Instituto Tecnológico y de Estudios Superiores de Monterrey

Campus Monterrey

School of Engineering and Sciences

Evaluation of *in vitro* antioxidant and antibacterial activities of extracts and hydrolysates from *Sphenarium purpurascens* of early and adult stage

A thesis presented by

Martha Selene Marín Morales

Submitted to the
School of Engineering and Sciences
in partial fulfillment of the requirements for the degree of

Master of Science

In

Biotechnology

Monterrey Nuevo León, December 6th, 2021

Dedication

To God for the great opportunity, he gave me to be able to fulfill this dream that I had tried in previous years.

To my little son, who was the reason for carrying out this project, because my professional and personal improvement will have an impact on him.

To my mother and my father, despite the circumstances, they were attentive and, in their own way, supported me.

Acknowledgments

First, thank everyone who participated in this project. Thank you for the professional and personal experience you have contributed to me.

To my advisor, **Dra. Celeste Concepción Ibarra Herrera** for giving me the confidence and the opportunity to belong to your work team, for the great learning that I obtained from her, and especially the human quality that characterizes her.

I thank **Dra Alma Cuellar Sanchez** for the support she gave me during my stay at the university.

To my lab colleagues for the help and patience they gave me in special to **MC. Alicia Reyes Herrera.**

To **Tecnológico de Monterrey** and **CONACyT CVU** 769152 **for** their support through scholarships.

Contents

List of Figures

List of Tables

Chapter 1

Introduction and Thesis Generalities

1. Introduction

Entomophagy is a feeding practice that is spread in many countries as a common practice and an attractive alternative in terms of nutritional quality and low cost. Several advantages can promote entomophagy as a quotidian practice, e.g., the generation of waste and greenhouse gases by livestock can be reduced. Furthermore, insects can be raised at relatively low economic and environmental costs; for example, insects use 50% to 90% less land per kilogram of protein produced and 40% to 80% less food per kilogram of edible insect. The environmental impact of insect farming is one of the main assets of entomophagy. Unlike pork, poultry, and cattle, edible insects produce 100 times fewer greenhouse gases and provide the same amount of protein. Cockroaches, termites, beetles, and many insects lack a digestive system; therefore, these small animals do not produce methane (Imathiu, 2020). Another advantage of consuming edible insects is the variability of nutrients. A comparison of nutritional profiles of insects and different types of meat carried out using nutritional value score (NVS) resulted in a significantly healthier scores to crickets, red palm weevil larvae, and mealworms than to beef and chicken. This is due to the nutritional composition of insects which contain protein, mineral, fat and various nutrients compared to the most consumed meats; for this reason, the incorporation of edible insects to obtain a well-balanced diet is recommended (Payne et al., 2016).

According to the Food and Agriculture Organization of the United Nations (FAO), the insects with the highest consumption belong to the order Coleoptera, corresponding to the 40% of the species of edible insects. In Latin America, the most consumed insects are bees, wasps, and ants (Hymenoptera) which represent 14% of the species of edible insects. Following these are the grasshoppers, locusts, and

crickets (Orthoptera) representing 13% of species of edible insects (*Edible Insects*, n.d.).

1.1 Entomophagy in Mexico

Due certain conditions, the ancient inhabitants obtained protein from other sources such as insects, crustaceans, fish, and batrachians, in the absence of horses, and pigs. In Mexico, grasshoppers were an important seasonal food for the Aztecs (Viesca González & Romero Contreras, 2009).

Nowadays, insects are widely consumed in Oaxaca, Puebla, and Tlaxcala. They are left alive for a few days to purge, boiled in salted water, then fried or roasted and eaten by adding garlic, lemon, and chili sauce. It is well known that insects provide nutritional compounds since proteins, fatty acids, fiber, vitamins, and minerals such as potassium, calcium, phosphorus, and sodium, are included in their composition. Even though Mexico is one of the countries with the most incredible diversity of edible insects, most of the studies focus on the determination of proximal analysis, where it is reported that edible insects are a great source of proteins, fats, minerals, and crude fiber, making them an excellent option to be considered on human diet worldwide (Guzmán-Mendoza et al., 2016). Additionally, some studies on Mexican species such as red maguey worm (*Comadia redtembacheri H.)*, maguey worm *(Aegiale hesperiaris W*), and escamoles (*Liometopum apiculatum M*.) determined the proximal chemical composition, minerals, and caloric intake, in different stages such as eggs, larvae or pupas where its nutritional importance is indicated (Ramos-Rostro et al., 2012).

Regarding studies of *Sphenarium purpurascens*, analysis of fatty acid profile where gamma-linoleic and linoleic acids were found in high concentrations is reported (Torruco-Uco et al., 2019), also the impact of changing diet of insects on their chemical composition is reported (Ibarra-Herrera et al., 2020); and the use of grasshopper in paste for the fortification of mole to increase its nutritional value is found (Arcos-Estrada et al., 2020).

1.2 Bioactivity of extracts obtained from edible insects

According to literature, some extracts obtained from edible insects presented different bioactivities such as antioxidant activity, anti-inflammatory activity, antimicrobial properties, among others (Nongonierma & FitzGerald, 2017). For example, in aqueous extracts of *Acheta domesticus*, antioxidant capacity was found. Studies in insects such as the central American locust, *Schistocerca piceifrons (Walker*), where different solvents were evaluated such as water and alcohols, reported the presence of bioactive compounds for example antioxidants, phenolic compounds, flavonoids, etc., and concluded that these extracts could be employed as a source of nutraceuticals (Pérez-Ramírez et al., 2019).

The use of enzymatic hydrolysis of proteins on extracts can generate favorable changes, e.g., improvement of techno-functional properties and enhancement of their bioactivity. However, there is limited evidence of their use in improvement of food products or any other commercial use. The exploration of functional and nutritional properties of protein hydrolysates could derive on their use on food products. Most of the studies on Mexican edible insects focus on proximal analysis and determination of compounds with bioactivity, but no studies have been reported on protein hydrolysates and their applications.

Considering that the grasshopper is the most popular and commonly consumed insect in Mexico, and according to its proximal studies, which corroborate that it is an excellent source of nutrients, especially proteins, it is presented a work that focuses on the analysis of in vitro biological activity on extracts and hydrolysates from *Sphenarium purpurascens.*

1.2.1 Proximal analysis

The proximal analysis involves a group of assays to determine the composition of the nutrients in food, which includes determining moisture, fat, fiber, ash, soluble carbohydrates, and protein. The content of protein, fat, minerals, and vitamins in

edible insects is high and generally satisfies food requirements based on a healthy diet (Izquierdo Córser et al., 2000). However, there is a considerable variation associated with insect species, sex, diet, life stage, collection site, method of processing, and rearing technology (Acosta-Estrada et al., 2021).

1.2.2 Total antioxidant capacity

The antioxidant properties of foods are generally given by the total and interaction of their molecules. The antioxidant compound can be classified mainly in vitamins, carotenoids, and polyphenols. Phenolic compounds are polar molecules with one hydroxyl group attached to an aromatic ring and present antioxidant properties. These compounds bring several benefits to human health; for example, they are used to treat and prevent cancer, cardiovascular diseases, and other inflammatory diseases. Polyphenols exhibit in their structure one or more hydroxyl groups attached to an aromatic ring. Among the polyphenols, it is possible to classify flavonoids, anthocyanins, tannins, among others (Neveu et al., 2010). It has shown that polyphenols have properties like vasodilator and possible intervention in oxidation of low-density lipoproteins. Phenolic compounds and polyphenols can be used as natural antioxidants in processed foods, increasing their nutritional value converting them into functional foods (Noreen et al., 2017).

The most common method for quantifying total phenolic compounds is Folin-Ciocalteu assay, which is a spectrophotometric method where the reagent reacts with oxidizable substances in an alkaline medium, developing blue coloration (see Fig. 1). Total phenolic compounds have been reported in edible insects like *Acheta domesticus* adult and *Tenebrio molitor* larvae, and the concentration of total phenolic compounds is around 3-50 mg gallic acid equivalents (GAE) (Navarro del Hierro et al., 2020). Although the quantification of TPC by this method is widely used as a preliminary way to characterize the antioxidant capacity of food, it should be considered that TPC measurement does not distinguish the proportion of each of the polyphenols present in a food. However, the TPC measurement is an excellent, practical, and straightforward approach to initially characterize a food in terms of its

antioxidant content. When the whole extract is used in these studies, it should be considered that the antioxidant capacity may also be due to the presence of compounds different from phenolic compounds or polyphenols. To avoid such an underestimation, it is essential that the characterization of the antioxidant richness of foods also includes the measurement of their "antioxidant activity" (Prior et al., 2005).

Most of the tests used to determine the antioxidant activity of food are based on the measurement of (1) the ability of antioxidant compounds to react with a determined free radical or reactive oxygen species (ROS), (2) the potential that such compounds have to reduce a complex formed between Fe(III) ions and the reagent TPTZ (2,4,6-tripyridyl-s-triazine) and (3) the capacity of donating or receiving electrons neutralizing radicals.

For *in vitro* determination of the antioxidant compounds, spectrophotometric methods are also used. Some methods used to measure antioxidant capacity are ABTS•+ (2,2´-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid)) and DPPH• (2,2-diphenyl-1-picrilhidrazil) and are based on the stabilization of metastable synthetic-free radicals, whose reaction with an antioxidant generates a color change that can be detected in a spectrophotometer. However, these radicals can be stabilized by hydrogen atoms transfer and electron transfer mechanisms, reactivity patterns are challenging to interpret when the chemical structure of the antioxidant is unknown (Miranda de Matos et al., 2021). Antioxidant activity depends on the type (position and number of hydroxyls in the molecule), the concentration of phenolic compounds, and the presence of transition metals, among other factors. In DPPH• assay (see Fig. 2), a change of color is observed from purple to light yellow in the presence of antioxidant compounds and it is measured at 517nm. Additionally, DPPH• is a stable nitrogen-centered radical that is far from resembling reactive species of biological importance; in fact, many antioxidants that react quickly with peroxyl radicals do not interact with DPPH•, due to the steric hindrance presented by the chemical structure surrounding the radical, which causes small substances to generally show higher activity (Marinova & V. Batchvarov, 2011)

On the other hand, ABTS•+ assay (see Fig. 3), it is necessary to add sodium persulfate to form ABTS•+ (radical) which presents a blue-green color, and in the presence of antioxidant compounds, ABTS•+ is reduced returning to its neutral colorless form (Gupta et al., 2009). This change of color is measured at 732 nm. Among the advantages of using this method, one advantage is that there isa wide range of foods where this method is reported, which allows establishing comparisons. In addition, it can be used in a wide range of pH and ionic strength, plus the fact that ABTS•+ is soluble in both aqueous and organic media allowing the evaluation of hydrophilic and lipophilic antioxidants. Among the disadvantages, there is that it is not a physiological radical, although it provides an approximation of the capacity of certain compounds to accept electrons under specific conditions. Both assays are used to evaluate the scavenging ability of antioxidants, and the results are reported as Trolox equivalent per gram of sample.

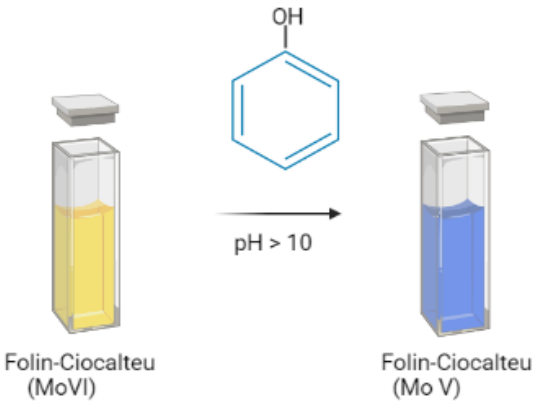

Figure 1 Total phenolic compounds assay.

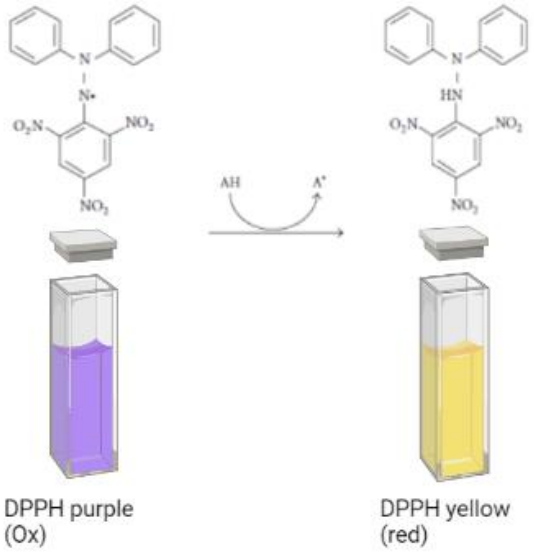

Figure 2 Antioxidant compound against DPPH radical.

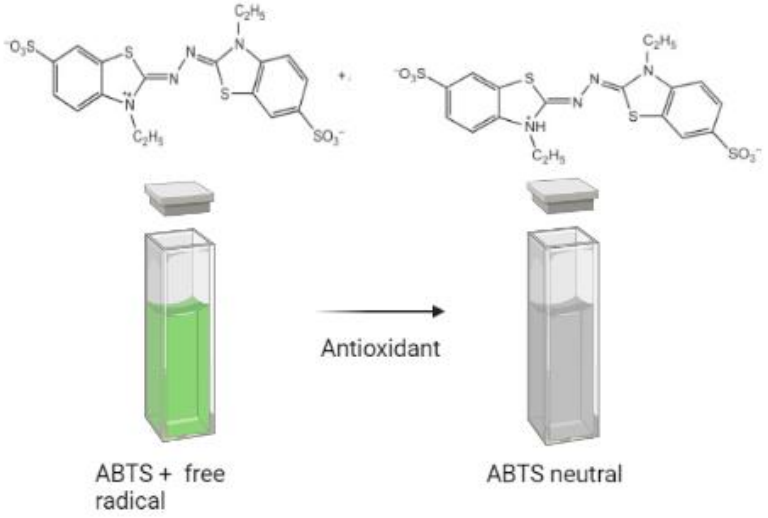

Figure 3 Antioxidant against ABTS radical.

Ferric reducing antioxidant power (FRAP) is a method used to measure the capacity of a compound to reduce Fe^{+3} contained into a standard solution of $FeCl_3$ until Fe^{+2}. Ferric tripyridyl triazine complex is reduced to its ferrous state in an acid medium by an antioxidant. This reaction produces a change in color that is measured spectrophotometrically at a wavelength of 595 nm. FRAP can also be determined by

preparing the sample in a 0.2 M phosphate buffer (pH 6.6) with potassium ferricyanide, trichloroacetic acid, and ferric chloride. The absorbance of the solution is measured at 700 nm (Zielińska et al., 2017).

1.2.3 Antimicrobial activity

The application of some insects in medicine is not a recent practice and it has been shown that it has beneficial aspects not only for nutrition but also for health areas. Antimicrobial peptides have been described in many species of organisms: fungi, plants, humans, and insects, the latter being the ones in which the most effective antimicrobial peptides have been described (Bastida et al., 2020). It is known that insects have an innate defense system that does not generate antimicrobial resistance, like antibiotics; these compounds are called antimicrobial peptides (Wu et al., 2018). These are classified according to the amino acid sequence of the peptide chain, and there are three groups: defensins, cecropins, and peptides with proline/glycine residues (Mudalungu et al., 2021). Defensins belong to the family arginine-rich peptides that have a good response against a wide range of microorganisms, mainly Gram-positive as *Staphylococcus aureus*. Most defensins contain 6-8 cysteine residues, and the length is 29-34 amino acids. The structure consisting of three disulfide bonds and β hairpin can bind to the cell membrane, provoking cell damage avoiding ion exchange. On the other hand, cecropins have a large structure that consists of 35-37 cysteine residues, and their microbial activity includes Gram positives and Gram-negatives (Wu et al., 2018). In Table 1, some antimicrobial peptides are briefly described. There are other compounds present in insects with antimicrobial and antifungal activity as fatty acids unsaturated and polyunsaturated found in *Sarcophaga carnaria* (Ishikawa et al., 1992). The chitosan from *Julus terrestris*, *Calliptamus barbarous,* and *Oedaleus decorus* has demonstrated antimicrobial activity against *Staphylococcus aureus, Pseudomonas aeruginosa, Enterobacter aerogenes, and Staphylococcus epidermidis* (Kaya et al., 2015).

Table 1 Antimicrobial peptides isolated from insects.

Antimicrobial peptide	Structure	Insect	Types	Reference
Cecropins	Without cysteine residues	*Hyalophora cecropia*	A, B, B_1, B_3, C, D	(Steiner et al., 1981)
Lebocins	Proline-rich/ O-glycosylated peptide	*Bombyx mori*	L1, L2, L3	(Hara & Yamakawa, 1995)
Drosocins	O- glycosylated peptide	*Drosophila. melanogaster*		(Bulet et al., 1993)
Diptericins	Glycine rich peptides	*Phormium terranovae*	A, B, C	(Ishikawa et al., 1992)
Prolixins		*Rhodnius prolixus*		(Ursic-Bedoya et al., 2011)
Metchnikowin	Proline-rich/	*Drosophila melanogaster*		(Levashina et al., 1995)
Ponericins		*Pachycondyla goeldii.*	G, L, and W	(Johnson et al., 2010)
Jelleines	8–9 amino acids	*Apis mellifera*	I, II, III and IV	(Romanelli et al., 2011)
Apisimin	54 amino acids rich Val and Ser	Honeybees		(Bíliková et al., 2002)
Pyrrhocoricin	proline-rich inducible AMP,	*Pyrrhocoris apterus*		Cociancich et al., 199(4)
Persulcatusin	three S-S bonds,	*Ixodes persulcatus*		(Saito et al., 2009)
Melittin	26 amino acid residues	Bee venom		(Lee & Bae, 2016)

One method to verify the antimicrobial activity is the spectrophotometric determination of bacterial growth inhibition, consist of a bacterial culture is adjusted according to the McFarland scale to the desired concentration, and subsequently, the compounds to be evaluated is added, and finally, the absorbance is measured

at different times (Christman, 2010). Inhibition of bacterial growth by the agar dilution method consists of adding a the compounds of interest and collocating in Petri dishes with agar which was inoculated with bacteria previously (Ruiz et al., 2009)

1.3 Obtention of extracts from *Sphenarium purpurascens*

1.3.1 Extraction generalities

The extraction process of compounds from edible insects has increasing in the last two decades since its focus is their use in the food industry to increase the nutritional value of these foods. As we know, edible insects are a great source of protein and fatty acids that can complement the daily intake in populations with nutrient deficiency. On the other hand, insects can also contain allergenic compounds such as chitin, which would attract counterproductive effects; for this reason, the study of different operations and processes for obtaining the compounds of interest is essential (Mintah et al., 2020).

One of the steps for improving protein extraction is defatting of samples, which is an efficient process to increase the protein concentration and minimize the lipid content of raw materials. It has been shown that in the defatting process, the use of solvents with different polarities can have an effect and influence specific characteristics, such as defatting efficiency and the techno-functional characteristics of compounds (Choi et al., 2017).

Most of the protein extraction processes are carried out in an aqueous phase at a pH higher than 7 (alkaline extraction) to increase the solubility of proteins. According to alkaline extractions in the aqueous phase using *Tenebrio molitor* it was shown that at pH between 4-6, the percentage of extraction was 29.6% w/w of the total protein. In comparison, an alkaline pH of 11 68.6% was obtained w/w of total proteins (Nongonierma & FitzGerald, 2017).

1.3.2 Enzymatic hydrolysis

Hydrolysis of extracts is commonly achieved using different types of enzymes. In this case, proteolytic enzymes isolated from bacteria and fungi are used, such as Alcalase®, which is a protease isolated from *Bacillus licheniformis* presents affinity by aromatic amino acids as tryptophane, phenylalanine, and tyrosine (Miranda de Matos et al., 2021). Alcalase® (E.C.3.4.21.14) is a serine endopeptidase consisting mainly of subtilisin A suitable for hydrolysis of proteins (Fig. 4). It has an optimal pH of 7.0-8.5. (*ALCALASE® Enzyme, Bacillus Licheniformis | 126741*, n.d.)

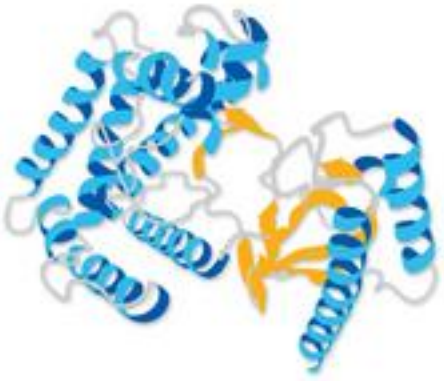

Figure 4 Protease (E.C. 3.4.21.14) from *Bacillus licheniformis.*

To determine enzymatic activity, it is necessary to measure the degree of hydrolysis (DH). DH is defined as the proportion of cleaved peptide bonds in a protein hydrolysate; pH and temperature conditions are crucial factors influencing the DH. There are several methods for measuring DH; determination of free amino groups by the reaction of 2,4,6-trinitrobenzene sulfonic acid solution (TNBS) (Sousa et al., 2020). DH can also be determined by the soluble fraction of the hydrolysate. When trichloroacetic acid is added, the proteins are precipitated, leaving the peptide fractions in the supernatant, which can be quantified by the total nitrogen Kjeldahl method; this determination is known as the soluble nitrogen-trichloroacetic acid index (SN-TCA). The SN-TCA index has become a parameter of practical use (Margot et al., 1994).

1.3.3 Ultrafiltration

Sometimes the hydrolysates are separated into small fractions to determine which of them has the greatest antioxidant potential. Ultrafiltration is a simple and cheap method that can be used to concentrate antioxidant compounds, peptides, and others. According to research, there is greater antioxidant capacity in ultrafiltered hydrolysate fractions (Wiriyaphan et al., 2015). In this way, ultrafiltration can be used to fractionate extracts and hydrolysates.

1.4 Experimental model

As mentioned above, edible insects are a promising source of food; additionally, specific compounds with in vitro biological activity can be obtained from them and used to improve processed foods. This work aims to determine if there are differences in the concentrations of compounds with *in vitro* antioxidant and antibacterial activities obtained from grasshopper extracts, hydrolysates, and fractions of the hydrolysates, as well as differences due to the metamorphic age of the insect.

1.4.1 *Sphenarium purpurascens*

One of the seasonal insects with the most incredible geographic abundance within Mexican territory is the grasshopper *Sphenarium purpurascens,* which belongs to the order of Orthoptera, family Pyrgomorphidae, genus Sphenarium, and species *purpurascens* Ch (Table 2). Due to its nutritional composition, the extraction of nutritional components such as proteins and lipids from edible insects for later use as food ingredients have been proposed to improve consumer appeal (Rodríguez-Miranda et al., 2019). According to several authors, grasshoppers contain between 43.9% to 77.1% of protein. Besides, these insects have essential amino acids necessary for a well-balanced diet, according to essential amino acid index (EAAI)

reported by Ibarra-Herrera et al.,(2020) for *Sphenarium purpurascens*, results of grasshopper fed with maize (65.44%) and grasshoppers fed with alfalfa (75.19%),are found into the range (70%-90%) to be considered as a useful source of protein(Ibarra-Herrera et al., 2020). There is a study where nutritional profiles of Mexican grasshoppers were compared, this included to *Sphenarium bistro*, *Sphenarium purpurascens*, *Melanoplus femurrubrum*, *Taeniopoda equse,* and *Schistocerca spp,* all were recollected in stage adult (Ruiz et al., 2015). So far, no study has been reported comparing the impact of the stage on the nutritional profiles of *Sphenarium purpurascens*. But in grasshoppers from Niger was found that the protein content also depends on the food source; grasshoppers fed with bran showed higher levels of essential fatty acids, they have almost twice the protein content in comparison with grasshoppers that fed with maize (Huis, 2013). In addition, the protein content found in *Zonocerus variegatus* adult stage was higher than other instars; however, other parameters (ashes, fiber, fat, and carbohydrates) were not different. This also depends on the stage of metamorphosis, and also some environmental conditions could have influence in the nutritional composition of edible insects (Ademolu & Idowu, 2010).

In the case of grasshoppers, *Sphenarium purpuranscens* is considered a devastating pest in Central and Southern Mexico. The states of Puebla, Tlaxcala, Oaxaca, Hidalgo, Mexico, Querétaro, Michoacán, and Guanajuato contributes approximately 350 thousand tons of grasshoppers in the breeding season (Fig. 5). Mainly, the crops of corn (*Zea mays L.*), alfalfa (*Medicago sativa L.*), and beans (*Phaseolus sp.*) are infested by this endemic species. In this regard, it is known that if 4 tons/ha are collected, with the infestation of grasshoppers, only one hectare of the crop is suitable for further use. This is the main reason producers collect and commercialize these insects. For the industrialization and formal commercialization of grasshopper, it must become a safe product, free of contaminants, enteric bacteria, and other components that would be harmful to the health of people. In Mexico, the edible insect industry is based on informality and lack of safety. However, the use of insects in human food allows promoting sustainability and food safety (Arcos-Estrada et al., 2020)

Table 2 Taxonomic classification of *S. purpurascens*

Sphenarium purpurascens	
Common name	Chapulin de milpa
Orden	Orthoptera
Sub Orden	Caelifera
Superfamily	Acridoidea
Family	Pyrgomorphidae
Sub Family	Pyrgomorphinae
Gender	Sphenarium

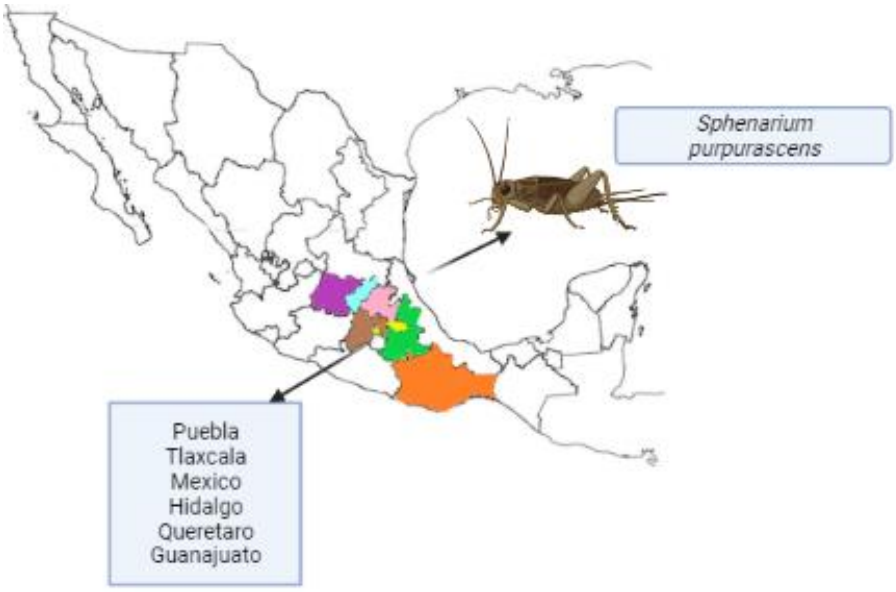

Figure 5 Geographical distribution of *Sphenarium purpurascens.*

1.5 Hypothesis

Extracts, hydrolysates and hydrolysate fractions from early-stage and adult-stage of grasshopper *Sphenarium purpurascens* will present different in vitro antioxidant and antibacterial activities.

1.6 General objective

The general objective of this work is to determine antioxidant and antibacterial activities of extracts, hydrolysates, and hydrolysate fractions from *Sphenarium purpurascens* of early and adult stages by *in vitro* methods.

1.7 Specific objectives

The hypothesis and the general objective mentioned above can be addressed by achieving these specific objectives:

1) Determine proximal analysis of early-stage and adult-stage *Sphenarium purpurascens,* including protein content, ash, chitin content, and ethereal extract.
2) Obtain protein extract from early-stage and adult-stage *Sphenarium purpurascens* through alkaline extraction.
3) Carry out enzymatic hydrolysis of the extracts using protease from *Bacillus licheniformis* and quantify the degree of hydrolysis.
4) Separate the hydrolysates in three fractions: > 30 kDa, >10 kDa, and <10 kDa using ultrafiltration.
5) Evaluate the antioxidant activity *in vitro* of the extracts, hydrolysates, and hydrolysate fractions using qualitative and quantitative assays.
6) Evaluate the antimicrobial activity of the extracts, hydrolysates, and hydrolysate fractions.

1.8 Thesis structure

As mentioned previously, this thesis work focuses on **evaluating the antioxidant and antibacterial activities of the compounds extracted from the early and adult stages of *Sphenarium purpurascens* by *in vitro* assays**. This thesis

consists of 3 chapters. In the first chapter, is briefly mentioned the advantages of entomophagy and its positive impact and benefits in the environmental sector in specific in greenhouse gases emission by livestock and food industry where it is explained that edible insects are a good source of several nutrients as proteins, fat, mineral and so on and compared with conventional food as meet. In addition, it explained the importance of proximal analyses; it is included a brief explanation about bioactive compounds found in edible insects and methods applied for their quantification. Furthermore, it is described *Sphenarium purpuranscens* as an experimental model, hypothesis, and objectives. The second chapter has described the methodologies to be followed, obtained results, and discussion. The third chapter mentions the general conclusion and some recommendations of this work.

1.9 References

Acosta-Estrada, B. A., Reyes, A., Rosell, C. M., Rodrigo, D., & Ibarra-Herrera, C. C. (2021). Benefits and Challenges in the Incorporation of Insects in Food Products. *Frontiers in Nutrition*, *8*, 344. https://doi.org/10.3389/fnut.2021.687712

Ademolu, K., & Idowu, A. B. (2010). Nutritional Value Assessment of Variegated Grasshopper, Zonocerus variegatus (L.) (Acridoidea: Pygomorphidae), During Post-Embryonic Development. *African Entomology*, *18*, 360–364. https://doi.org/10.4001/003.018.0201

ALCALASE® Enzyme, Bacillus licheniformis | 126741. (n.d.). Retrieved November 27, 2021, from https://www.merckmillipore.com/MX/es/product/ALCALASE-Enzyme-Bacillus-licheniformis,EMD_BIO-126741

Amarender, R. V., Bhargava, K., Dossey, A. T., & Gamagedara, S. (2020). Lipid and protein extraction from edible insects – Crickets (Gryllidae). *LWT*, *125*, 109222. https://doi.org/10.1016/j.lwt.2020.109222

Arcos-Estrada, E. G., Díaz-Ramírez, M., Jiménez-Guzmán, J., Fabela-Morón, M. F., Rayas-Amor, A. A., León-Espinosa, E. B., García-Garibay, M., & Cruz-Monterrosa, R. G. (2020). Análisis fisicoquímico y proteínico de la pasta de chapulín (Sphenarium purpurascens Charpentier). *Agro Productividad*, *13*(6), Article 6. https://doi.org/10.32854/agrop.vi.1729

Baigts-Allende, D., Doost, A. S., Ramírez-Rodrigues, M., Dewettinck, K., Van der Meeren, P., de Meulenaer, B., & Tzompa-Sosa, D. (2021). Insect protein

concentrates from Mexican edible insects: Structural and functional characterization. *LWT*, *152*, 112267. https://doi.org/10.1016/j.lwt.2021.112267

Bastida, A. Z., Costeira-Huerta, C., Herrera-Soto, I. A., Martínez, M. A., Soto-Simental, S., & López, J. O. (2020). Péptidos de insectos: Una alternativa antibacteriana. *Boletín de Ciencias Agropecuarias del ICAP*, *6*(12), 5–8. https://doi.org/10.29057/icap.v6i12.5532

Bíliková, K., Hanes, J., Nordhoff, E., Saenger, W., Klaudiny, J., & Šimúth, J. (2002). Apisimin, a new serine–valine-rich peptide from honeybee (Apis mellifera L.) royal jelly: Purification and molecular characterization1. *FEBS Letters*, *528*(1–3), 125–129. https://doi.org/10.1016/S0014-5793(02)03272-6

Black, M. M., & Schwartz, H. M. (1950). The estimation of chitin and chitin nitrogen in crawfish waste and derived products. *Analyst*, *75*(889), 185–189. https://doi.org/10.1039/AN9507500185

B. Nongonierma, A., Lamoureux, C., & J. FitzGerald, R. (2018). Generation of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides during the enzymatic hydrolysis of tropical banded cricket (Gryllodes sigillatus) proteins. *Food & Function*, *9*(1), 407–416. https://doi.org/10.1039/C7FO01568B

Botella-Martínez, C., Lucas-González, R., Pérez-Álvarez, J. A., Fernández-López, J., & Viuda-Martos, M. (2021). Assessment of chemical composition and antioxidant properties of defatted flours obtained from several edible insects. *Food Science and Technology International = Ciencia Y Tecnologia De Los Alimentos Internacional*, *27*(5), 383–391. https://doi.org/10.1177/1082013220958854

Boulos, S., Tännler, A., & Nyström, L. (2020). Nitrogen-to-Protein Conversion Factors for Edible Insects on the Swiss Market: T. molitor, A. domesticus, and L. migratoria. *Frontiers in Nutrition*, *7*, 89. https://doi.org/10.3389/fnut.2020.00089

Bulet, P., Dimarcq, J. L., Hetru, C., Lagueux, M., Charlet, M., Hegy, G., Van Dorsselaer, A., & Hoffmann, J. A. (1993). A novel inducible antibacterial peptide of Drosophila carries an O-glycosylated substitution. *The Journal of Biological Chemistry*, *268*(20), 14893–14897.

Choi, B. D., Wong, N. A. K., & Auh, J.-H. (2017). Defatting and Sonication Enhances Protein Extraction from Edible Insects. *Korean Journal for Food Science of Animal Resources*, *37*(6), 955–961. https://doi.org/10.5851/kosfa.2017.37.6.955

Christman, J. M. (2010). *Antimicrobial Activity of Casein Hydrolysates against Listeria monocytogenes and Escherichia coli O157:H7*. 93.

Cociancich, S., Dupont, A., Hegy, G., Lanot, R., Holder, F., Hetru, C., Hoffmann, J. A., & Bulet, P. (1994). Novel inducible antibacterial peptides from a hemipteran insect, the sap-sucking bug Pyrrhocoris apterus. *The Biochemical Journal*, *300 (Pt 2)*, 567–575. https://doi.org/10.1042/bj3000567

Cultivo y consumo de insectos, opción ante la creciente demanda de alimentos. (2020, March 17). PortalAmbiental.com.mx. https://www.portalambiental.com.mx/sustentabilidad/20200317/cultivo-y-consumo-de-insectos-opcion-ante-la-creciente-demanda-de-alimentos

Daroit, D. J., & Brandelli, A. (2021). In vivo bioactivities of food protein-derived peptides – a current review. *Current Opinion in Food Science*, *39*, 120–129. https://doi.org/10.1016/j.cofs.2021.01.002

Di Mattia, C., Battista, N., Sacchetti, G., & Serafini, M. (2019). Antioxidant Activities in vitro of Water and Liposoluble Extracts Obtained by Different Species of Edible Insects and Invertebrates. *Frontiers in Nutrition*, *6*, 106. https://doi.org/10.3389/fnut.2019.00106

Edible insects. (n.d.). Retrieved August 9, 2021, from http://www.fao.org/3/i3253e/i3253e00.htm

Gupta, R., Sharma, M., Lakshmy, R., Prabhakaran, D., & Reddy, K. S. (2009). Improved method of total antioxidant assay. *IJBB Vol.46(1) [February 2009]*. http://nopr.niscair.res.in/handle/123456789/3333

Guzmán-Mendoza, R., Calzontzi-Marín, J., Salas-Araiza, M. D., Martínez-Yáñez, R., Guzmán-Mendoza, R., Calzontzi-Marín, J., Salas-Araiza, M. D., & Martínez-Yáñez, R. (2016). La riqueza biológica de los insectos: Análisis de su importancia multidimensional. *Acta zoológica mexicana*, *32*(3), 370–379.

Hall, F., Johnson, P. E., & Liceaga, A. (2018). Effect of enzymatic hydrolysis on bioactive properties and allergenicity of cricket (Gryllodes sigillatus) protein. *Food Chemistry*, *262*, 39–47. https://doi.org/10.1016/j.foodchem.2018.04.058

Hara, S., & Yamakawa, M. (1995). A novel antibacterial peptide family isolated from the silkworm, Bombyx mori. *The Biochemical Journal*, *310 (Pt 2)*, 651–656. https://doi.org/10.1042/bj3100651

Hurd, K. J., Shertukde, S., Toia, T., Trujillo, A., Pérez, R. L., Larom, D. L., Love, J. J., & Liu, C. (2019). The Cultural Importance of Edible Insects in Oaxaca, Mexico. *Annals of the Entomological Society of America*, *112*(6), 552–559. https://doi.org/10.1093/aesa/saz018

Ibarra-Herrera, C. C., Acosta-Estrada, B., Chuck-Hernández, C., Serrano-Sandoval, S. N., Guardado-Félix, D., & Pérez-Carrillo, E. (2020). Nutritional content of edible grasshopper (Sphenarium purpurascens) fed on alfalfa (Medicago sativa) and maize (Zea mays). *CyTA - Journal of Food*, *18*(1), 257–263. https://doi.org/10.1080/19476337.2020.1746833

Imathiu, S. (2020). Benefits and food safety concerns associated with consumption of edible insects. *NFS Journal*, *18*, 1–11. https://doi.org/10.1016/j.nfs.2019.11.002

Insectos: La alimentación del futuro. (2021, September 22). The Food Tech. https://thefoodtech.com/columnistas/insectos-la-alimentacion-del-futuro/

Ishikawa, M., Kubo, T., & Natori, S. (1992). Purification and characterization of a diptericin homologue from Sarcophaga peregrina (flesh fly). *The Biochemical Journal*, *287 (Pt 2)*, 573–578. https://doi.org/10.1042/bj2870573

Izquierdo Córser, P., Torres Ferrari, G., Barboza de Martínez, Y., Márquez Salas, E., & Allara Cagnasso, M. (2000). [Proximal analysis, fatty acids profile, essential amino acids and mineral content in 12 species of fishes of commercial importance in Venezuela]. *Archivos Latinoamericanos De Nutricion*, *50*(2), 187–194.

Jantzen da Silva Lucas, A., Menegon de Oliveira, L., da Rocha, M., & Prentice, C. (2020). Edible insects: An alternative of nutritional, functional and bioactive compounds. *Food Chemistry*, *311*, 126022. https://doi.org/10.1016/j.foodchem.2019.126022

Johnson, S. R., Copello, J. A., Evans, M. S., & Suarez, A. V. (2010). A biochemical characterization of the major peptides from the Venom of the giant Neotropical hunting ant Dinoponera australis. *Toxicon: Official Journal of the International Society on Toxinology*, *55*(4), 702–710. https://doi.org/10.1016/j.toxicon.2009.10.021

Kaya, M., Baran, T., Asan-Ozusaglam, M., Cakmak, Y. S., Tozak, K. O., Mol, A., Mentes, A., & Sezen, G. (2015). Extraction and characterization of chitin and chitosan with antimicrobial and antioxidant activities from cosmopolitan Orthoptera species (Insecta). *Biotechnology and Bioprocess Engineering*, *20*(1), 168–179. https://doi.org/10.1007/s12257-014-0391-z

Kunatsa, Y., Chidewe, C., & Zvidzai, C. J. (2020). Phytochemical and anti-nutrient composite from selected marginalized Zimbabwean edible insects and vegetables. *Journal of Agriculture and Food Research*, *2*, 100027. https://doi.org/10.1016/j.jafr.2020.100027

Lee, G., & Bae, H. (2016). Anti-Inflammatory Applications of Melittin, a Major Component of Bee Venom: Detailed Mechanism of Action and Adverse Effects. *Molecules (Basel, Switzerland)*, *21*(5), E616. https://doi.org/10.3390/molecules21050616

Levashina, E. A., Ohresser, S., Bulet, P., Reichhart, J. M., Hetru, C., & Hoffmann, J. A. (1995). Metchnikowin, a novel immune-inducible proline-rich peptide from Drosophila with antibacterial and antifungal properties. *European Journal of Biochemistry*, *233*(2), 694–700. https://doi.org/10.1111/j.1432-1033.1995.694_2.x

Margot, A., Flaschel, E., & Renken, A. (1994). *Continuous Monitoring of Enzymatic Whey Protein Hydrolysis. Correlation of Base Consumption with Soluble Nitrogen Content*. https://doi.org/10.1016/0032-9592(94)80066-9

Marinova, G., & V. Batchvarov. (2011). *EVALUATION OF THE METHODS FOR DETERMINATION OF THE FREE RADICAL SCAVENGING ACTIVITY BY DPPH*. *17*, 11–24.

Mexico Could Be the Top Source of Edible Insects. Here's Why It's Not. (2019, August 5). OZY. https://www.ozy.com/news-and-politics/mexico-could-be-the-top-source-of-edible-insects-heres-why-its-not/95545/

Mintah, B. K., He, R., Agyekum, A. A., Dabbour, M., Golly, M. K., & Ma, H. (2020). Edible insect protein for food applications: Extraction, composition, and functional properties. *Journal of Food Process Engineering*, *43*(4), e13362. https://doi.org/10.1111/jfpe.13362

Miranda de Matos, F., Novelli, P., & Janser, R. (2021). Enzymatic hydrolysis of black cricket (Gryllus assimilis) proteins positively affects their antioxidant properties. *Journal of Food Science*, *86*. https://doi.org/10.1111/1750-3841.15576

Mishyna, M., Martinez, J.-J. I., Chen, J., & Benjamin, O. (2019). Extraction, characterization and functional properties of soluble proteins from edible grasshopper (Schistocerca gregaria) and honey bee (Apis mellifera). *Food Research International*, *116*, 697–706. https://doi.org/10.1016/j.foodres.2018.08.098

Mudalungu, C. M., Tanga, C. M., Kelemu, S., & Torto, B. (2021). An Overview of Antimicrobial Compounds from African Edible Insects and Their Associated Microbiota. *Antibiotics*, *10*(6), 621. https://doi.org/10.3390/antibiotics10060621

Muñoz-Velázquez, E. E., Rivas-Díaz, K., Loarca-Piña, M. G. F., Mendoza-Díaz, S., Reynoso-Camacho, R., & Ramos-Gómez, M. (2012). Comparación del contenido fenólico, capacidad antioxidante y actividad antiinflamatoria de infusiones herbales comerciales. *Revista mexicana de ciencias agrícolas*, *3*(3), 481–495.

Navarro del Hierro, J., Gutiérrez-Docio, A., Otero, P., Reglero, G., & Martin, D. (2020). Characterization, antioxidant activity, and inhibitory effect on pancreatic lipase of extracts from the edible insects Acheta domesticus and Tenebrio molitor. *Food Chemistry*, *309*, 125742. https://doi.org/10.1016/j.foodchem.2019.125742

Neveu, V., Pérez-Jiménez, J., Vos, F., Crespy, V., Chaffaut, L., Mennen, L., Knox, C., Eisner, R., Cruz, J., Wishart, D., & Scalbert, A. (2010). Phenol-Explorer: An online comprehensive database on polyphenol contents in foods. *Database : The Journal of Biological Databases and Curation*, *2010*, bap024. https://doi.org/10.1093/database/bap024

Nongonierma, A. B., & FitzGerald, R. J. (2017). Unlocking the biological potential of proteins from edible insects through enzymatic hydrolysis: A review. *Innovative Food Science & Emerging Technologies*, *43*, 239–252. https://doi.org/10.1016/j.ifset.2017.08.014

Noreen, H., Semmar, N., Farman, M., & McCullagh, J. S. O. (2017). Measurement of total phenolic content and antioxidant activity of aerial parts of medicinal plant Coronopus didymus. *Asian Pacific Journal of Tropical Medicine*, *10*(8), 792–801. https://doi.org/10.1016/j.apjtm.2017.07.024

Payne, C. L. R., Scarborough, P., Rayner, M., & Nonaka, K. (2016). Are edible insects more or less 'healthy' than commonly consumed meats? A comparison using two nutrient profiling models developed to combat over- and undernutrition. *European Journal of Clinical Nutrition*, *70*(3), 285–291. https://doi.org/10.1038/ejcn.2015.149

Pérez-Ramírez, R., Torres-Castillo, J. A., Barrientos-Lozano, L., Almaguer-Sierra, P., & Torres-Acosta, R. I. (2019). Schistocerca piceifrons piceifrons (Orthoptera: Acrididae) as a Source of Compounds of Biotechnological and Nutritional Interest. *Journal of Insect Science*, *19*(5). https://doi.org/10.1093/jisesa/iez088

Prior, R. L., Wu, X., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry*, *53*(10), 4290–4302. https://doi.org/10.1021/jf0502698

Purschke, B., Meinlschmidt, P., Horn, C., Rieder, O., & Jäger, H. (2018). Improvement of techno-functional properties of edible insect protein from migratory locust by enzymatic hydrolysis. *European Food Research and Technology*, *244*(6), 999–1013. https://doi.org/10.1007/s00217-017-3017-9

Ramos-Rostro, B., Quintero-Salazar, B., Ramos-Elorduy, J., Pino Moreno, J., Campos, S., Pérez, A. G., & García, V. D. B. (2012). Chemical and nutritional analysis of three edible insects of commercial interest at the archaeological site of the municipality of san juan teotihuacan and at otumba, state of Mexico. *Interciencia*, *37*, 914–920.

Rodríguez-Miranda, J., Alcántar-Vázquez, J. P., Zúñiga-Marroquín, T., & Juárez-Barrientos, J. M. (2019). Insects as an alternative source of protein: A review of the potential use of grasshopper (Sphenarium purpurascens Ch.) as a food ingredient. *European Food Research and Technology*, *245*(12), 2613–2620. https://doi.org/10.1007/s00217-019-03383-0

Romanelli, A., Moggio, L., Montella, R. C., Campiglia, P., Iannaccone, M., Capuano, F., Pedone, C., & Capparelli, R. (2011). Peptides from Royal Jelly: Studies on the antimicrobial activity of jelleins, jelleins analogs and synergy with temporins. *Journal of Peptide Science: An Official Publication of the European Peptide Society*, *17*(5), 348–352. https://doi.org/10.1002/psc.1316

Ruiz, V., SandovalTrujillo, H., Quirino-Barreda, C., SnchezHerrera, K., DazGarca, R., & CalvoCarrillo, C. (2015). Chemical composition and amino acids content of five

species of edible Grasshoppers from Mexico. *Emirates Journal of Food and Agriculture*, *17*. https://doi.org/10.9755/ejfa.2015.04.093

Ruiz, V., Silva, P. G., & Laciar, A. L. (2009). Comparison of microplate, agar drop and well diffusion plate methods for evaluating hemolytic activity of. *Afr. J. Microbiol. Res.*, 6.

Saito, Y., Konnai, S., Yamada, S., Imamura, S., Nishikado, H., Ito, T., Onuma, M., & Ohashi, K. (2009). Identification and characterization of antimicrobial peptide, defensin, in the taiga tick, Ixodes persulcatus. *Insect Molecular Biology*, *18*(4), 531–539. https://doi.org/10.1111/j.1365-2583.2009.00897.x

See, S. F., Hoo, L., & Babji, A. (2011). Optimization of enzymatic hydrolysis of salmon (Salmo salar) skin by Alcalase. *International Food Research Journal*, *18*, 1359–1365.

Silvestre-De-León, R., Espinosa-Ramírez, J., Pérez-Carrillo, E., & Serna-Saldívar, S. O. (2021). Extruded chickpea flour sequentially treated with alcalase and α-amylase produces dry instant beverage powders with enhanced yield and nutritional properties. *International Journal of Food Science & Technology*, *56*(10), 5178–5189. https://doi.org/10.1111/ijfs.15199

Sousa, P., Borges, S., & Pintado, M. (2020). Enzymatic hydrolysis of insect Alphitobius diaperinus towards the development of bioactive peptide hydrolysates. *Food & Function*, *11*(4), 3539–3548. https://doi.org/10.1039/D0FO00188K

Steiner, H., Hultmark, D., Engström, Å., Bennich, H., & Boman, H. G. (1981). Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature*, *292*(5820), 246–248. https://doi.org/10.1038/292246a0

Torruco-Uco, J. G., Hernández-Santos, B., Herman-Lara, E., Martínez-Sánchez, C. E., Juárez-Barrientos, J. M., & Rodríguez-Miranda, J. (2019). Chemical, functional and thermal characterization, and fatty acid profile of the edible grasshopper (Sphenarium purpurascens Ch.). *European Food Research and Technology*, *245*(2), 285–292. https://doi.org/10.1007/s00217-018-3160-y

Ursic-Bedoya, R., Buchhop, J., Joy, J. B., Durvasula, R., & Lowenberger, C. (2011). Prolixicin: A novel antimicrobial peptide isolated from Rhodnius prolixus with differential activity against bacteria and Trypanosoma cruzi. *Insect Molecular Biology*, *20*(6), 775–786. https://doi.org/10.1111/j.1365-2583.2011.01107.x

Viesca González, F. C., & Romero Contreras, A. T. (2009). La Entomofagia en México. Algunos aspectos culturales. *El Periplo Sustentable*, *16*, 57. https://doi.org/10.21854/eps.v0i16.922

Villaseñor, V. M., Enriquez-Vara, J. N., Urías-Silva, J. E., & Mojica, L. (2021). Edible Insects: Techno-functional Properties Food and Feed Applications and Biological

Potential. *Food Reviews International*, *0*(0), 1–27. https://doi.org/10.1080/87559129.2021.1890116

WANG, Z., WANG, J., ZHANG, Y., WANG, X., ZHANG, X., LIU, Y., XI, J., TONG, H., WANG, Q., JIA, B., & SHEN, H. (2017). Karasinekteki (Musca domestica) Antimikrobiyal Peptidler Salmonella pullorum ile Enfekte Tavuklarda Bağırsak Lactobacillus acidophilus ve Mukozal Epitel Hücrelerini Etkiler. *Kafkas Universitesi Veteriner Fakultesi Dergisi*. https://doi.org/10.9775/kvfd.2016.16901

Wiriyaphan, C., Xiao, H., Decker, E. A., & Yongsawatdigul, J. (2015). Chemical and cellular antioxidative properties of threadfin bream (Nemipterus spp.) surimi byproduct hydrolysates fractionated by ultrafiltration. *Food Chemistry*, *167*, 7–15. https://doi.org/10.1016/j.foodchem.2014.06.077

Wu, Q., Patočka, J., & Kuča, K. (2018). Insect Antimicrobial Peptides, a Mini Review. *Toxins*, *10*(11), 461. https://doi.org/10.3390/toxins10110461

Xu, Y., Galanopoulos, M., Sismour, E., Ren, S., Mersha, Z., Lynch, P., & Almutaimi, A. (2020). Effect of enzymatic hydrolysis using endo- and exo-proteases on secondary structure, functional, and antioxidant properties of chickpea protein hydrolysates. *Journal of Food Measurement and Characterization*, *14*(1), 343–352. https://doi.org/10.1007/s11694-019-00296-0

Zielińska, E., Karaś, M., & Jakubczyk, A. (2017). Antioxidant activity of predigested protein obtained from a range of farmed edible insects. *International Journal of Food Science & Technology*, *52*(2), 306–312. https://doi.org/10.1111/ijfs.13282

Chapter 2

Evaluation of *in vitro* antioxidant and antibacterial activities of extracts and hydrolysates from *Sphenarium purpurascens* of early and adult stage

Abstract

Edible insects have become a promising food source since it has been shown that they are rich in protein, fatty acids, minerals, among others. *Sphenarium purpurascens* is considered an edible insect endemic from Mexican culture and its geographical distribution includes Tlaxcala, Puebla, Guanajuato, Queretaro, Hidalgo y México. Furthermore, grasshoppers contain several nutritional compounds in which the percentage of protein stands out. Obtaining innovative and alternative ingredients with biological activity from edible insect proteins has increased the demand for which it is necessary to advance research regarding entomophagy.

The present study aimed was determined the antioxidant properties of the extract obtained from *Sphenarium purpurascens* early and adult age and evaluate the differences between them. It was carried out analyses compared proximal analyses of different stages of development of the insect and have been reported slightly different which are attributed to the age of the grasshoppers. Besides, the application of enzymatic hydrolysis using the *Bacillus licheniformis* 2.4L protease caused an increase in antioxidant capacity against DPPH• and ABTS•+. Once fractionated by ultrafiltration, the fractions that presented the highest antioxidant activity against DPPH• and ABTS•+ were ≤10 kDa. In conclusion, the enzymatic treatment with endoproteases proved to be an effective way to improve the concentration of compounds from the grasshopper, thus generating a viable source of possible nutraceuticals.

Keywords: protein, bioactivity, hydrolysis, grasshopper, edible insects

2.1 Introduction

The importance that researchers have taken on edible insects is limited to knowing that they are an excellent alternative for nourishing due to their nutritional content. According to the organization of the United Nations, for the year 2050, the growth of the world population will oscillate around 9,000 million people, therefore the demand for food will increase and with this other food sources must be sought. (*Insectos*, 2021). One of the main advantages of consuming edible insects is the development of a sustainable diet since the large-scale production of insects could satisfy a part of the daily intake of individuals, reducing the emission of greenhouse gases by part of livestock, saving drinking water for raising livestock, reducing deforestation, among others (*Edible Insects*, n.d.).

In Mexico, there are about 549 species of insects that are safe to eat. In some regions, especially in the Southeast of the country, culinary traditions involve the consumption of these insects such as ant eggs or dried worms which are prepared with salt and chili powder to make a condiment, among others (*Mexico Could Source of Edible Insects. Here is Why It's Not*, 2019). In several rural and urban communities of Oaxaca and Puebla, edible insects are collected, processed, and retail such as grasshoppers (*Sphenarium purpurascens Charpentier* (Pyrgomorphidae, Orthoptera), *Melanoplus mexicanus* (Saussure) (Acrididae, Orthoptera), chicatanas (*Atta mexicana* (F. Smith) (Formicidae, Hymenoptera), maguey worms *Comadia redtenbacheri* (Hammerschmidt) (Cossidae, Lepidoptera) and mealybug *Dactylopius coccus Costa* (Dactylopiidae, Hemiptera). Entomophagy in these places is widely common because access to other foods of animal origin has been limited; thus, insects are an important nutritional component of daily intake that today also translates into a significant economic value for hundreds of families in rural areas (Hurd et al., 2019). *Sphenarium purpurascens* is considered an edible insect endemic from Mexico and its geographical distribution include Tlaxcala, Puebla, Guanajuato, Queretaro, Hidalgo y México. Furthermore, grasshoppers contain several nutritional compounds in which protein content stands out (Ibarra-Herrera et al., 2020). In the case of grasshoppers, for every two grams of weight, they provide

one gram of food, while beef, for every 108 grams of body mass, provides only one edible gram (*Cultivo y consumo de insectos*, 2020).

Even though the great nutritional value of edible insects has been demonstrated, there is no total acceptance by the population to include them in the daily diet; for this reason, some authors propose to carry out the extraction of main nutrients to be used as ingredients in processed foods. One of the main purposes of extracting protein from edible insects is to be able to use this extract to increase the amount of protein in food products and thus be able to integrate insects into the daily diet. (Amarender et al., 2020). Alkaline extraction has shown a higher efficiency in extraction of proteins due to the higher solubility of proteins in that condition (Amarender et al., 2020). Furthermore, some studies have shown that compounds with bioactivity can be extracted from edible insects, such as compounds with antioxidant, anti-inflammatory, antimicrobial, or antihypertensive activity. These can be used to improve the nutritional value of processed foods, including eradicating the use of additives that are not beneficial to human health (Jantzen da Silva Lucas et al., 2020).

Thanks to the use of enzymatic hydrolysis applied to insect proteins, it is possible to obtain bioactive compounds; for example, through enzymatic hydrolysis of extract from the tropical cricket (*Gryllodes sigillatus*) generated peptides with inhibitory activity dipeptidyl peptidase IV, which is a modulator of conditions such as diabetes or inflammation (B. Nongonierma et al., 2018). Similarly, proteins from crickets (*G. sigillatus*), mealworm larvae (*T. molitor*), and desert locusts (*S. gregaria*) have also shown anti-inflammatory effects after protein hydrolysis and peptide fractionation (Zielińska et al., 2017).

Although the properties of some insect proteins and hydrolysates have been identified, the number of studies is still quite limited, especially in Mexican edible insects. In the present study, an analysis of the antioxidant capacity of the extracts, hydrolysates, and hydrolysate fractions from early-stage and adult-stage *Sphenarium purpurascens* was performed.

2.2 Experimental section

2.2.1 Reagents and materials.

Sodium Hydroxide, Chloride Acid, Cupric Sulfate, Sulfuric Acid, Sodium Carbonate, Potassium Sulfate, Boric Acid, Methyl Red, Methylene Blue Soy Flour, N-Hexane, Methanol, Ethanol, 1 N Folin-Ciocalteu Reagent, Gallic Acid, 2,2-diphenyl-1-picrilhidrazil DPPH•, 2,2´-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS•+,), (S)-6-Methoxy-2,5,7,8-tetramethylchromane-2-carboxylic acid Trolox, Sodium Persulfate, Ferric Chloride Trichloroacetic Acid, Potassium Ferricyanide, Phosphate Buffer, Potassium Permanganate, Protease from *Bacillus licheniformis,* Luria Agar, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Enterobacter aerogenes* ATCC 13048 *Salmonella sp*., *Pseudomonas aeruginosa* ATCC 77853. Common material of laboratory.

The grasshopper samples were taken from cornfields in the town of Coronado, Puebla. The geographical coordinates are the parallels 19º 06'36 " and 19º 10'42 " of north latitude and the meridians 98º 14'54 " and 98º 19'40 " of western longitude to 2 180 meters above sea level. The recollection took place in September 2020 for early grasshopper (EG) and November 2020 for adult grasshopper (AG) (Fig. 6). The grasshopper samples were cleaned and washed with potable water and then with distilled water, later they were frozen at -80°C for later use.

Figure 6 Sampling and washing and preparing of grasshoppers samples.

2.2.2 Equipment

Lyophilizer Labconco FreeZone® 4.5 Liter was used for preservation of the samples, Kjeldahl Distiller RapidStill™ I Model 6500000 was used for determination of Nitrogen Total, Spectrophotometer Hach DR600 was used for all spectrophotometric measuring, Centrifuge Eppendorf 5702 R was used for phase separation, pH meter Hanna Instruments HI 2216 was used for adjusting pH of the samples, Bath Water Memmert was used for hydrolysis, Analytical Balance Ohaus *Pioneer.*

2.2.3 Proximal analysis of early-stage and adult-stage *Sphenarium purpurascens*.

The proximal analysis of the freeze-dried samples of early grasshopper (EG) and adult grasshopper (AG) was performed according to AOAC methodologies: ash content 942.05, ethereal extract 945.39, percentage of protein by total nitrogen Kjeldahl 991.20, where 6.25 was used to obtain the percentage of protein in grasshopper, and percentage humidity 964.22. sample triplicates were used for all processes. The methodology used to determine chitin is described by Acosta-Estrada *et al.* (2021), with slight modifications (Black & Schwartz, 1950).

2.2.4 Extraction from early-stage and adult-stage *Sphenarium purpurascens*

Grasshopper samples were lyophilized. It was weighed 2.6 g taken for each replica; later, hexane was added in a ratio of 1: 5 w/v and stirred at 150 rpm at 25 °C for 27 hours. It was allowed the sedimentation for one hour, and the fatty phase was removed by simple decantation. The solid residue was left to dry in an oven at 35°C for 72 hours to remove the hexane. Distilled water was added at a ratio of 1:3 w/v, the pH was adjusted to 9.0 by adding 4M of NaOH, and it was stirred for 2 hours. Then it was filtered using a small pore gauze, and the filtrate was frozen at -80°C

and lyophilized. The extracts obtained were frozen at -80°C and freeze-dried for later use. Protein determination using the total nitrogen Kjeldahl method was performed.

2.2.5 Enzymatic hydrolysis

The hydrolysis of the extracts from grasshopper was prepared as described by Silvestre-De-León et al. (2021). Protease from *Bacillus licheniformis* 2.4 U/g was used. Briefly, Freeze-dried extract from early-stage and adult-stage grasshopper was dissolved with distilled water in 1:20 w/v, and the pH was adjusted with 0.1 N NaOH to pH 8, then 0.6% of protease was added. Subsequently, they were incubated in a water bath at a temperature of 60°C for 30 minutes. Inactivation of the enzyme was achieved by incubating at 90°C for 15 minutes. Once the samples were at room temperature, they were centrifuged at 4,000 rpm for 20 min. The degree of hydrolysis was determined as follows: to 1 mL of the hydrolysate, 1 mL of 10% trichloroacetic acid was added, and total nitrogen was determined using total nitrogen Kjeldahl of AOAC 991.20 method. The percentage of DH was calculated using the following equation:

$$\% \text{ DH} = \frac{(mg\ soluble\ Nitrogen\ of\ sample)}{mg\ NTK} * 100$$

The hydrolysates were frozen and then lyophilized. They were stored at -20°C until use. Subsequently, a fractionation by ultrafiltration was carried out using10 and 30 kDa molecular weight cut-off membranes (Merck). The samples were centrifuged at 4,000 rpm for 30 min. The fractions were freeze-dried and stored until use.

2.2.6 Determination of percentage of soluble proteins

For protein quantification by the Bradford method, a calibration curve should be constructed with a standard of bovine serum albumin in a working range of 0.1 to 1.4 mg/mL. For standards or samples, take 10 µL of sample and add 200 µL of

Bradford reagent, incubate for 15 minutes, and read at 595 nm; this method was used a 96-well plate.

2.2.7 Determination of antimicrobial activity

The methodology carried out was the one proposed by WANG et al. (2017) with slight modifications. First, the strains *E. coli* ATCC 25922, *S. aureus* ATCC 25923, E. *aerogenes* ATCC 13048 *Salmonella sp*., *P. aeruginosa* ATCC 77853 were inoculated in Luria broth and incubated for 48 hours at 35°C. Once there was growth (presence of turbidity), the test was performed using Luria agar; the paper discs were soaked in the samples for 30 seconds, then placed on the agar plate and incubated for 48 hours at 35°C. As a control, amikacin 1:10 v/v was used. The interpretive criteria were as follows: low susceptible, inhibition zone diameter ≤10 mm; intermediate, 10 to 14 mm; susceptible, 14 to 19 mm; and highly susceptible ≥19 mm.

2.2.8 Detection of total phenolic compound with colorimetric tests

The detection was carried out according to the methodology proposed by Pérez-Ramírez et al. (2019). To detect phenolic compounds with $KMnO_4$ was carried out by diluting a 100 µL sample with an additional 100 µL of distilled water; detection was performed by adding 30 µL of 0.1% $KMnO_4$. The reaction was considered positive when the purple color turned yellowish or greenish. Distilled water was used as a control. To detect phenolic compounds with $FeCl_3$ was carried out to 100 µL sample with 100 L of distilled water and adding to 30 µL of 0.5% $FeCl_3$. The reaction was deemed positive when dark green, blue to dark blue, or even blackish tones appeared in the mixture.

2.2.9 Total phenolic compounds quantification

To quantify total phenolic compounds, a gallic acid standard was used. From each sample, 100 µL were taken, and 400 µL of distilled water were added, later 1250 µL of 20% sodium carbonate was added, and then 250 µL of 1N Folin-Ciocalteu reagent was added. They were left to incubate for 2 hours, and then the samples were read in a UV-VIS spectrophotometer at 760 nm.

2.2.10 DPPH• assay

For the evaluation of the antioxidant capacity of the samples, 2,2-diphenyl-1-picrilhidrazil (DPPH•) assay was used. Stock solutions of DPPH and 5µM Trolox were prepared. Twenty-five µL of each sample were taken, and 75 µL of the corresponding stock solution was added. They were left to incubate for 30 min in the dark at room temperature, and then the reading was done at 517 nm.

2.2.11 ABTS•+ assay

Free radical scavenging capacity against ABTS•+ radical was carried out with a 2,2´-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS•+) solution according to published by Pérez-Ramírez et al. (2019) with slight modifications. ABTS solution was prepared, then potassium persulfate was added; this solution was allowed to settle for 12 to 16 hours. It was diluted with ethanol to obtain a working solution with an absorbance of 0.7 to 732 nm. A standard of 5µM Trolox diluted in ethanol was used to construct a calibration curve. From each sample, 20 µL were mixed with 2 ml of the stock solution; they were left incubating for 10 minutes at room temperature and in the dark. Later the reading was done at 732nm.

2.2.12 Ferric reducing power

The ferric reducing power was determined according to Zielińska et al. (2017) with slight modification. To 960 µL of the sample, 1 mL of 0.2 M phosphate buffer (pH 6.6) and 1 mL of 1% potassium ferricyanide (w/v) were added. The samples were incubated at 50°C for 20 min, cooled, and mixed with 1 mL of 10% TCA (v/v). In the next stage, the mix was centrifugated at 4,000 rpm for 10 minutes. To the supernatant, 1 mL of distilled water and 0.2 mL 0.1% ferric chloride (w/v) were added. After a reaction time of 10 minutes, the absorbance of the solution was measured at 700 nm.

The reducing power was calculated using the formula:

$$\text{Reducing power} = A_{sample} - A_{control} \qquad (1)$$

Where $A_{control}$ is the absorbance of solution without a sample (distilled water instead of the sample); A_{sample} is the absorbance of the solution with a sample.

2.2.13 Statistical analyses

The data obtained in each quantitative assay were compared between samples by t-test in the Minitab® 18 program. All samples were analyzed in triplicate, and their standard deviation was calculated.

2.3 Results and discussion

2.3.1 Proximal analyses

The chemical characterization of early grasshopper (EG) and adult grasshopper (AG) is presented in Table 3, where a significant difference between the samples was observed. In addition, humidity analyses and the results obtained were 78.0% ± 1.3 for EG and 70.1% ± 0.64, corresponding to the AG sample. For the calculation of the protein percentage, the conversion factor 6.25 was used. When results on the

amino acid profiles are not available, protein determination based on total nitrogen content by Kjeldahl or a similar method by a general factor is considered acceptable. Currently, protein conversion factors have been reported for certain specific insects *Achaeta domesticus* (5.25), *Locusta migratoria* (5.33), and *Tenebrio molitor* (5.41); for these insects, the total nitrogen, the amide nitrogen, and the amino acid profile were determined. (Boulos et al., 2020). Although the factors found are similar in different insects, if it is necessary to calculate that of certain insects in specific Regarding protein content found in EG, 49.4%, and in AG, 57.3% to obtain these result 6.25 factor was used, compared with similar edible insects as black cricket *Gryllus assimilis,* where is reported 36% of protein (Miranda de Matos et al., 2021), both samples of grasshoppers contain a higher quantity of protein. Insects like *Schistocerca piceifrons p.* contains 80.26% of protein, fat 6.21%, ashes 3.35%, and chitin 11.88% (Pérez-Ramírez et al., 2019); where the percentage of protein is higher in comparison to a grasshopper. Furthermore, *S. purpurascens* has a high quantity of ashes, 11.5% in EG and 7.94% in AG compared with 3.35% of ashes reported in *Schistocerca piceifrons p.* For fat percentage, 9.35% obtained in EG and 13.1% in AG are higher than the fat percentage of *S. piceifrons p.* 6.21%. Mexican edible insects such as white worm *Aegiale hesperiasis W.,* red worm *Comadia redtembacheri H*., and the escamol *Liometopum apiculatum M.,* presented 37.79%, 31.23%, and 36.98% of protein, respectively, which lower than those obtained in this work. On the contrary, fat content reported for these insects were 34.94%, 58.54%, and 36.90% respectively (Ramos-Rostro et al., 2012); this is three to five times more than the fat content of EG and AG.

Comparing EG and AG samples, AG had a higher content of fat (13.1%), protein (57.3%), and chitin (15.6%), and lower percentage of ashes than obtained in EG. We can conclude that the stage of the grasshoppers influences their contents of protein, chitin, ashes, and ethereal extract.

Table 3 Results obtained from proximal analyses performed on grasshopper samples.

Parameters	Early grasshopper*	Adult grasshopper*
% Ash	$11.5^{a} \pm 0.32$	$7.94^{b} \pm 0.13$
% Ethereal Extract	$9.35^{a} \pm 0.08$	$13.1^{b} \pm 0.09$
% Protein	$49.4^{a} \pm 0.65$	$57.3^{b} \pm 1.4$
% Chitin	$10.5^{a} \pm 0.15$	$15.6^{b} \pm 0.81$

**Contents expressed in g/100 g of dry matter. All values are means ± SD by triplicate.*
[a,b] Different letter between columns indicates a statistically significant difference, p-value < 0.05 according to t-test.

The content of protein obtained on the extract of EG is 56.5% ±1.2, and for AG, 60.7% ±0.15, which had an increase of 14.37% and 5.93% compared to the full insect, respectively. In the alkaline extraction applied to *Schistocerca gregaria* the protein content in extract increased 14.8% compared with protein content in the insect (Mishyna et al., 2019). Although an increase in the content of the protein was obtained in the AG extract, it was lower compared to the EG extract.

2.3.2 Antimicrobial activity assay

Even though antimicrobial peptides/polypeptides (AMP) are an innate component of immunity to insects found in their hemolymph and have important biological activity against fungi, viruses, parasites, and, most importantly, antibiotic-resistant bacteria, in this study, samples of extracts, hydrolysate extracts, and hydrolysate fractions did not present inhibition halo indicating that there is no presence of compounds with antimicrobial activity (Fig 8).

In some cases, the production of these compounds must be stimulated. For example, the housefly larvae were inoculated with a suspension of *S. pullorum* cells to obtain AMP (WANG et al., 2017). Another example is the crickets, which were infected with *Photorhabdus asymbiotica,* and from that infection glidobactin A, luminmycin, and luminmycin D have been isolated (Mudalungu et al., 2021). Considering that the crickets and the grasshoppers belong to the Orthoptera family,

it is possible to isolate antimicrobial compounds applying the conditions reported in other works.

Figure 7 Antimicrobial activity test for extract, hydrolysates, and hydrolysate fractions from *S. purpurascens* in the qualitative assay using antibiogram technique, where there is no inhibition halo observed, indicating nonantimicrobial activity.

2.3.3 Qualitative test for determination of total phenolic compounds

Extracts, hydrolysates, and hydrolysate fractions samples from EG and AG were analyzed for the detection of phenolic compounds with $KMnO_4$ (purple color) and $FeCl_3$ (yellow color). As it is observed in the image (Fig. 9), a positive response was obtained in the test with $KMnO_4$ since this in contact with the samples changes its coloration. On the other hand, for $FeCl_3$ tests, extracts, hydrolysates, and hydrolysate fractions were diluted 1:100 so that its color did not mask the color change, although, change in color perception was deficient (Fig. 10). In an aqueous extract from *Schistocerca piceifrons p.*, colorimetric detection of Total Phenolic Compound was positive in both cases (Pérez-Ramírez et al., 2019).

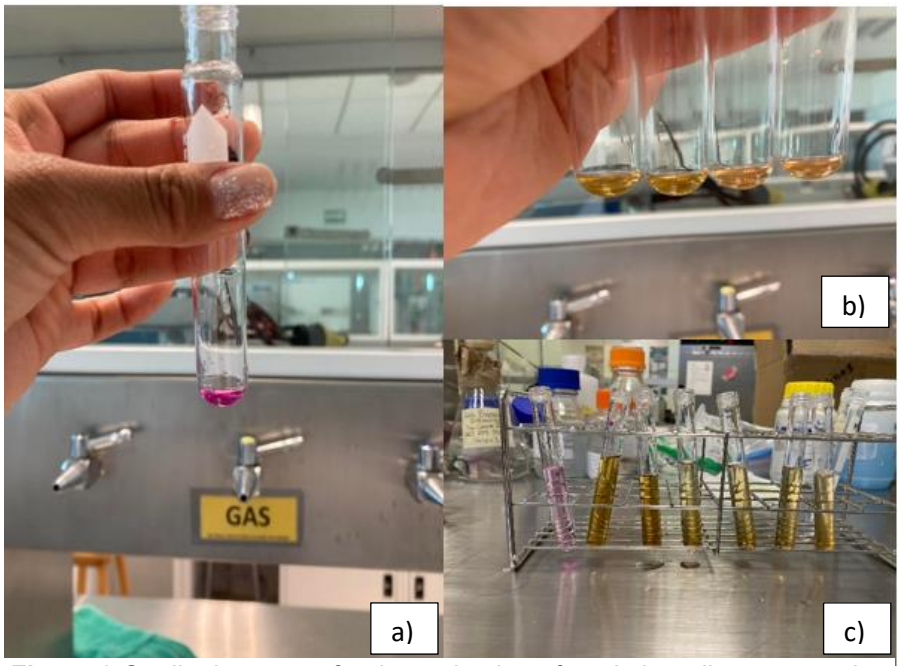

Figure 8 Qualitative assay for determination of total phenolic compounds using $KMnO_4$ as detector agent a) Blank (distilled water) b) and c) samples from *S. purpuracenses*. Change in color indicates total phenolic compounds presence.

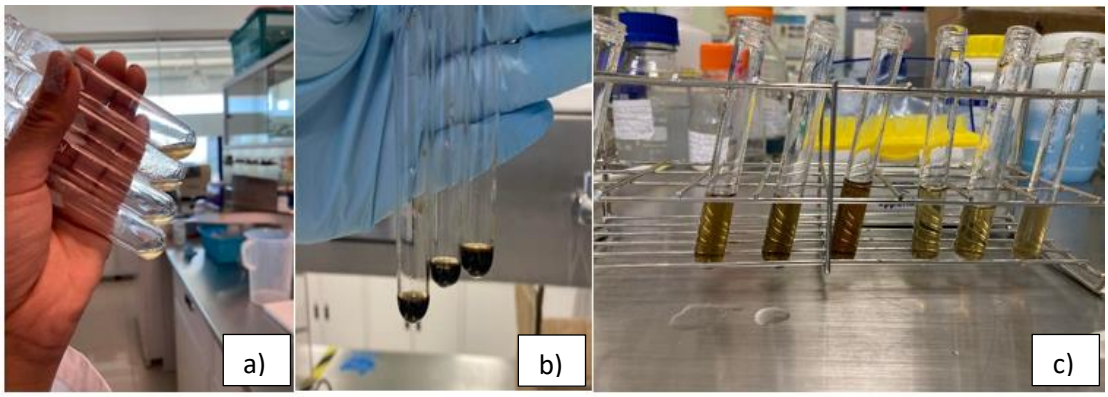

Figure 9 Determination of total phenolic compounds with FeCl3 a) the sample do not have change of color due total peholic compound were not detected b) concentrated sample with high total phenolic concentration c) diluted sample can observe a light green color due to reaction of sample and ferric chloride demonstrated presence of phenolic compounds.

2.3.4. Degree of hydrolysis

For *S. purpurascens*, the results obtained were: EG was 12.6% ±0.64 and AG was 13.1% ±0.6. Results reported by Purschke et al. (2018), where hydrolysis process applied in *Locusta migratoria* using the treatment with Alcalase® at 0.05, 0.5, and

1% E/S during 30 min they obtained 7.3%, 9.5%, and 11.6% respectively. In this work, results obtained in EG and AG showed a higher DH. Other examples are: See *et al.*, (2011) hydrolysates Salmon (*Salmo salar*) skin using Alcalase® 2.4L, and obtained a DH of 76.43%. On the other hand, the hydrolysis of chickpea protein isolate with Alcalase® showed DH of 25.8% (Xu et al., 2020). Samples of *Grilloides sigillatus* were also treated with Alcalase®, to obtain a similar DH of 15.2 %, the conditions were: 0.25% E:S during 10 min to 50°C pH 8 (Hall et al., 2018). The examples mentioned above show that hydrolysis depends on temperature and time conditions and the source where the protein was extracted(Zielińska et al., 2017).

Due to hydrolysis, the molecular properties of proteins change, producing a decrease in molecular weight, an increase in charge, and the release of hydrophobic groups, among other phenomena (Purschke et al., 2018). As a result of the molecular changes of proteins, functional properties have also been affected. Possible functional properties are increasing by the release of bioactive peptides. For this reason, the hydrolysates were fractionated and analyzed to determine antioxidant capacity.

2.3.5 Fractionation of hydrolysates

As it is observed protein fractionation in Table 4., fractions 1 and 3 contain a higher amount of protein than fractions 2; this is in both EG and AG samples. Ultrafiltration is a tool to refine hydrolysates and increase their specific activity in the perspective of the modernization of the by-products industry to produce bioactive ingredients for human and animal feed. Then fractionation according to the molecular weights of compounds is a good alternative to purify them and increase their bioactive properties (Miranda de Matos et al., 2021).

Table 4 Protein distribution in hydrolysate fractions.

Sample	% of protein EG	% of protein AG
Fraction 1 (>30 kDa)	33.01[a] ±0.03	43.17[b] ±0.04
Fraction 2 (>10 kDa)	18.82[a] ±0.05	13.52[b] ±0.03
Fraction 3 (<10 kDa)	32.16[a] ±0.03	39.41[b] ±0.03

All values are means±SD by triplicate.
[a,b] Different letters between columns indicates a statistically significant difference, p-value< 0.05 according to the t-Student test.

2.3.6 Total phenolic compounds

The enzymatic hydrolysis of the EG and AG extracts proved to be an efficient process for obtaining compounds with higher biological activity than the samples without hydrolysis. EG hydrolysate raised almost double of total phenolic compounds than EG extract, and AG hydrolysate had a rising of nearly double in comparison with AG extract (see Fig. 11). Additionally, the hydrolysate fraction that obtained the highest concentration of phenolic compounds in EG and AG was fraction 1 (< 10 kDa), where 111.7 mg GAE were obtained in EG and 77.02 mg GAE in AG. It is reported that smaller compounds from hydrolysates presented higher bioactivity due to higher exposure to amino acids (Daroit & Brandelli, 2021).

The concentration of total phenolic compounds can be compared to aqueous extract from *Henicus whellani* and *Macrotermes facilger* that reported 7.7 mg GAE and 9.37mg GAE/dry base 100 g, respectively (Kunatsa et al., 2020); in the aqueous extract from *Sphenarium purpuranscens* 12.33 mg GAE and 10.92 mg GAE/dry base 100 g, in EG and AG samples were obtained, respectively. Mexican insects like jumiles *Ascra cordifera*, wasp *Brachygastra mellifica,* and *Hermetia illucens* presented 12.8 mg GAE, 10.9 mg GAE, 3.9 mg GAE of phenolic compounds, respectively (Baigts-Allende et al., 2021).

It is known that plants are excellent sources of phenolic compounds; for example, plants used to make infusions as chamomile presented 69.28 mg GAE and lemon 71.69 mg GAE, which can be compared with results obtained in EG extract (104.8

mg GAE) and in AG extract (84.98 mg). On the other hand, phenolic compounds concentration from plants like spearmint (231.85 mg GAE), arnica (173.3mg GAE), and boldo (312.71 mg GAE) (Muñoz-Velázquez et al., 2012) can be compared with results obtained in samples of hydrolysates EG (195.3 mg GAE) and AG (143.5 mg GAE).

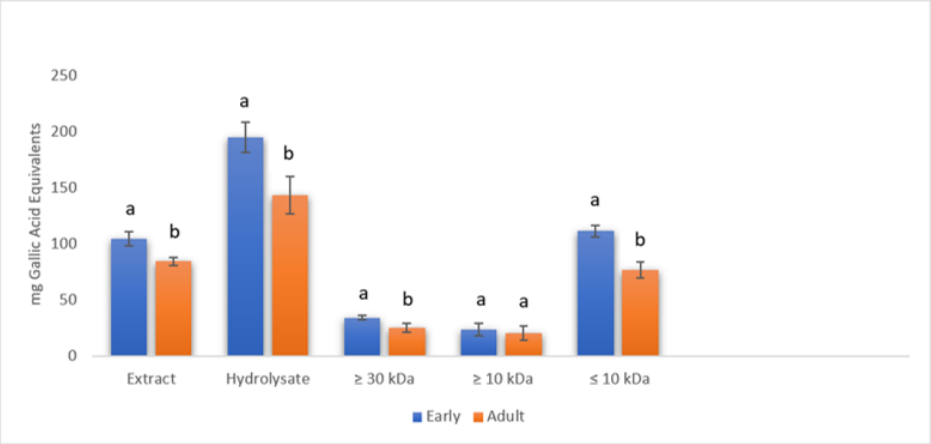

Figure 10 Total phenolic compounds in extract, hydrolysate, and hydrolysate fractions of *S. purpurascens*. Results obtained are reported in mg of gallic acid equivalents (GAE). Different letters between bars indicate significant difference (p-value<0.05).

2.3.7 Antioxidant activity against DPPH•, ABTS•+, and ferric reducing power

The hydrolysates EG (158.9 mM Trolox) and AG (138.3 mM Trolox) had higher antioxidant activity against DPPH• than extract EG (9.75 mM Trolox) and AG (10.9 mM Trolox), as shown in Figure 12. The concentration of compounds with antioxidant activity against DPPH had a considerable increase up to 15 times. Hydrolysate fractions with molecules < 10 kDa presented higher antioxidant activity (87.56 mM Trolox in EG and 67.76 mM Trolox in AG) than other fractions analyzed. In work carried out by Miranda de Matos et al. (2021), fractions with a molecular weight lower than 10 and 5 kDa were responsible for the highest biological activity for DPPH and ABTS radical scavenging activities.

Other edible insects show antioxidant activity against these radicals; the flour of the insect *Rhynchophorus ferrugineus* presented 2.03 mM Trolox against DPPH in

aqueous extract (Botella-Martínez et al., 2021). In this study, *S. purpurascens* presented 7.65 mM Trolox and 9.22 mM Trolox in EG and AG samples, respectively.

There are significant differences between the samples EG and AG. Then the better option is a source of antioxidant compounds in the hydrolysate from EG.

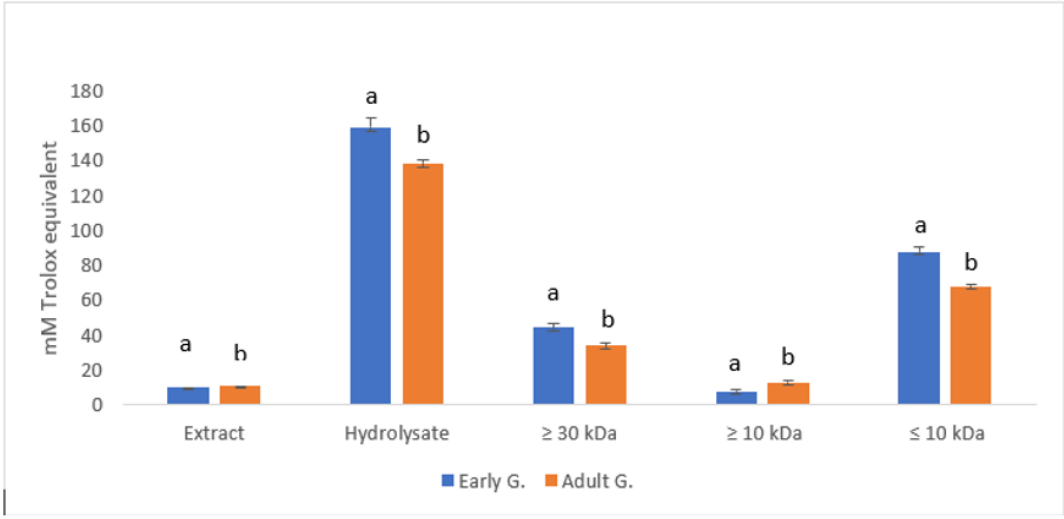

Figure 11 Antioxidant activity DPPH of extracts, hydrolysates, and hydrolysate fractions. Results are presented in mM Trolox equivalents. Different letters between bars indicate a significant difference (p-value <0.05).

Regarding ABTS•+, the results obtained were presented in Figure 13. Hydrolysate of EG (142.5 Mm Trolox) had a raising of ten times compared with EG extract (11.22 mM Trolox) and hydrolysate AG (252.7 mM Trolox) increased thirty times in comparison with AG extract (7.90 mM Trolox). These results can be a product of the type of compounds that had contact with the oxidant reagent (Villaseñor et al., 2021) since, in this case, the compounds from the AG had higher bioactivity than the EG.

The result obtained in this study can be compared with the hydrolysates from *Alphitobius diaperinus* where had 95.0 mM Trolox against ABTS•+ (Sousa et al., 2020). In *S. purpurascens,* 142.5 mM Trolox and 252.7 mM Trolox corresponding to EG, and AG hydrolysates were found, respectively, demonstrating higher values than *A. diaperinus*. The soluble extracts of grasshoppers (2.55 ± 0.05), silkworm (2.48 ± 0.19), and crickets (2.37 ± 0.03) contain higher values of antioxidant capacity,

expressed as mM Trolox, until five times higher than fresh orange juice (0.40 ± 0.01) (Di Mattia et al., 2019). If we compared these data with our results, the AG hydrolysate should be considered a great source of antioxidant compounds.

Furthermore, the hydrolysate fraction of AG with less molecular weight < 10 kDa presented higher antioxidant activity (103.9 mM Trolox) than other fractions, where the results were > 30 kDa (77.61 mM Trolox) and >10 kDa (53.48 mM Trolox). The highest values that could be determined with the method were found in hydrolysates. AG had a better response to this radical.

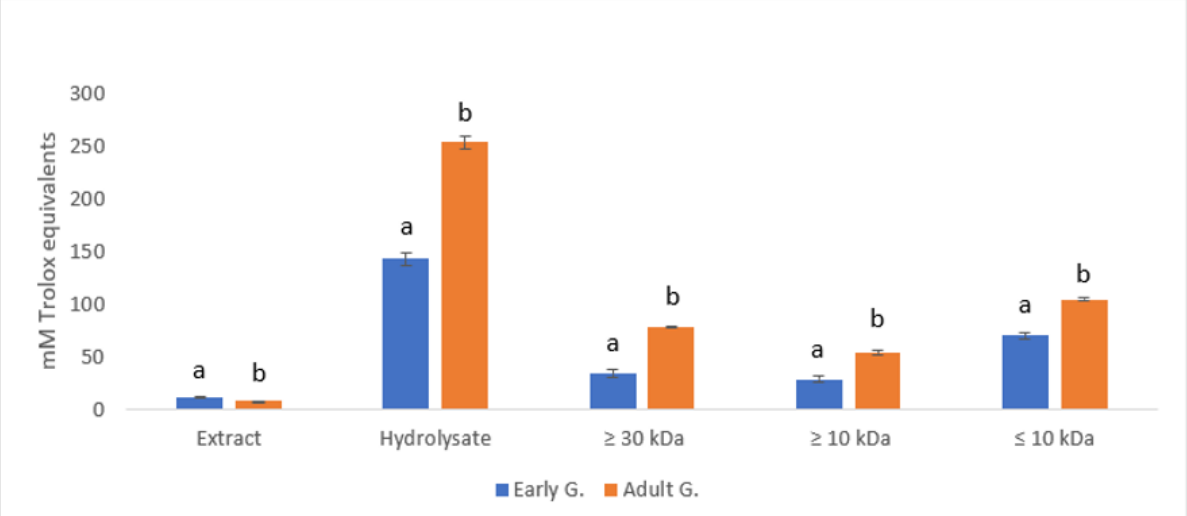

Figure 12 Antioxidant activity against ABTS radical of extracts, hydrolysates, and hydrolysate fractions. Results obtained are reported in mM Trolox equivalents. Different letters between bars indicate significant difference (p-value < 0.05).

The ferric reducing antioxidant power values obtained are represented in Figure 14, where EG extract had 0.465 abs and AG extract had 0.394 abs. While hydrolysates, EG had 0.635 abs, and AG had 0.587 abs. Similar results have been found in edible insects such as the hydrolysate of cricket *A. annulipes* 0.652 abs, *Z. morio* 0.522 abs, and *G. portentosa* 0.485 abs (Zielińska et al., 2017). In phaseolin, the hydrolysate was obtained 0.062 absorbance units (Carrasco-Castilla et al., 2012), approximately ten times less than hydrolysates from edible insects. The evaluation of each hydrolysate fraction showed that <10 kDa of EG (0.308 abs) and AG (0.299 abs) had higher absorbances concerning values obtained in >10 kDa, EG (0.101 abs) and AG (0.086 abs), and >30 kDa EG (0.212 abs) and AG (0.153 abs). Even

though there is a significant difference between EG and AG fractions, in the case of fraction three, there is no significant difference (p-value > 0.05).

In conclusion, edible insect hydrolysates can be considered electron donor compounds and counteract the oxidative effect of radicals. These differences in antioxidant activities can be mainly related to the compound structures of each protein and the enzyme used.

Thus, also a slight variation in the hydrolysis condition can significantly change the profile of the hydrolysates.

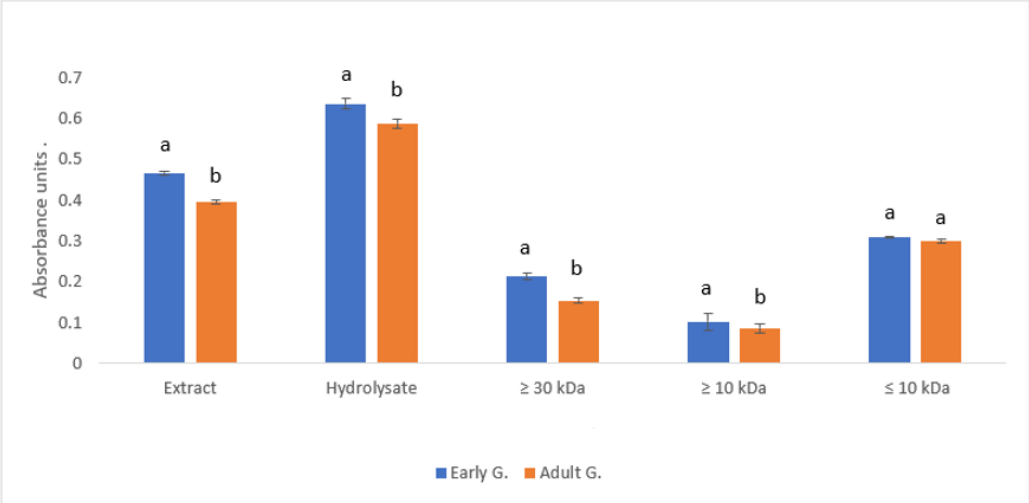

Figure 13 Ferric reducing antioxidant power of extracts, hydrolysates, and hydrolysate fractions. Results are reported in absorbance unit at a wavelength of 700 nm. Different letters between bars indicate a significant difference (p-value <0.05).

2.4 Conclusions

Due to the great demand for food worldwide and the search for alternative food sources, the interest in insects has increased, even as a source of additives that can be used in the industry. Under the working conditions of this research, the grasshopper *Sphenarium purpurascens* presented a great nutritional value; according to proximal analyses, there is a significant difference between early-stage and adult stages. This can be attributed to the insect's own metamorphic stage as well as environmental conditions, diet, and sex; therefore, the collection times are a

factor that must be considered for future studies. Adult grasshoppers can be employed for fortifying certain foods since it contains a high quantity of proteins and fats, contributing to the nutritional improvement of food. Besides, grasshoppers are an excellent source of compounds with biological activity that might increase the nutritional value of foods. Total phenolic compounds and the free radical scavenging capacity of compounds found in grasshoppers are associated with biological activities such as oxidative stress, antidiabetic, anti-inflammatory, antimicrobial activities. In evaluating phenolic compounds, ferric reducing power, and the antioxidant capacity for DPPH•, the early grasshopper presented a higher antioxidant activity. Adult grasshopper samples gave a better antioxidant activity for ABTS•+, turning them into an excellent choice of antioxidant compounds. It is important to emphasize that although DPPH• and ABTS•+ are not radicals present in living organisms, the use of *in vitro* methods gives us an idea of their concentration of antioxidants compounds, and with this, we could go to tests in cell lines.

The concentration of antioxidant compounds can be increased by applying enzymatic hydrolysis to the samples, as demonstrated in this study. Due to the increase of antioxidant compounds, the hydrolysates from early grasshoppers can be used as a possible source of nutraceuticals. Among the quantified antioxidants, there could be other compounds of a non-phenolic nature, for which it is necessary to characterize them. Authors have used insect flours for the fortification of some food-like flours. Due to the refining processes of the flours, certain nutrients are lost, which is why it is important to fortify them. However, most of these additives are inorganic sources; for this reason, the use of organic sources of additives like flour of grasshopper increases the nutrient value of the foods.

Nowadays, commercially available foods are formulated, including insect ingredients. Nevertheless, research about the biological potential of compounds derived from Mexican edible insects is limited.

2.5 Acknowledgements

This work was supported by the School of Engineering and Sciences at Tecnologico de Monterrey. Martha Selene Marín Morales would like to thank CONACyT for grant no. 769152.

2.6 References.

Amarender, R. V., Bhargava, K., Dossey, A. T., & Gamagedara, S. (2020). Lipid and protein extraction from edible insects – Crickets (Gryllidae). LWT, 125, 109222. https://doi.org/10.1016/j.lwt.2020.109222

B. Nongonierma, A., Lamoureux, C., & J. FitzGerald, R. (2018). Generation of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides during the enzymatic hydrolysis of tropical banded cricket (Gryllodes sigillatus) proteins. Food & Function, 9(1), 407–416. https://doi.org/10.1039/C7FO01568B

Botella-Martínez, C., Lucas-González, R., Pérez-Álvarez, J. A., Fernández-López, J., & Viuda-Martos, M. (2021). Assessment of chemical composition and antioxidant properties of defatted flours obtained from several edible insects. Food Science and Technology International = Ciencia Y Tecnologia De Los Alimentos Internacional, 27(5), 383–391. https://doi.org/10.1177/1082013220958854

Carrasco-Castilla, J., Hernández-Álvarez, A. J., Jiménez-Martínez, C., Jacinto-Hernández, C., Alaiz, M., Girón-Calle, J., Vioque, J., & Dávila-Ortiz, G. (2012). Antioxidant and metal chelating activities of peptide fractions from phaseolin and bean protein hydrolysates. Food Chemistry, 135(3), 1789–1795. https://doi.org/10.1016/j.foodchem.2012.06.016

Cultivo y consumo de insectos, opción ante la creciente demanda de alimentos. (2020, March 17). PortalAmbiental.com.mx. https://www.portalambiental.com.mx/sustentabilidad/20200317/cultivo-y-consumo-de-insectos-opcion-ante-la-creciente-demanda-de-alimentos

Di Mattia, C., Battista, N., Sacchetti, G., & Serafini, M. (2019). Antioxidant Activities in vitro of Water and Liposoluble Extracts Obtained by Different Species of Edible Insects and Invertebrates. Frontiers in Nutrition, 6, 106. https://doi.org/10.3389/fnut.2019.00106

Ibarra-Herrera, C. C., Acosta-Estrada, B., Chuck-Hernández, C., Serrano-Sandoval, S. N., Guardado-Félix, D., & Pérez-Carrillo, E. (2020). Nutritional content of edible

grasshopper (Sphenarium purpurascens) fed on alfalfa (Medicago sativa) and maize (Zea mays). CyTA - Journal of Food, 18(1), 257–263. https://doi.org/10.1080/19476337.2020.1746833

Insectos: La alimentación del futuro. (2021, September 22). The Food Tech. https://thefoodtech.com/columnistas/insectos-la-alimentacion-del-futuro/

Jantzen da Silva Lucas, A., Menegon de Oliveira, L., da Rocha, M., & Prentice, C. (2020). Edible insects: An alternative of nutritional, functional and bioactive compounds. Food Chemistry, 311, 126022. https://doi.org/10.1016/j.foodchem.2019.126022

Kunatsa, Y., Chidewe, C., & Zvidzai, C. J. (2020). Phytochemical and anti-nutrient composite from selected marginalized Zimbabwean edible insects and vegetables. Journal of Agriculture and Food Research, 2, 100027. https://doi.org/10.1016/j.jafr.2020.100027

Margot, A., Flaschel, E., & Renken, A. (1994). Continuous Monitoring of Enzymatic Whey Protein Hydrolysis. Correlation of Base Consumption with Soluble Nitrogen Content. https://doi.org/10.1016/0032-9592(94)80066-9

MARINOVA, G., & V. BATCHVAROV. (2011). EVALUATION OF THE METHODS FOR DETERMINATION OF THE FREE RADICAL SCAVENGING ACTIVITY BY DPPH. 17, 11–24.

Mexico Could Be the Top Source of Edible Insects. Here's Why It's Not. (2019, August 5). OZY. https://www.ozy.com/news-and-politics/mexico-could-be-the-top-source-of-edible-insects-heres-why-its-not/95545/

Miranda de Matos, F., Novelli, P., & Janser, R. (2021). Enzymatic hydrolysis of black cricket (Gryllus assimilis) proteins positively affects their antioxidant properties. Journal of Food Science, 86. https://doi.org/10.1111/1750-3841.15576

Mudalungu, C. M., Tanga, C. M., Kelemu, S., & Torto, B. (2021). An Overview of Antimicrobial Compounds from African Edible Insects and Their Associated Microbiota. Antibiotics, 10(6), 621. https://doi.org/10.3390/antibiotics10060621

Müller, L., Fröhlich, K., & Böhm, V. (2011). Comparative antioxidant activities of carotenoids measured by ferric reducing antioxidant power (FRAP), ABTS bleaching assay (αTEAC), DPPH assay and peroxyl radical scavenging assay. Food Chemistry, 129(1), 139–148. https://doi.org/10.1016/j.foodchem.2011.04.045

Ng'ang'a, J., Fombong, F., Kiiru, S., Kipkoech, C., & Kinyuru, J. (2021). Food safety concerns in edible grasshoppers: A review of microbiological and heavy metal hazards. International Journal of Tropical Insect Science, 41(3), 2103–2111. https://doi.org/10.1007/s42690-020-00372-9

Nino, M. C., Reddivari, L., Ferruzzi, M. G., & Liceaga, A. M. (2021). Targeted Phenolic Characterization and Antioxidant Bioactivity of Extracts from Edible Acheta domesticus. Foods, 10(10), 2295. https://doi.org/10.3390/foods10102295

Pérez-Ramírez, R., Torres-Castillo, J. A., Barrientos-Lozano, L., Almaguer-Sierra, P., & Torres-Acosta, R. I. (2019). Schistocerca piceifrons piceifrons (Orthoptera: Acrididae) as a Source of Compounds of Biotechnological and Nutritional Interest. Journal of Insect Science, 19(5). https://doi.org/10.1093/jisesa/iez088

Purschke, B., Meinlschmidt, P., Horn, C., Rieder, O., & Jäger, H. (2018). Improvement of techno-functional properties of edible insect protein from migratory locust by enzymatic hydrolysis. European Food Research and Technology, 244(6), 999–1013. https://doi.org/10.1007/s00217-017-3017-9

See, S. F., Hoo, L., & Babji, A. (2011). Optimization of enzymatic hydrolysis of salmon (Salmo salar) skin by Alcalase. International Food Research Journal, 18, 1359–1365.

Silvestre-De-León, R., Espinosa-Ramírez, J., Pérez-Carrillo, E., & Serna-Saldívar, S. O. (2021). Extruded chickpea flour sequentially treated with alcalase and α-amylase produces dry instant beverage powders with enhanced yield and nutritional properties. International Journal of Food Science & Technology, 56(10), 5178–5189. https://doi.org/10.1111/ijfs.15199

Sousa, P., Borges, S., & Pintado, M. (2020). Enzymatic hydrolysis of insect Alphitobius diaperinus towards the development of bioactive peptide hydrolysates. Food & Function, 11(4), 3539–3548. https://doi.org/10.1039/D0FO00188K

Sudan, R., Bhagat, M., Gupta, S., Singh, J., & Koul, A. (2014). Iron (FeII) Chelation, Ferric Reducing Antioxidant Power, and Immune Modulating Potential of Arisaema jacquemontii (Himalayan Cobra Lily). BioMed Research International, 2014, e179865. https://doi.org/10.1155/2014/179865

Vadivel, V., & Brindha, P. (2015). Antioxidant property of solvent extract and acid/alkali hydrolysates from rice hulls. Food Bioscience, 11, 85–91. https://doi.org/10.1016/j.fbio.2015.06.002

WANG, Z., WANG, J., ZHANG, Y., WANG, X., ZHANG, X., LIU, Y., XI, J., TONG, H., WANG, Q., JIA, B., & SHEN, H. (2017). Karasinekteki (Musca domestica) Antimikrobiyal Peptidler Salmonella pullorum ile Enfekte Tavuklarda Bağırsak Lactobacillus acidophilus ve Mukozal Epitel Hücrelerini Etkiler. Kafkas Universitesi Veteriner Fakultesi Dergisi. https://doi.org/10.9775/kvfd.2016.16901

Yusof, N. B., Hasan, M. H., Armayni, U. A., Bin, M. S., Mohsin, H. F., & Wahab, I. A. (n.d.). The Ferrous Ion Chelating Assay of Pandanus Extracts. 1.

Zielińska, E., Karaś, M., & Jakubczyk, A. (2017). Antioxidant activity of predigested protein obtained from a range of farmed edible insects. International Journal of Food Science & Technology, 52(2), 306–312. https://doi.org/10.1111/ijfs.13282

Chapter 3

Conclusions and recommendations

The data presented in this work confirm that a grasshopper is an excellent option considering functional foods. Results obtained in the proximal analysis showed that grasshoppers have great quantities of nutrients, including proteins and fats. The results of the proximal analysis also were significantly different due to the grasshopper stage. To consider that it is a quality protein, it is suggested to carry out in vitro digestibility analysis and the determination of the amino acid profile.

Regarding the obtention of extracts, it is suggested defatting with other solvents since, according to the literature, several authors have obtained a higher percentage of proteins using solvents less aggressive than hexane. Then, it is recommended defatting the samples with other solvents such as ethanol.

In this study, protein extraction in alkaline conditions was used obtained good results. However, tests with additional technologies such as sonication may increase the efficiency of the extraction. Therefore, it would be a good option to apply these techniques in grasshopper samples and compare the results to know which process has the highest efficiency.

In the evaluation of phenolic compounds and the antioxidant capacity for DPPH• and ABTS•+, there is a significant difference between the early and adult grasshopper samples. Therefore, if the primary purpose is to obtain more antioxidant compounds, the best option is early grasshoppers. Also, considering the antioxidant capacity of grasshoppers; it is proposed to evaluate anti-inflammatory activity employing methods like lipoxygenase inhibitory activity and cyclooxygenase inhibitory activity assay. Once *in vitro* antioxidant capacity has been demonstrated, an area of opportunity would be to use these compounds in cell lines such as cancer cells to determine their antioxidant capacity against this cancer.

Furthermore, quantification of phenolic compounds and their identification, relying on other methodologies to characterize them, like FTIR, is proposed.

The hydrolysates obtained in this work presented good antioxidant capacities. Different hydrolysis conditions can be used, including other enzymes (mixtures), to evaluate the antioxidant activities and compare them with those found in this study, thus determining which one could obtain a greater quantity of the compounds.

In this study, hydrolysate fractions using ultrafiltration were obtained; although good results were obtained, an alternative is to use a chromatographic method to fractionate and possibly characterize the compounds.

To be able to separate the proteins from the rest of the compounds present in the extracts and hydrolysates, we can apply strategies to precipitate proteins, such as a change in pH to change isoelectric point, an increase in temperature, the addition of solvents, charged molecules, etc., the method of precipitation by salting with $(NH_4)_2SO_4$, can be used. This can be complemented with an SDS page to know the molecular weight of the samples. Regarding the analysis of antimicrobial activity, it is suggested to include microorganisms such as molds and yeasts to identify antifungal activity, in addition to including other Gram-negative and Gram-positive bacteria.

To conclude, most of the investigation that has been carried out in Mexican edible insects focuses on proximal analyzes. Therefore, the determination of biological activities represents an exciting opportunity, which can originate in a new generation of nutraceuticals that can be employed in food and pharmaceutical industries.

Vita

Martha Selene Marín Morales was born in Puebla, Puebla, México on September 20th, 1990. She earned the in-Biotechnology Engineering degree from *Universidad Politécnica de Puebla*, in April 2012. She has worked in the food industry in the quality and inspection departments. In addition to working in the branch of environmental engineering in laboratories and wastewater treatment plants.

She was accepted in the Master of Sciences in Biotechnology program at Tecnológico de Monterrey. This thesis presents the results of this program's work.

This document was typed in using Microsoft Word by Martha Selene Marín Morales.