CHIA PROTEIN CONCENTRATE (*SALVIA HISPANICA* L.) ANTI-INFLAMMATORY AND ANTIOXIDANT ACTIVITY

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ABSTRACT

Objective: The objective of the study was to evaluate the antioxidant and anti-inflammatory activity of chia proteins concentrates in *in vitro* conditions.

Methods: Chia seeds (*Salvia hispanica*) were utilized to obtain chia protein concentrate at different pH 2.0, pH 3.0, pH 4.0, pH 5.0, and pH 6.0 using the isoelectric precipitation method and to evaluate the *in vitro* anti-inflammatory and *in vitro* inhibition of peroxidation lipid of chia protein concentrates.

Results: The best treatment to obtain chia protein concentrate was at pH 3.0 with a value of 25.53% yield using water as solvent and 38.13% yield using NaCl 1M as solvent. The higher protein content was found in the chia protein concentrate at pH 4.0 with values of 91.18% and 57.87% of protein content. All chia protein concentrates presented high *in vitro* anti-inflammatory activity and inhibition peroxidation lipid. Chia protein concentrate at pH 3.0 (100–100 μg/ml) presented anti-inflammatory activity with values ranging from 56.32% to 103.00% in a form dependent doses. Chia protein concentrate at pH 6.0 presented a value range of 92.80–95.98% of inhibition of peroxidation lipid.

Conclusion: This study suggests that chia protein concentrates possess potent antioxidant and anti-inflammatory activities in *in vitro* conditions.

Keywords: *Chia, Salvia hispanica* L., Chia protein concentrate, Proteins.

INTRODUCTION

The genus *Salvia* has around 900 species. The *Lamiaceae* chia seeds (*Salvia hispanica* L.) are a tropical and subtropical climates herbaceous plant which belongs to the family Lamiaceae and originated from southern Mexico and northern Guatemala. Food seeds have been important for human nutrition since 1500 BC [1,2]. Conventionally, chia seeds were used as food, in a wide range of traditional medicines, primary cosmetics, and as part of religious rituals in pre-Columbian civilizations [3]. Aztecs and Mayas used chia seeds in the preparation of folk medicines, food, and canvases. Chia seed composition is 32–39 g/100 g oil, 26–41 g/100 g of carbohydrates, 18–30 g/100 g of dietary fiber, 4–6 g/100 g of ash, and 22–24 g/100 g protein contents. Chia seeds have an important content of vitamins, minerals, and antioxidants. Chemical composition and nutritional value of the chia seed can vary according to the climatic condition and location of growing [4]. In South America (Brazil, Ecuador, Bolivia, and Argentina), chia seeds are usually consumed mainly in the form of flour and seeds that can be added to preparations such as fruit, yogurt, cakes, and among others. At present, its consumption has increased due to its beneficial effects related to obesity, cardiovascular diseases, diabetes, and some types of cancer. Free radicals, and different species of free radicals, are reactive oxygen species (ROS) such as O_2, H_2O_2, and OH^-. ROS produce oxidative damage in human body and biochemical compounds such as enzymes. Oxidative stresses can be a cause of different diseases such as diabetes, arteriosclerosis, thrombosis, inflammation, and various types of cancers [5-7]. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are used as food additives and preservatives [8,9]. However, due to the possible toxic and carcinogenic effects associated to BHT and BHA, research is being conducted to find natural sources [10,11]. Medicinal plants have been utilized for millennia by humans, because these plants have phytochemical components for the prevention and treatment of different diseases and ailments such as inflammation processes. Food

proteins have antioxidant and anti-inflammatory activity. Hen egg white lysozyme presents antioxidant activity when ORAC and thiobarbituric acid reactive substances (TBARS) methods are used. Hen egg white lysozyme presents anti-inflammatory activity in mice with a reduction of edema paw [12,13]. The aim of this work was to obtain chia protein concentrate and evaluate its *in vitro* antioxidant and anti-inflammatory activity.

METHODS

Defatted chia flour (DCF) preparation
DCF was produced according to the method of Sze-Tao and Sathe [14]. Chia (*S. hispanica* L.) seeds were purchased from the supermarket shelves in Ecuador. Walnuts were ground in a windmill. The flour was defatted with hexane (flour/hexane ratio of 1:10 [w/v]) under constant magnetic stirring for 3 h. The slurry was vacuum filtered through a paper filter and the residue was used for subsequent extraction. Hexane extractions were repeated until the filtrate was clear. Residue from the last extraction and filtration step was air dried in a fume hood. DTF was stored at −20°C until further use.

Preparation of protein isolates from chia seeds
Chia protein concentrate was prepared according to the process described by Barrio and Añón [15] with minor modifications. DCF was extracted by stirring for 2 h at room temperature (about 25°C) with deionized water adjusted to pH 8.0 with 1 M NaOH (flour:water ratio, 1:20 [w/v]). The slurry was centrifuged at 5000 g for 30 min at 25°C in an Eppendorf centrifuge (USA). The insoluble chia protein pellet was reslurried with pH adjusted deionized water as above and centrifuged again. The supernatants mixed together were adjusted to pH 3.0; pH 4.0; pH 5.0; and pH 6.0 (isoelectric point), kept for 48 h at 4°C with 1 M HCl and subsequently centrifuged at 5000 g for 30 min at the same temperature. The precipitate was washed with deionized water, resolubilized in deionized water, neutralized to pH 7 with 1 M NaOH

at room temperature, then dialyzed against water and lyophilized. All lyophilized protein samples were stored in airtight plastic bottles at −20°C until further use.

Proximate analysis of chia seeds proteins

Protein, moisture, fat, ash, and fiber contents were determined according to AOAC methods [16], numbers 920.152, 950.46, 930.09, 920.153, and 934.10, respectively. Determination of soluble solids of the materials was made according to the ISO 2173:2003 method [17]. The protein contents of samples were determined by the micro-Kjeldahl method (AACC, 2000) using a protein-nitrogen coefficient of 6.25. Carbohydrates were determined according to the Zhu *et al.* [18] method. The contents were expressed on a dry weight basis. Each analysis was carried out in triplicate, and data were reported as means ± standard deviation.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE electrophoresis of chia (*S. hispanica* L.) proteins, proteins fractions, and finally products of enzymatic hydrolysis of proteins, was carried out according to the method proposed by Laemmli [19]. Runs were performed in mini-protean (Bio-Rad, Hercules, CA, USA). In SDS-PAGE system gels of 12 g acrylamide/100 mL of resolving gel and 4 g acrylamide/100 mL of stacking gels were used. Relative molecular masses of protein were determined by comparison to the molecular weight marker with polypeptide SDS-PAGE standard (MW 6.5–200 kDa) (Bio-Rad) kDa GE Healthcare Life Sciences. Gels were fixed and stained with Coomassie Brilliant Blue G-250 (Sigma-Aldrich, St. Louis, MO) for 16 h.

Evaluation of *in vitro* anti-inflammatory activity

Anti-inflammatory activity of chia protein concentrate at pH 2.0, pH 3.0, pH 4.0, pH 5.0, and pH 6.0 was evaluated with the protein denaturation method [20,21]. Diclofenac sodium, a powerful non-steroidal anti-inflammatory drug (NSAID), was used as a standard drug. The reaction mixture consisting of 2 mL of different concentrations of protein concentrate (100–1000 µg/mL) or standard diclofenac sodium (100 and 1000 µg/mL) and 2.8 mL of phosphate-buffered saline (pH 6.4) was mixed with 2 mL of egg albumin (from fresh hen egg) and incubated at 27°C for 15 min. Denaturation was induced by keeping the reaction mixture at 70°C in a water bath for 10 min. After cooling, the absorbance was measured at 660 nm using double-distilled water as blank. Each experiment was done in triplicate and the average was calculated. The percentage inhibition of protein denaturation was calculated using the following formula: % inhibition of protein denaturation = $1-(At-Ac/Ac) \times 100$.

TBARS assay

Chia protein concentrate were used to evaluate the inhibition of lipid peroxidation. At 0.5 g of olive oil was rusty by heat at 65° during 8 days, after were added Chia protein concentrate until to obtain at concentrations of 0.02; 0.04; 0.1; and 0.2 mg/mL of milk proteins and were incubated at 30°C during 24 h. BHT was used such as positive control at concentrations of 0.02; 0.04; 0.1; and 0.2 mg/mL of BHT. Olive oil without sample (antioxidant) was used such negative control. 1 mL of sample was mix with 1 mL of the 1% TBA, the solution was heated at 95°C during 1 h, and cooled down during 15 min. Then, absorbance of the final solution containing milk digests was measured at 532 nm using a spectrophotometer (Thermo Scientific Evolution 200). The decrease

of absorbance indicates an increase of antioxidant activity. The values of antioxidant activity were expressed as the percentage of inhibition of lipid peroxidation in larvae homogenate as follows: % inhibition of lipid peroxidation = $(1-[Ab-As]/Ab \times 100)$, where Ab is the absorbance of blank and As is the absorbance of the sample [22].

Statistical analysis

Results are presented as means ± standard deviation from three replicates of each experiment. Differences between mean values were determined by the analysis of variance (ANOVA). The *post hoc* analysis was performed by the Tukey test. All tests were considered significant at $p < 0.05$. Statistical analysis was performed using the software package Prism 4 for Windows, version 6.0 (GraphPad Software Inc., www.graphpad.com).

RESULTS

Proximate analysis

The results of proximate analysis of chia flour and chia protein concentrate obtained at pH 4.0 are shown in Table 1. Chia flour has a content of protein of 19.78% compared to the chia protein concentrate of Ecuador with a content of 61.32% of protein measured using the Kjeldahl method. Carbohydrates content in chia flour were 31.46% of total content. Chia protein concentrates present 21.74% of carbohydrates showing that this biomolecule presence decreased. Lipid content of chia flour was of 16.06% of lipid compared to the lipid content of chia protein concentrate, which had a 9.63% content of lipid after being defatted with hexane. Lipid content decreased in chia protein concentrate. Ullah *et al.*, 2015 reported a protein content ranging between 15% and 25% in chia seeds. Our value is in between these limits [23]. The protein content of chia seed grown in different habitats ranges from 18.8% to 21.5%. The variation in protein content was probably due to agronomic, climatic, and soil conditions [24]. The content of fat in the chia seeds was 16.06%. Ixtaina *et al.*, 2008 reported 30–33% of fat in chia seeds. Our value is lower than that value. In Argentina, different values of content of oil in chia have been reported with values ranging 32.2–36.8% compared to 35.6–38.6% [25]. The difference in oil content could be connected to the difference in climatic conditions, agronomic practices, fertilization regimes, irrigation practices, etc. [26]. The higher values of tocopherols can have a positive impact on the storage stability of chia oil. Content of fiber was 27.88%. Protein, lipids, and fiber are biocompounds having a good acceptance among consumers. European Union has accepted chia as a functional food. Chia seeds are considered to be safer without the harmful effects on health. Chia seeds can be used in baked goods, nutritional supplements, cereal bars, cookies, bread, snacks, etc. Chia seeds are used as a component of several foods in Mexico, Argentina, Chile, Ecuador, Canada, USA, and Japan and are also used for different purposes. The USA dietary guidelines recommend consuming chia seeds for health reasons as a primary source of good food. Chia sprouts are used in salads, chia seeds are also used in beverages, cereal based foods, and finally chia seeds can be consumed in raw form [27].

Yield of chia protein concentrate

Table 2 summarizes yield of chia protein concentrate obtained using water and NaCl as solvents to isoelectric precipitation. Chia seeds were used in our study to obtain the chia defatted flour. The defatted chia flour was used to obtain chia concentrate protein using the isoelectric precipitation method at different pHs (pH 2.0; pH 3.0; pH 4.0; pH 5.0; and pH 6.0) with water as solvent. The highest yield was obtained at

Table 1: Proximate analysis of chia flour and chia protein concentrate at pH 4.0

Content (%)	Protein	Lipid	Carbohydrates	Fiber	Moisture	Ash
Flour	19.78±0.015[a]	16.06±0.038[b]	31.46±0.062[c]	27.88±0.021[d]	5.71±0.032[e]	4.82±0.025
Concentrate pH 4.0	61.32±0.019[g]	9.63±0.025[h]	21.74±0.005[i]	0.52±0.003[j]	2.86±0.011[k]	3.93±0.020[l]

Results represent the average of three determinations of±SD (n=3). Different letters show statistical difference between the groups (p<0.05) ANOVA and Tukey's test. SD: Standard deviation

pH 3.0 with a value of 25.53% using water solvent and 38.13% using NaCl solvent. Yields for pH 2.0 and pH 3.0 with water solvent presented no statistical differences. Yields for pH 3.0, pH 4.0, and pH 6.0 with NaCl solvent present no statistical differences with p<0.05. The contents of protein in the chia seed concentrate protein were analyzed using the Biuret and BCA methods. The treatment at pH 4.0 presented a higher content of protein with both methods with a 91.81% obtained by Biuret method and a 57.87% obtained by BCA method (Table 3). For all pHs, there are statistical differences with p<0.05 when both methods are compared. These differences can be fundamental in the measurement of each method. The Biuret method consists of the formation of purple complexes between Cu^{+2} and the amine group of the peptide bonds (NH_2). The BCA method is based on the measure of Cu^{+1} formatted for the purple complex produced by the reduction of Cu^{+2} by the peptide bonds into Cu^{+1}.

SDS-PAGE electrophoresis

All chia protein concentrates were analyzed by SDS-PAGE electrophoresis. The gel of polyacrylamide shows the bands of chia protein concentrate obtained with water solvent. The range of molecular weights is between 40 and 70 kDa. All the concentrates of chia present the same profile of proteins with identical bands. All the bands present high expression at all pHs tested. The majority of the bands identified were globulins and albumins (Fig. 1).

In vitro anti-inflammatory activity

The inhibitory effect at different concentrations of chia protein concentrate using the protein denaturation method is shown in Table 4. The chia protein concentrates obtained at pH 2.0, pH 3.0, pH 4.0, pH 5.0, and pH 6.0 were tested at 100–1000 μg/ml concentration. Diclofenac at 100–1000 μg/ml concentration was used as a positive control, diclofenac being a strong anti-inflammatory medicine of the AINES (anti-inflammatory non-steroid or NSAIDs in the US) type. Table 4 summarizes a significant inhibition of denaturation of egg albumin on a concentration-dependent manner. The positive control diclofenac sodium presented a high anti-inflammatory activity with values of 46.97–100.00% of inhibition of denatured protein. The effect was dependent on doses. The *in vitro* anti-inflammatory activity of chia proteins concentrate was comparable to the diclofenac sodium anti-inflammatory activity. Chia protein concentrate at pH 2.0 was the most effective sample reducing the percentage of denature protein. The effect was dependent on doses. Chia protein concentrate also presented a high activity with values between 60.97% and 97.66% of inhibition of denatured protein. Chia protein concentrate at pH 6.0 presented 106.98% of anti-inflammatory activity compared to the positive control, diclofenac sodium.

Inhibition of peroxidation lipid (TBARS)

In vitro antioxidant activity of chia protein concentrate was evaluated using the TBARS method. Chia protein concentrate at pH 2.0, pH 3.0, pH 4.0, pH 5.0, and pH 6.0 at concentrations of 100, 200, 500, and 1000 μg/ml were used. Synthetic antioxidant BHT was used as positive control for all assays at concentrations of 100, 200, 500, and 1000 μg/ml. BHT presented high antioxidant activity with values between 93.94% and 97.43% of inhibition of lipid peroxidation. This antioxidant activity was dependent on doses, the higher the dose the higher concentrations, and hence, the higher antioxidant activity. All chia protein concentrate samples presented a high antioxidant activity with values ranging 86.97–95.98% of inhibition of lipid peroxidation on a dependent doses effect. Chia protein concentrate at pH 6.0 was the most effective sample to inhibit the lipid peroxidation with values ranging 92.80–95.98% of inhibition of lipid peroxidation using the TBARS method (Table 5).

DISCUSSION

In vitro and *in vivo* studies on inflammation have become the focus of global scientific research dye to its implication in important to all human and animal diseases or aliments. Plant-based drugs used in the practice of traditional treatment of many diseases including inflammation have become the objective of current research because the plants are easy production, cheap, and have a big therapeutic potential. They can be included in the diet of human and animals. The NSAIDs possess anti-inflammatory action for treatment of many inflammatory conditions such as rheumatoid arthritis, osteoarthritis, and musculoskeletal disorders, but the continuous use of NSAIDs has some associated negative effects such as nausea, fluid retention, bleeding, and gastric lesion (gastric ulcer and gastritis) [28,29]. For this reason, new anti-

Table 2: Percentage of yield of chia protein concentrate obtained with water and NaCl (1M) solvents

Sample	Solvents	
	Water (%)	NaCl 1M (%)
pH 2.0	24.82±0.757[d]	28.21±0.726[c]
pH 3.0	25.53±0.911[d]	38.13±0.148[a]
pH 4.0	12.30±0.238[g]	38.03±0.698[a]
pH 5.0	19.12±0.743[e]	34.73±0.103[b]
pH 6.0	14.62±0.186[f]	37.40±0.326[a]

Results represent the average of three determinations of±SD (n=3). Different letters show statistical difference between the groups (p<0.05) ANOVA and Tukey's test. SD: Standard deviation

Table 3: Content of protein of chia protein concentrate using Biuret and BCA method

Protein (%)	pH 2.0	pH 3.0	pH 4.0	pH 5.0	pH 6.0
Biuret	61.21±1.705[d]	81.03±2.385[c]	91.81±2.092[a]	89.05±3.057[ab]	84.89±1.063[bc]
BCA	45.10±1.429[c]	44.91±2.041[c]	57.87±1.830[a]	51.23±2.424[b]	48.21±0.429[bc]

Results represent the average of three determinations of±SD (n=3). Different letters show statistical difference between the groups (p<0.05) ANOVA and Tukey's test. SD: Standard deviation

Table 4: *In vitro* anti-inflammatory activity of chia protein concentrate using denaturation of albumin protein method

Inhibition (%)	Concentrations			
	100 μg/ml	200 μg/ml	500 μg/ml	1000 μg/ml
Diclofenac	47.46±0.523[a]	77.64±0.485[a]	98.34±0.159[a]	100.00±0.558[a]
pH 2.0	56.32±0.653[b]	69.22±0.405[b]	80.57±0.211[b]	103.00±0.670[a]
pH 3.0	60.97±0.388[c]	68.31±0.239[b]	74.94±0.575[c]	97.66±0.138[a]
pH 4.0	54.00±0.138[d]	54.41±0.211[c]	56.04±0.833[d]	58.76±0.239[b]
pH 5.0	50.36±0.319[e]	53.33±0.211[c]	53.79±0.239[e]	69.75±0.211[c]
pH 6.0	43.76±0.211[f]	45.49±0.159[d]	67.19±0.780[f]	106.98±0.211[a]

Data are expressed as the mean±SD (n=3). Values in the same column having different letters differ significantly (p<0.05). ANOVA and Tukey's test, SD: Standard deviation

Table 5: Inhibition of peroxidation lipid (TBARS) of chia protein concentrate. Time of incubation 24 h

Inhibition (%)	Concentrations			
	100 µg/ml	200 µg/ml	500 µg/ml	1000 µg/ml
BHT	93.94±0.034[a]	94.05±0.056[a]	96.18±0.032[a]	97.43±0.078[a]
pH 2.0	86.97±0.278[b]	91.45±0.382[b]	95.01±0.201[a]	95.42±0.219[b]
pH 3.0	90.86±0.041[c]	91.09±0.077[b]	92.63±0.089[b]	95.57±0.053[b]
pH 4.0	91.55±0.188[c]	91.84±0.107[b]	92.36±0.118[b]	92.65±0.045[c]
pH 5.0	91.75±0.244[c]	92.30±0.036[b]	92.91±0.015[b]	94.30±0.096[b]
pH 6.0	92.80±0.265[c]	94.51±0.056[a]	95.86±0.160[a]	95.98±0.027[b]

Data are expressed as the mean±SD (n=3). Values in the same column having different letters differ significantly (p<0.05). ANOVA and Tukey's test. SD: Standard deviation, TABRS: Thiobarbituric acid reactive substances, BHT: Butylated hydroxytoluene
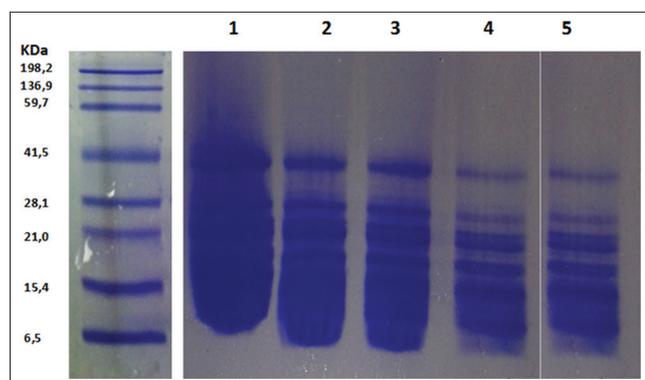

Fig. 1: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of chia protein concentrate. Lane 1: Chia protein concentrate at pH 2.0, lane 2: Chia protein concentrate at pH 3.0, lane 3: Chia protein concentrate at pH 4.0, lane 4: Chia protein concentrate at pH 5.0, and lane 5: Chia protein concentrate at pH 6.0

inflammatory drugs without these side effects are being researched such as alternatives to NSAID without a lot of side effects associated with the use of drugs synthetic such as diclofenac and ibuprofen.

Chen *et al.* [30] have reported fish and shellfish protein hydrolysate with high capacity to inhibit lipid peroxidation. These peptides are considered good candidates in search of effective non-toxic substances with differing antioxidant mechanisms. Even though the exact mechanism of peptides to act as an antioxidant is not clearly known, some aromatic amino acids and histidine are reported to play a key role in this activity. Proteins have been shown to have antioxidative activities against the peroxidation of lipids and/or fatty acids on hydrolysis. Chen *et al.*, 1995 [31] reported six antioxidative peptides from the proteolytic digests of a soybean protein. Chen *et al.*, 1996 reported that a small peptide with the sequence Leu-Leu-Pro-His-His was used to obtain 28 synthetic peptides and their antioxidative activities against the peroxidation of linoleic acid were compared in an aqueous system [32]. In this study, chia protein was effective to inhibit lipid peroxidation of olive oil. The result of this study could explain the efficiency in medicinal use of these proteins of chia for the antioxidant and anti-inflammation purpose.

CONCLUSION

Chia seeds (*S. hispanica*) have a good proportion of protein to obtain using the isoelectric method at different pHs to collect chia protein concentrate. Chia protein concentrate presents a high *in vitro* anti-inflammatory activity and inhibition peroxidation lipid. Chia seeds can be used to obtain vegetal proteins with biological activities. Chia proteins can be used as ingredients to elaborate functional foods. Chia proteins can be a good source of vegetal protein in Ecuador.

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