease of PBMC (Bors *et al*., 2012). Some compounds isolated from plants have an anti-lymphoproliferative effect (Meng *et al*.,2013), but most have a proliferative effect (Yeap *et al*., 2007). On the other hand, some proteins possess multifunctional properties including the immunomodulatory effect in PBMC, lectin of *Microgramma vacciniifolia* is an example (de Siqueira Patriota *et al*., 2017). These results are related to effect shown by a protein isolated from *Solanum tuberosum*, which is capable of producing significant changes in haematological parameters; such as the reduction of neutrophils and the increase of lymphocytes in mice (Lynch *et al*., 2012).

**CONCLUSIONS**

In this work, a high yield protein was found in PF-FPLC that is comparable to that reported in other plants. The fraction contains at least 7 fragments with molecular masses of 18-112 kDa. Additionally, this fraction displays enzymatic activity and has antioxidant effects. On the other hand, we observed that PF-FPLC has no toxic effect in *A. salina*, but reduces the viability of PBMC. Finally, this fraction shows a lower antimicrobial effect against the microorganisms tested.

**ACKNOWLEDGMENTS**

YSB acknowledge to CONACYT-Mexico for the support with a doctoral fellowship (200567) to JGC which strengthens the program in Science and Technology of Food as well as to academic research team (UCOAH-CA-27-PRODEP).

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