INSTITUTO POLITÉCNICO NACIONAL

CENTRO INTERDISCIPLINARIO DE INVESTIGACIÓN PARA EL DESARROLLO INTEGRAL REGIONAL
Unidad Sinaloa

DEPARTAMENTO DE BIOTECNOLOGÍA AGRÍCOLA
LABORATORIO DE ALIMENTOS FUNCIONALES

CARACTERIZACIÓN PROTEÓMICA Y FUNCIONAL DE LAS PRINCIPALES FRACCIONES PROTEÍNICAS DE LA TORTA RESIDUAL DE *JATROPHA CURCAS* NO TÓXICA

MC. ANDRÉS LEÓN VILLANUEVA

TESIS

PRESENTADA COMO REQUISITO PARCIAL
PARA OBTENER EL GRADO DE

DOCTOR EN CIENCIAS EN BIOTECNOLOGÍA

GUASAVE, SINALOA, 2018

INSTITUTO POLITÉCNICO NACIONAL

CENTRO INTERDISCIPLINARIO DE INVESTIGACIÓN PARA EL DESARROLLO INTEGRAL REGIONAL
Unidad Sinaloa

DEPARTMENT OF AGRICULTURAL BIOTECHNOLOGY
FUNCTIONAL FOODS LABORATORY

PROTEOMIC AND FUNCTIONAL CHARACTERIZATION OF THE MAJOR PROTEIN FRACTIONS OF NON-TOXIC *JATROPHA CURCAS* BYPRODUCT CAKE

MC. ANDRÉS LEÓN VILLANUEVA

THESIS

PRESENTED AS A PARTIAL REQUIREMENT
TO OBTAIN THE DEGREE

DOCTOR IN SCIENCE IN BIOTECHNOLOGY

GUASAVE, SINALOA, 2018

INSTITUTO POLITÉCNICO NACIONAL

SECRETARÍA DE INVESTIGACIÓN Y POSGRADO

CARTA CESIÓN DE DERECHOS

En la Ciudad de Guasave, Sinaloa el día 12 del mes de septiembre del año 2018, el que suscribe Andrés León Villanueva alumno del Programa de Doctorado en Ciencias en Biotecnología con número de registro B110362, adscrito al Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional, manifiesta que es el autor intelectual del presente trabajo de Tesis bajo la dirección del Dr. Sergio Medina Godoy y la Dra. Ana Paulina Barba de la Rosa y cede los derechos del trabajo titulado "Caracterización proteómica y funcional de las principales fracciones proteínicas de la torta residual de *Jatropha curcas* no tóxica", al Instituto Politécnico Nacional para su difusión, con fines académicos y de investigación.

Los usuarios de la información no deben reproducir el contenido textual, gráficas o datos del trabajo sin el permiso expreso del autor y/o directores del trabajo. Este puede ser obtenido escribiendo a las siguientes direcciones: loeweing@gmail.com, smedinam@ipn.mx y apbarba@ipicyt.edu.mx. Si el permiso se otorga, el usuario deberá dar el agradecimiento correspondiente y citar la fuente del mismo.

Andrés León Villanueva

Nombre y firma del Alumno

SIP-13-BIS

INSTITUTO POLITÉCNICO NACIONAL
SECRETARIA DE INVESTIGACIÓN Y POSGRADO

ACTA DE REGISTRO DE TEMA DE TESIS
Y DESIGNACIÓN DE DIRECTORES DE TESIS

Guasave, Sinaloa a 11 de septiembre del 2018

El Colegio de Profesores de Estudios de Posgrado e Investigación de CIIDIR-SINALOA en su sesión ordinaria No. 9 celebrada el día 06 del mes de septiembre conoció la solicitud presentada por el(la) alumno(a):

León	Villanueva	Andrés
Apellido paterno	Apellido materno	Nombre (s)

Con registro: B 1 1 0 3 6 2

Aspirante de: **Doctorado en Ciencias en Biotecnología**

1.- Se designa al aspirante el tema de tesis titulado:
"Caracterización proteómica y funcional de las principales fracciones proteínicas de la torta residual de *Jatropha curcas* no tóxica".

De manera general el tema abarcará los siguientes aspectos:
Obtener las principales fracciones proteínicas a partir de un subproducto de semillas de *Jatropha curcas* no tóxica mediante la extracción secuencial basada en criterios de solubilidad, generar los mapas de gel bidimensionales e identificar las proteínas a través de LC-MS /MS. Caracterizar las propiedades funcionales de las fracciones proteínicas bajo condiciones relevantes para los sistemas alimentarios.

2.- Se designan como Directores de Tesis a los Profesores:
Dr. Sergio Medina Godoy y a la Dra. Ana Paulina Barba de la Rosa

3.- El trabajo de investigación base para el desarrollo de la tesis será elaborado por el alumno en:
Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional CIIDIR-Sinaloa

que cuenta con los recursos e infraestructura necesarios. Proyecto aprobado y financiado en la convocatoria de Investigación Científica Básica 2008 SEP-CONACYT con registro CB-2008-01 #103601.

4.- El interesado deberá asistir a los seminarios desarrollados en el área de adscripción del trabajo desde la fecha en que se suscribe la presente hasta la aceptación de la tesis por la Comisión Revisora correspondiente:

Directores de Tesis

Dr. Sergio Medina Godoy

Dra. Ana Paulina Barba de la Rosa

MC. Andrés León Villanueva
Aspirante

Dr. Héctor Manuel Esparza Leal
Presidente del Colegio

SIP-14-BIS

INSTITUTO POLITÉCNICO NACIONAL
SECRETARÍA DE INVESTIGACIÓN Y POSGRADO

ACTA DE REVISIÓN DE TESIS

En la Ciudad de Guasave, Sinaloa siendo las 11:00 horas del día 12 del mes de septiembre del 2018 se reunieron los miembros de la Comisión Revisora de la Tesis, designada por el Colegio de Profesores de Estudios de Posgrado e Investigación del: Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional Unidad Sinaloa

para examinar la tesis titulada:

"Caracterización proteómica y funcional de las principales fracciones proteínicas de la torta residual de *Jatropha curcas* no tóxica".

Presentada por el alumno:

León	Villanueva	Andrés
Apellido paterno	Apellido materno	Nombre(s)

Con registro:

B	1	1	0	3	6	2

aspirante de:

DOCTORADO EN CIENCIAS EN BIOTECNOLOGÍA

Después de intercambiar opiniones los miembros de la Comisión manifestaron ***APROBAR LA TESIS***, en virtud de que satisface los requisitos señalados por las disposiciones reglamentarias vigentes.

LA COMISIÓN REVISORA

Directores de tesis

Dr. Sergio Medina Godoy

Dra. Ana Paulina Barba de la Rosa

Dr. Hervey Rodríguez González

Dr. César Marcial Escobedo Bonilla

Dra. Laura Gabriela Espinosa Alonso

PRESIDENTE DEL COLEGIO DE PROFESORES

Dr. Héctor Manuel Esparza Leal

This thesis work was developed at CIIDIR-IPN Sinaloa's Functional Food laboratory of Agricultural Biotechnology Department; the Proteomics and Molecular Biomedicine Laboratory at IPICYT, San Luis Potosí; and the Physicochemistry and Protein Engineering Laboratory of Biochemistry Department at UNAM's Medicine Faculty in Mexico City.

The research was supported by SEP-CONACYT GRANT CB-2008-01 No. 103601. CONACYT-MEXICO GRANT 56787 (Laboratory for Nanoscience and Nanotechnology Research-LINAN), INFRA-2013-01 No. 204373. ALV also thanks to CONACYT for the doctoral fellowship CVU 171851 and academic exchanges 290673, 290841.

"Sweet are the uses of adversity,
Which, like the toad, ugly and venomous,
Wears yet a precious jewel in his head;
And this our life, exempt from public haunt,
Finds tongues in trees, books in the running brooks,
Sermons in stones, and good in every thing."
William Shakespeare

I dedicate this thesis to my family.

Acknowledgment

First, I want to give a special thanks to my supervisor Dr. Sergio Medina Godoy to support me throughout my thesis, for providing me the opportunity to explore a new area of knowledge and allow me to work in my own way.

I wish to express my special and sincere appreciation to Dra. Ana Paulina Barba de la Rosa for her advice, guidance and supervision of the research, contribution to the manuscript and for seeing this thesis through to its conclusion.

My gratitude goes to the members of my thesis committee: Dr. Hervey Rodríguez, Dr. Cesar M. Escobedo. and Dra. Laura G. Espinosa, for their support, valuable help and advices during this research.

I would also like to thank Dr. José A. Huerta for his unquantifiable assistance during my experiments, as well as Dr. Alejandro Fernández for receiving me in his team as an academic exchange student.

I gratefully thank MC. Alberto Barrera and Dra. Isabel Velázquez, for the technical support and their help during my research.

My appreciation goes to each and every one of colleagues and friends at Laboratorio De Proteómica y Biomedicina Molecular, IPICYT; Laboratorio de Fisicoquímica e Ingeniería de Proteínas, UNAM; Laboratorio de Alimentos Funcionales, CIIDIR-IPN Unidad Sinaloa.

I especially thank my friends for your warm company and numerous adventures during my studies and lifetime, especially to Paola, Karla, Mónica, Iván, Glenda, Daniel, Alicia, Lucrecia, Felipe, Xiomara, Emma, Lucy, and Marizel.

I thank Dr. Leticia E. Verdugo for her friendship and emotional support during this period of my professional training, and for showing me what constitutes congruence and honesty.

Finally, my love and appreciation go to my family; my parents Andrés León Soto and Gloria Villanueva Franco, my sister Nolaska, my brother-in-law Joel, and my nephew Vincent, thank you for your patient, support and understanding throughout my studies; and most of all to God who makes everything possible.

List of contents

List of tables

List of figures

RESUMEN

En México se han reportado genotipos no tóxicos de *Jatropha curcas*, la torta residual obtenida después de la extracción de aceite se presenta como una fuente potencial de proteína para usos alimentarios. Sin embargo, la caracterización de las proteínas de la torta aún se desconoce. El objetivo de este trabajo fue llevar a cabo la caracterización molecular de las proteínas de almacenamiento de semilla de *J. curcas*. Las proteínas de la torta se fraccionaron previamente de acuerdo con el procedimiento de Osborne. La principal fracción proteica en la torta de *J. curcas* estuvo representada por glutelinas, el análisis electroforético mostró que las glutelinas y las globulinas tienen el mismo perfil, lo que indica que el proceso de extracción de aceite podría tener efecto sobre la aglomeración de las globulinas. Las fracciones proteicas se analizaron mediante electroforesis bidimensional en gel y espectrometría de masas; los resultados proporcionaron un nuevo conjunto de datos de especies de proteínas o proteoformas que se acumulan en el endospermo. Las albúminas, proteínas hidrosolubles, son ricas en diferentes especies relacionadas con el metabolismo de carbohidratos y otras proteínas involucradas en el metabolismo de lípidos, nitrógeno y purinas. También se detectaron proteínas relacionadas con la desintoxicación de xenobióticos. La identificación de proteínas tóxicas como la curcina, podría indicar que esta tiene un papel importante en las semillas, aunque la cantidad presente puede no ser suficiente para causar efectos tóxicos. Se identificaron proteínas reguladoras tales como las subunidades del proteosoma, 14-3-3 y Nucleósido-Difosfato quinasas, que son componentes de las vías de señalización. También se identificó un grupo de diferentes especies de proteínas de choque térmico y de defensa contra el estrés, así como proteasas relacionadas con la actividad inhibidora de la Dipeptidil Peptidasa IV; esto podría apoyar el uso potencial de la torta de *J. curcas* como alimento nutracéutico. Además, se investigaron las propiedades fisicoquímicas y funcionales de la harina desgrasada y las principales fracciones proteicas. La harina desgrasada (JCDM) y sus cuatro fracciones contenían la mayoría de los aminoácidos esenciales. Las subunidades de las fracciones proteicas se encontraron en pesos moleculares que oscilan entre 20.0 y 70.0 kDa. En general, la harina desgrasada y las fracciones tuvieron el mejor rendimiento en condiciones alcalinas (por encima de pH 9), presentando una elevada solubilidad, propiedades emulsificantes y espumantes. En comparación con el aislado proteico de soya, las fracciones de proteína y la harina desgrasada exhibieron una mejor capacidad de absorción de aceite y propiedades emulsificantes, pero menor capacidad de retención de agua. La harina desgrasada mostró la mayor capacidad de formación de espuma a pH 9, mientras que la fracción de glutelinas mostró la mejor estabilidad del volumen de la espuma en todos los rangos de pH evaluados. Los resultados revelaron que podrían producirse harina desgrasada y fracciones proteicas con propiedades funcionales adecuadas para utilizarse como un buen ingrediente en sistemas alimentarios. Sin embargo, son necesarios más estudios para diseñar un procedimiento que permita extraer estas proteínas con calidad alimentaria y contribuir a mejorar la viabilidad financiera y la sostenibilidad de una industria de biodiesel a partir de *Jatropha*.

SUMMARY

Jatropha curcas non-toxic genotypes have been reported in Mexico; the residual press-cake obtained after oil extraction, represents a potential of new source of protein for food and feed uses. However, the characterization of the press-cake proteins is still unknown. The aim of this work was to carry out the molecular characterization of *J. curcas* seed storage proteins. Proteins in press-cake were pre-fractionated according to the classical Osborne procedure. The main protein fraction in *J. curcas* cake was represented by glutelins, the electrophoretic analysis showed that glutelins and globulins have the same profile, indicating that oil extraction process could have effect on globulins agglomeration. Protein fractions were analysed by two-dimensional gel electrophoresis and mass spectrometry, results provide a new dataset of protein species or proteoforms that are accumulated in *J. curcas* endosperm. The albumins, water-soluble proteins, are rich on different protein species related with carbohydrate metabolism and other proteins involved in lipids, nitrogen, and purines metabolism. Proteins related with detoxification of xenobiotics were also detected. The identification of toxic proteins such as curcin, could represent that this protein has important roles in seeds, although the amount present may be not enough to cause toxic effects. Regulatory proteins such as proteasome subunits, 14-3-3 and Nucleoside-diphosphate kinases, which are components of signaling pathways were identified. A group of different heat shock and stress defence protein species were also identified, as well as Proteases related with inhibitory activity against dipeptidyl peptidase IV; this could support the potential use of *J. curcas* cake as nutraceutical food. In addition, the physicochemical and functional properties of the defatted meal and major protein fractions were investigated. Defatted Meal (JCDM) and its four fractions contained most of the essential amino acids. The subunits of protein fractions were found with molecular weight ranging from 20.0 to 70.0 kDa. In general, defatted meal and protein fractions had the best performance at alkaline conditions (above pH 9), presenting a high solubility, emulsifying and foaming properties. As compared with soy protein isolate, the protein fractions and defatted meal exhibited better oil absorption capacity and emulsifying properties, but less water holding capacity. Defatted meal showed the highest foam capacity at pH 9, while the glutelin fraction showed the best foam volume stability in all the pH range evaluated. The results revealed that defatted meal and protein fractions with suitable functional properties could be produced to be used as a good protein ingredient in food systems. However, further studies are needed to devise a procedure to extract these proteins in a food-grade manner and contribute to enhance the financial viability and sustainability of a *Jatropha*-based biodiesel industry.

Chapter 1

Introduction and thesis outline

1.1. Introduction

Biomass, bioenergy, and biofuel

Energy has proven to be a highly effective driver of economic progress. Nevertheless, conventional energy sources based on oil, coal and natural gas have many disadvantages, combustion of fossil fuels also causes environmental problems, especially with regards to CO_2 emissions leading to damage the environment and human health (Kamel et al., 2018). Meanwhile, the renewable energy sources can in principle meet the world's energy demand with the advantages of preserving the environment and the abundance all over the world (Guan et al., 2017; Qian et al., 2010; Zabeti et al., 2009).

Currently, non-renewable fossil-based fuels supply about 85% of the primary energy resources. Until the year 2050, due to the increase of the world population, the global primary energy consumption is anticipated to increase almost three-fold (Sanders et al. 2007). This may lead to the depletion of fossil feedstock and, therefore, an increase in oil prices. Given this scenario, several countries in the world have started to use and develop more efficient and cleaner renewable energies from biomass.

Biomass can be used as raw materials for food, feed, chemicals, materials, and energy. The energy from biomass is called bioenergy, while the crops that are used to produce bioenergy are called energy crops. In 2016, biomass contribute with a 4.69% to Mexico energy production (SENER, 2017).

Biofuels, such as bioethanol, biodiesel, and biooil, is the form of bioenergy that is specifically used for transportation and may be classified into two categories: primary and secondary biofuels. The primary biofuels are the natural biofuels directly produced from firewood, plants, forest, animal waste, and crop residue. The secondary biofuels are directly generated from plants and microorganisms and may be further divided into three generations.

The first generation of biofuels is the production of ethanol from starch rich food crops like wheat, barley, corn, potato, sugarcane, or biodiesel from soybean, sunflower and animal fat. While the second generation of biofuels is the production of bioethanol and biodiesel from several species of plants such as jatropha, cassava, miscanthus, straw, grass and wood. The third generation of biofuels is the production of biodiesel from microalgae and microbes (Rodionova et al., 2017).

Global bioethanol production in 2007 was 49.7 million m^3, reached 95.3 million in 2015, and is projected to reach 168 million by 2022. According to OECD-FAO, Mexico produced 1.15 million m^3 of ethanol in 2014, with sugar mills contributing 0.6 million and the rest came from independent alcoholic beverage and other producers. In Mexico, only sugarcane, sugarbeet, and sweet sorghum can be used for ethanol production (Galicia-Medina et al., 2018).

The production of energy from agricultural resources, such as biodiesel, must not compete with the production of foodstuff. Therefore, the focus is now towards the use of non-edible oils such as *Jatropha*, *Karanja*, sea mango and algae oil, waste cooking oils, low-quality animal fats and side-streams from oil refining (Demirbas, 2009; Kamel et al., 2017).

Jatropha curcas

Jatropha belongs to the family *Euphorbiaceae* and has 175 species. It originated from tropical America and distributes all over the tropics and subtropics of Asia and Africa. Its high oil content, rapid growth, easy propagation, drought tolerant nature, ability to grow and reclaim various types of land, need for less irrigation and agricultural inputs, pest resistance, short gestation periods and suitable traits for easy harvesting (Edrisi et al., 2015) makes it ideal for use as energy or fuel source in marginal soils not suitable for food crops.

The oil from *J. curcas* seeds has the appropriate qualities for use in the production of biodiesel, such as low acidity, good oxidation stability in comparison e. g. to the oil from soybean, lower viscosity than the oil from castor, and better cold properties in comparison to palm oil; contains mainly linoleic, stearic, oleic, palmitic and arachidic acids that can be converted to their methyl esters during the transesterification reaction (Kamel et al., 2018).

Besides to its potential as feedstock for biodiesel production, *Jatropha* has been traditional used as cooking/lighting fuel, medicine, bio-pesticide, and for soap making, as well as the seed cake, an oil extraction by-product, used as organic fertilizer, combustible fuel, or for biogas production.

Worldwide there are two groups of germplasm, the toxic and the non-toxic, and most of the biofuels programs are based on the first one. Recent initiatives proposed that the global economy of *Jatropha* could be improved if non-toxic varieties would be developed or if its by-products could be detoxified. However, instead of invest in industrial detoxification, it has been proposed as easier and cheaper the use of the non-toxic germplasm (Vera-Castillo et al., 2014).

Current situation of *Jatropha curcas* in Mexico

J. curcas is a new species from the commercial point of view, it is not cultivated in an extensive way, the National Research Institute for Forestry, Agricultural and Livestock (INIFAP) estimated that the potential lands for *Jatropha* cultivation in Mexico were more than 2.6 million hectares (Valdés Rodríguez et al., 2014), experimental crops were carried out in some states such as Nuevo León, Morelos, Sinaloa and Puebla, but the first commercial jatropha plantings began in 2007 and were located in the states of Chiapas and Michoacán, entities that promote, with social, private and public participation, development programs for the production of biofuels. Seed yields expectations ranged from 1500 to 5000 kg ha^{-1} a^{-1} (Zamarripa et al., 2012).

In Michoacán, the state together with international companies, supported the establishment of cultivars and one commercial bio-refinery to produce and sell biodiesel from *Jatropha* to the rest of the country with a capacity of nine million liters per year. The governments of Yucatan, Veracruz and Quintana Roo sought the collaboration of foreign investors in the oil processing chain, while promoting the establishment of plantations of this species among local farmers, with the state providing the seedlings and the economic stimulus. Sinaloa, through the Council for the Economic Development (CODESIN, 2016), decided to lead and coordinate a project with the support of the National Council of Science and Technology and Produce Foundation, the main objective was to generate a sustainable development of the *Jatropha curcas*' agro-industrial chain, the rescue of the marginalized mountain area of the northwest of Mexico with scope in the states of Sonora and Nayarit. The Secretary of Agriculture, Livestock, Rural Development, Fisheries and Food (SAGARPA, 2017) maintains a program to support research projects on biofuels and the propagation of oily plants, with *Jatropha* being among them.

In Mexico, non-toxic genotypes of *J. curcas* has been identified (Makkar et al., 1997; Martínez-Herrera et al., 2006), although the yield and quality of the oil from seeds of these genotypes are similar to those of the toxic ones, they are not used for oil extraction for biodiesel production. These genotypes look similar to the toxic ones, have similar protein, fiber, and oil concentration but contain no or a very low concentration of toxic phorbol esters (PEs). This condition is advantageous since the seed cake can be used without any prior treatment for animal feed without a health risk to livestock.

The quick development of the biofuel program based on *J. curcas* contrasts with the lack of knowledge about the species, which is undergoing an incipient domestication process and it shows the characteristics of an underutilized crop (Vera-Castillo et al., 2014). Some of the studies that need to be undertaken are comparative evaluation between genotypes, with respect to disease susceptibility and nutrient and water requirements as well as breeding studies to produce cultivars that are adapted to different agroclimatic conditions with reduced toxins as well as the protection of the non-toxic accessions.

Jatropha's protein potential

Protein serves significant roles in many biological processes, and from a nutritional perspective, seed storage proteins have always been major players in supplying global protein needs and food energy. The larger need for proteins in the livestock sector has accentuated the search for new protein sources that do not conflict with human food security interests.

Protein has also many functional properties that are useful for food and non-food applications, for examples as emulsifier e.g. in mayonnaise, as foaming agent, or be used in drug encapsulation. Nowadays soy protein, wheat gluten and casein have been produced commercially. Soy protein is an example of industrial protein derived from oilseed crop. Soy protein products are sold in the market in the form of protein

concentrate or protein isolate. Protein is also a potential source for amino acids, which can be used as food supplements, animal feed, and pharmaceutical applications.

The raw seed cake (obtained by passing seeds through a screw press to extract oil) produced from *Jatropha curcas* seeds has a variable nutrient composition, but it is considered a good source of protein (Félix-Bernal et al., 2014). Therefore, efforts have been made to prepare protein isolate or seed meal to remove fibre from the seed cake. This cake has antinutritional constituents such as trypsin inhibitors, lectins and phytates along with high levels of PEs. Because of the presence of these compounds, the seed kernels of the toxic variety are inedible to animals and humans. Nevertheless, trypsin inhibitors and lectins are heat labile and can be inactivated by heat treatment, while adverse effects of phytates can be mitigated by adding phytase; so, the main challenge for detoxification of *Jatropha* residues has been the removal/inactivation of PEs from them.

Jatropha protein isolates have been prepared using the principle of iso-electric precipitation or by using organic solvents that precipitate proteins (Makkar et al., 2012) and seed meals have been prepared from seed cake using a process of sieving to remove shells that contain higher level of fibre. The Jatropha kernel is very soft and the extrusion process (mechanical pressing) is ineffective at extracting oil from kernels. To recover oil from extruded Jatropha kernel cake, it is further subjected to solvent extraction, giving kernel meal. Kernel meal from the non-toxic genotype has been successfully used in feeding trials with rats and fish and could be considered as a suitable animal feed ingredient (Makkar, 2016).

Jatropha **perspectives and challenges**

Based on the assumption of the 10% substitution of fossil fuel for transportation, Sanders et al. (2007) estimated additional annual production of 100 million tonnes of proteins as byproduct of the biofuel production, which is about four times protein requirement for the global human population.

Jatropha curcas byproduct cake could be among these new protein sources but is necessary to analyze if its proteins have chemical and functional properties comparable to soy, canola, rapeseed, or sunflower, and contribute to enhancing the financial viability and sustainability of a *Jatropha*-based industry.

At a molecular level, the modus operandi in investigating the seed sub-region proteomes of *Euphorbiaceae* species has open the availability of some relevant proteomic studies conducted till date. Seed proteomic studies lay the foundations to understand basic information on the biosynthetic pathways associated with synthesis of toxic diterpenes, fatty acids and triacylglycerols and deposition of storage proteins during seed development. As *Jatropha* seeds are prospective resource of biodiesel generation. These studies provide an important glimpse into the enzymatic machinery devoted to the production of carbon (C) and nitrogen (N) sources to sustain seed development and quality.

Given that most of the studies related to these aspects have been carried out with toxic genotypes, the analysis and comparative study with non-toxic genotypes acquire great importance due to its natural advantages.

1.2. Justification

During the last decades, there is a growing awareness of environmental problems; in that sense, alternative energy sources are gaining importance. The concept of biodiesel produced from energy plants became a focus of interest. *Jatropha curcas* L. seeds had a high content of oil that can be used for biodiesel production and two byproducts, namely the protein rich cake and the shells, also offer opportunities for adding value to the crop.

There is a variety of studies on protein nutritional quality, feeding value and others technical and biotechnological usefulness of *Jatropha* proteins, mainly from toxic genotypes, after removing phorbol esters by diverse detoxification treatments.

Nevertheless, non-toxic genotypes have drawn less attention, therefore the extraction, identification and functional characterization of the proteins from these sources will help to obtain valuable information to determine their techno-functional properties and potential uses; moreover, to enhance the profitability and sustainability of the biodiesel production process and the introduction of these genotypes as a viable alternative to the toxic ones.

1.3. Hypothesis and research objectives

1.3.1. Hypothesis

Jatropha curcas L. non-toxic byproduct seed cake is mainly composed by glutelins and globulins with similar structural and functional properties to those found in economically important legumes.

1.3.2. Objectives

The aim of this research was to identify and functionally characterize the main protein fractions of non-toxic *Jatropha curcas* byproduct seed cake.

The specific objectives were:

- To obtain the seed storage proteins from a non-toxic *Jatropha curcas* byproduct seed cake by sequential extraction based on the solubility criteria.
- To obtain the two-dimensional gel maps from the main protein fractions of non-toxic *Jatropha curcas* byproduct seed cake and identify the protein spots through LC-MS/MS.

- To characterize the functional properties of the main protein factions of non-toxic *Jatropha curcas* byproduct cake over a wide range of conditions that are relevant to food systems.

1.4. Thesis outline

Chapter 1 presents an introduction to this thesis, with a general overview related to the emergence of bioenergetics, the crops associated with their generation and in particular the place occupied by *Jatropha curcas* within them, as well as the potential of their by-products as a new source of good quality proteins. It also presents the justification, hypotheses, and objectives on which this research is based.

Chapter 2 is a review on the state-of-the-art of *Jatropha curcas* under a new approach, general characteristics, its role as a feedstock for biodiesel and the potential uses of byproducts through a biotechnological approach. In order to provide a comprehensive summary, all the information was collected from peer-reviewed literature, conference proceedings and books.

Chapter 3 addresses the proteomic analysis in non-toxic *Jatropha curcas* byproduct cake after oil extraction, where the majority of proteins were unique while some housekeeping proteins were common to specific sub-regions earlier reported. The identified proteins revealed the predominance of protein inhibitor, metabolism, ROS regulated, transport, development and protein degradation related proteins.

Chapter 4 gives an overview of the functional properties of these proteins extracted under solubility criteria and explore its possible techno-functional application as food ingredients or feed supplementation.

Finally, Chapter 5 gives a general conclusion of the results and some recommendations of the future utilization of *Jatropha*.

1.5. References

Demirbas, A., 2009. Progress and recent trends in biodiesel fuels. Energy Conversion and Management 50, 14-34.

Desarrollo Sustentable de la Cadena Agroindustrial de *Jatropha curcas* para el Rescate de la Zona Serrana Marginada del Noroeste de México. http://www.codesin.mx/wp-content/uploads/2015/03/compendio_jatropha.pdf. Accessed September 2016.

Edrisi, S.A., Dubey, R.K., Tripathi, V., Bakshi, M., Srivastava, P., Jamil, S., Singh, H.B., Singh, N., Abhilash, P.C., 2015. Jatropha curcas L.: A crucified plant waiting for resurgence. Renewable and Sustainable Energy Reviews 41, 855-862.

Félix-Bernal, J.A., Angulo-Escalante, M.A., Estrada-Angulo, A., Heredia, J.B., Muy-Rangel, D., López-Soto, M.A., Barreras, A., Plascencia, A., 2014. Feeding value of nontoxic *Jatropha curcas* seed cake for partially replacing dry-rolled corn and soybean meal in lambs fed finishing diets. Animal Feed Science and Technology 198, 107-116.

Galicia-Medina, M., Barrios-Estrada C., Esquivel-Hernández D.A., Rostro-Alanís, M.J., Torres, A., Parra-Saldívar, R., 2018. Current state of bioethanol fuel blends in Mexico. Biofuels, Bioproducts and Biorefining 12, 338-347.

Guan, Q., Li, Y., Chen, Y., Shi, Y., Gu, J., Li, B., Miao, R., Chen, Q., Ning, P., 2017. Sulfonated multi-walled carbon nanotubes for biodiesel production through triglycerides transesterification. RSC Advances 7, 7250-7258.

Kamel, D.A., Farag, H.A., Amin, N.K., Fouad, Y.O., 2017. Biodiesel synthesis from non-edible oils by transesterification using the activated carbon as heterogeneous catalyst. International Journal of Environmental Science and Technology 14, 785-794.

Kamel, D.A., Farag, H.A., Amin, N.K., Zatout, A.A., Ali, R.M., 2018. Smart utilization of jatropha (*Jatropha curcas* Linnaeus) seeds for biodiesel production: Optimization and mechanism. Industrial Crops and Products 111, 407-413.

Makkar, H.P.S., 2016. State-of-the-art on detoxification of *Jatropha curcas* products aimed for use as animal and fish feed: A review. Animal Feed Science and Technology 222, 87-99.

Makkar, H.P.S., Becker, K., Sporer, F., Wink, M., 1997. Studies on Nutritive Potential and Toxic Constituents of Different Provenances of *Jatropha curcas*. Journal of Agricultural and Food Chemistry 45, 3152-3157.

Makkar, H.P.S., Kumar, V., Becker, K., 2012. Use of detoxified jatropha kernel meal and protein isolate in diets of farm animals. In: Makkar, H.P.S. (Ed.), Biofuel Co-Products as Livestock Feed–Opportunities and Challenges. FAO, Rome, Italy, pp. 351–378.

Martínez-Herrera, J., Siddhuraju, P., Francis, G., Dávila-Ortíz, G., Becker, K., 2006. Chemical composition, toxic/antimetabolic constituents, and effects of different treatments on their levels, in four provenances of *Jatropha curcas* L. from Mexico. Food Chemistry 96, 80-89.

National Energy Balance reports 2016. Mexico Ministry of Energy (SENER). https://www.gob.mx/cms/uploads/attachment/file/288692/Balance_Nacional_de_Energ_a_2016_2_.pdf Accessed September 2017.

Qian, J., Shi, H., Yun, Z., 2010. Preparation of biodiesel from *Jatropha curcas* L. oil produced by two-phase solvent extraction. Bioresource Technology 101, 7025-7031.

Sanders, J., Scott, E., Weusthuis, R., Mooibroek, H., 2007. Bio-Refinery as the Bio-Inspired Process to Bulk Chemicals.

Secretary of Agriculture, Livestock, Rural Development, Fisheries and Food (SAGARPA). Innovation and Technological Development in Agricultural Research Program. http://www.sagarpa.gob.mx/ProgramasSAGARPA/2017/fomento%20_agricultura/energ%C3%ADas_renovables/Paginas/Conceptos_de_apoyo.aspx. Accessed October 2017.

Valdés Rodríguez, O., Vázquez, A., Muñoz Gamboa, C., 2014. Drivers and Consequences of the First Jatropha curcas Plantations in Mexico. Sustainability 6, 3732.

Vera-Castillo, Y.B., Cuevas, J.A., Valenzuela-Zapata, A.G., Urbano, B., González-Andrés, F., 2014. Biodiversity and indigenous management of the endangered non-toxic germplasm of Jatropha curcas L. in the Totonacapan (Mexico), and the implications for its conservation. Genetic Resources and Crop Evolution 61, 1263-1278.

Zabeti, M., Wan Daud, W.M.A., Aroua, M.K., 2009. Activity of solid catalysts for biodiesel production: A review. Fuel Processing Technology 90, 770-777.

Zamarripa, C.A.; Diaz, P.G., 2008. Áreas de Potencial Productivo de Piñón: Jatropha curcas L., Como Especie de Interés Bioenergético en México; Oleaginosas en Cadena: Mexico City, Mexico.

Zamarripa-Colmenero, A., Solis-Bonilla, J.L., Martínez-Valencia, B.B. , Pecina-Quintero V., 2012. Agronomic and biochemical study of *Jatropha curcas* L. in Mexico. BIT Congress, Inc (Ed.), BIT's 2nd annual world congress of bioenergy. April 25th-28th; XI'an China, BIT Congress, Inc, XI'an. China, p. 115

Chapter 2

A new approach on *Jatropha curcas* – A review.

Abstract

Jatropha curcas, a multipurpose, drought resistant, perennial plant belonging to *Euphorbiaceae* family have been subject of research and commercial interest, due to its importance as a feedstock in the biodiesel production. In the future, this will result in the availability of high amounts of pressed seed cake or kernel meal as byproducts, which are rich in proteins of high quality. Two types of varieties are used for oil production: toxic and non-toxic varieties, in function of toxic phorbol esters in seeds. Unfortunately, the *Jatropha*'s press cake from toxic varieties are restricted for food or feed, however non-toxic varieties have been evaluated, showing similar or better than the conventional toxic *Jatropha* varieties in seed yield per plant and seed oil content. Investigation on press cake and oil detoxification have been developed, as well as protein isolates extraction and characterization from defatted seed meal as a strategy to reduce the contents of antinutritional and toxic components to be used as an attractive protein and carbohydrate source for fermentation processes and/or for formulations for animal feeding. The state-of-the-art information provided here aimed to stimulate further research and development leading to more intensive, efficient, and sustainable utilization of *Jatropha*.

Keywords: *Jatropha curcas*, byproducts, non-toxic, protein, sustainable.

2.1. Introduction

Jatropha curcas L. (*Euphorbiaceae*) is a native crop to Mexico and Central America and it is now widespread all over the tropical and subtropical world such as; Africa, India, South-East Asia and China. *Jatropha* is a drought resistant, perennial plant that can grow in rocky lands, marginal lands, deserts, and even in saline soils (Kamel et al., 2018). Due to its high oil content; it serves as raw material for the production of soap, candles, cosmetics, and paraffins (Kumar and Sharma, 2008) and as a potential feedstock for oil production in order to obtain biodiesel (Meher et al., 2013).

Besides oil for biodiesel production, the *Jatropha* seeds provide proteins, which have a descriptive history in both nutrition and therapeutic applications (Mandal and K. Mandal, 2000). The larger need for proteins in the livestock sector has accentuated the search for new protein sources that do not conflict with human food security interests. In the current situation, oilseeds able to produce on marginal soils are the potential and preferred choice for protein and other nutrients for livestock.

Moreover, the expected large production of biodiesel from *Jatropha* seeds will generate pressed cake from seeds and kernel meal as by-products that could also be a source for various bioactive molecules having a wide range of activities (Wang et al., 2007). Jatropha proteins and peptides have been studied for their role in the plant metabolic activities and defence against predators as well as for their therapeutic and industrial or agricultural potential (Saikia et al., 2012), and from a pharmaceutical prospective, *Jatropha* species are also a rich source of phytochemicals.

In the present review an attempt has been made to discuss the *Jatropha*'s botany, its potential as a biodiesel source, the nutritional quality of seed proteins, they role and the potential application of its biologically active molecules, feeding value and others biotechnological usefulness.

2.2. Botanical and agroclimatic description

The genus *Jatropha* belongs to the tribe *Joannesieae* (or *Jatropheae*) of the subfamily *Crotonoideae* in the *Euphorbiaceae* family. Although still controversial, *J. curcas* is probably native of tropical America, with the original area of distribution in Mexico and in continental Central America (Belize, Costa Rica, El Salvador, Guatemala, Honduras, Nicaragua and Panama) (Maes et al., 2009). From tropical America, *J. curcas* was probably distributed to Africa and Asia by Portuguese seafarers via Cape Verde Islands and Guinea Bissau and, nowadays, it has a pantropical distribution **Figure 1.** (Achten et al., 2010).

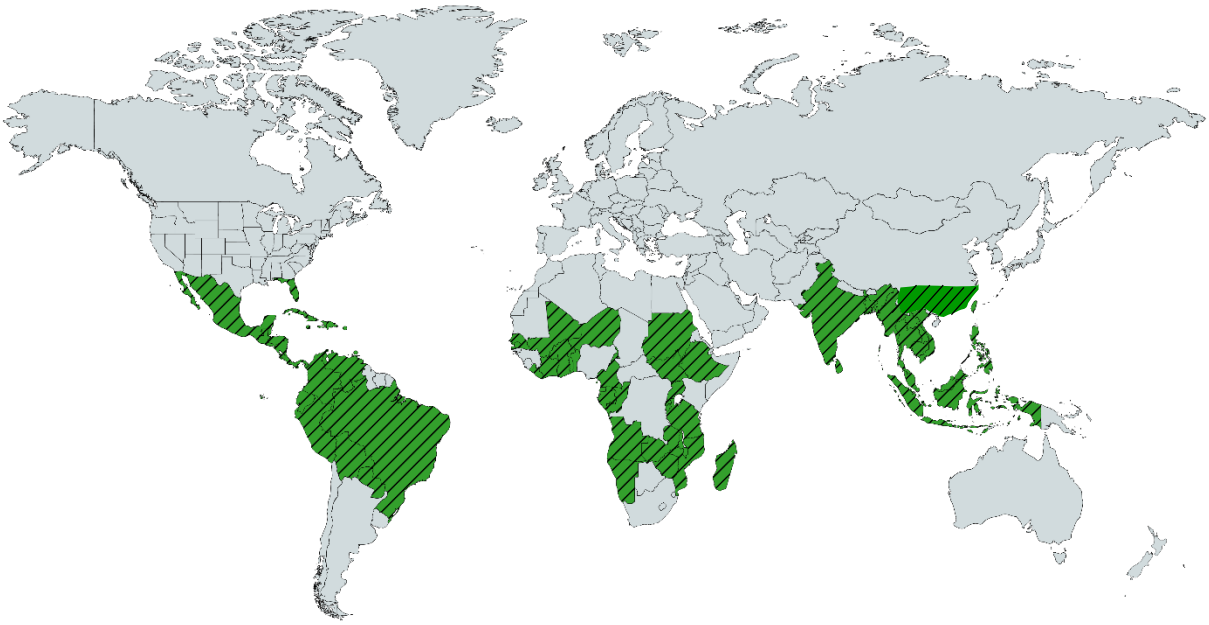

Figure 1. Approximate global distribution of *J. curcas*. (shaded regions).
(Source: Adapted from (Heller et al.).

According to Sabandar et al. (2013) the genus '*Jatropha*' comprises of about 170 species of small trees, shrubs, subshrubs or herbs widely distributed in the Old and New World tropics. The common species in *Jatropha* genes are *J. curcas* L.*, J. chevalieri* Beille*, J. elliptica* Muell*. Arg., J. gaumeri* Greenm*., J. glandulifera* Roxb*., J. gossypiifolia* L.*, J. grossidentata* Paxet. Hoffm.*, J. integerrima* Jacq.*, J. macrantha*, *J. mahafalensis* Jum and H. Perrier, *J. multifida* L.*, J. nana* Dalz, *J. podagrica* Hook, *J. pohliana* Muell. Arg.*, J. tanjorensis* Ellis and Saroja, *J. unicostate*, *J. weddelliana* Baillon and *J. platyphylla.* Some of them are indigenous and endemic representative of the *Jatropha* genus.

The name of *Jatropha curcas* is *J. curcas* Linnaeus (Euphorbiaceae). Linnaeus was the first to name the physic nut J. curcas L. The genus name *Jatropha* derives from the Greek word jatr´os (doctor) and troph´e (food), which implies its medicinal uses. The *J. curcas* is monoecious, flowers are unisexual and occasionally hermaphrodite flowers occur, each inflorescence yields a bunch of approximately 10 or more ovoid fruits. Flowering occurs during the wet season and two flowering peaks are often seen in summer and autumn(Kumar and Sharma, 2008).

The seeds of *J. curcas* are formed within seed pods (**Figure 2**). Each seed pod typically contains three seeds (**Figure 2C**) (King et al., 2009). The seeds mature about 3–4 months after flowering. The plant can be easily propagated from seeds or cuttings. It will grow under a wide range of rainfall regimes from 250 to over 1200 mm per annum.

The trees are deciduous, shedding the leaves in dry season. One major trait associated with the plant is its hardiness and sustainability in warm and arid climates, prefers well-drained alkaline soil (pH 6-9) for its growth (Kumar and Sharma, 2008).

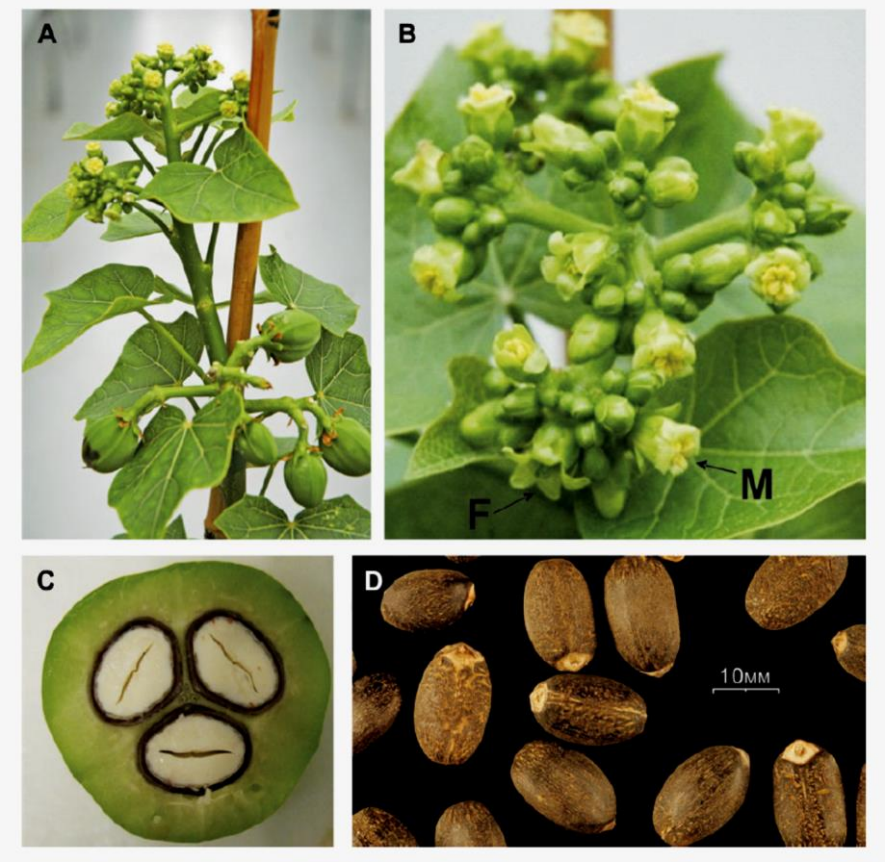

Figure 2. Images of *J. curcas*. (A) Young *J. curcas* plant with both flowers and developing seed pods. (B) *J. curcas* inflorescence containing both male staminate flowers (M) and female pistillate flowers (F). (C) Cross-section of a *J. curcas* seed pod containing three developing seeds. (D) Mature seeds of *J. curcas*. (Source: King et al. (2009)).

2.3. *Jatropha* as a biodiesel source

J. curcas seeds contain about 33.0–39.1% of oil per seed dry mass, which can be relative easily expelled or extracted (Jongschaap et al., 2007). The production of oil from *J. curcas* seeds requires several steps: (i) dehusking process, to separate seeds from husk, (ii)dehulling process, to separate kernel from shell, (iii) oil extraction process, to produce oil and seed cake as by-product, and (iv) oil cleaning process, to transform crude oil, with impurities, into pure oil (**Figure 3**)(Contran et al., 2013). Nowadays, two methods have been identified for the extraction of seed-oil: (i) mechanical extraction and (ii) chemical extraction; but the possibilities, procedures, and means are evolving rapidly (Achten et al., 2008).

Biodiesel is made from virgin or used vegetable oils (both edible and non-edible) and animal fats through transesterification and is a diesel substitute and requires very little or no engine modifications up to 20% blend and minor modification for higher percentage blends. *Jatropha* oil contains mainly linoleic, stearic, oleic, palmitic and arachidic acids that can be converted to their methyl esters during the transesterification reaction, hence, this oil is one of the most appropriate sustainable alternative feedstock for biodiesel production in terms of availability and cost (Kamel et al., 2018).

Pure *J. curcas* oil can be chemically modified into biodiesel through four primary methods: (i) blending, (ii) microemulsion, (iii) pyrolysis, and (iv) transesterification (Koh and Mohd. Ghazi, 2011). Alkaline catalysis transesterification reaction is the preferable as it has many advantages; short reaction time and high biodiesel production yield. Heterogeneous solid base catalysts are advantageous due to ease of recovery, regeneration, reusing and potential facilitation of continuous processing (Semwal et al., 2011), but its high preparation cost of base always hinders their usage in industry ((Ali et al., 2015).

J. curcas biodiesel has comparable properties with those of fossil fuel and conforms to the latest standards for biodiesel, such as European (EN 14214:2003) and USA (ASTM D 6751), but standardization according to Country defined standards for biodiesel is a prerequisite for successful market introduction and penetration (Parawira, 2010).

Until now, the cultivation of *J. curcas* for biodiesel production was carried out in China, Mali, Cambodia, Indonesia, Thailand, Brazil, Nicaragua, Costa Rica, Belize, El Salvador, Guatemala, Honduras, Mexico, Egypt, Tanzania, Ghana and India, and certainly other countries could be added to the list. However, the main criterion for a successful biodiesel production from *J. curcas* should be the sustainability of the projects. This sustainability involves the environmental, as well as social and economic criteria (Castro Gonzáles, 2016).

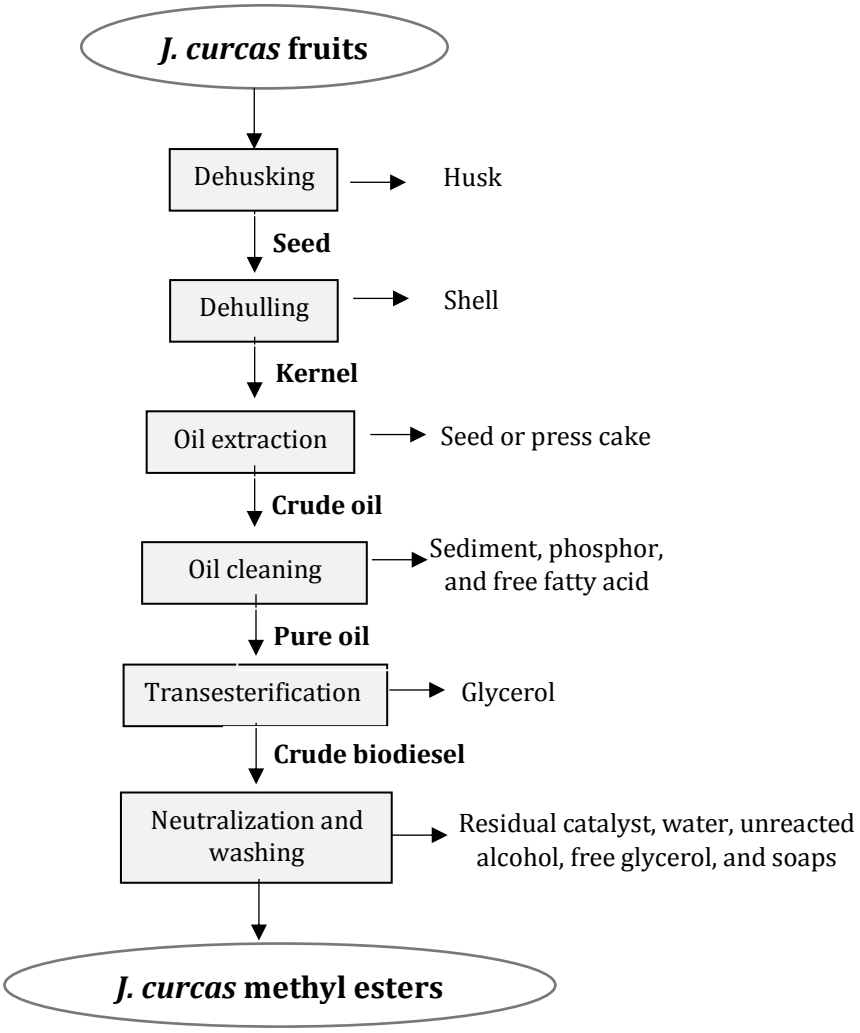

Figure 3. Processes for *J. curcas* oil and biodiesel production (Source: Contran et al., 2013).

2.4. Chemical and nutritional quality

The seeds of *J. curcas* are composed of kernel and shell with an average ratio of 62.2:37.7. The kernel has higher crude protein (22–28%) and oil contents (54–58%) compared to the shell (4–6% crude protein and 0.8–1.4% oil)(Makkar et al., 1998).

The seeds of the *Jatropha* accessions occurring naturally in Africa and Asia are toxic due to their high content of phorbol esters (PEs), because of the presence of these compounds, their kernels are inedible to animals and humans; but in Mexico, there are nontoxic variants used in traditional dishes in the states of Veracruz and Puebla (Makkar et al., 1997; Martínez-Herrera et al., 2006). The lack of variation in the content

of other antinutrients such as curcin in the toxic and edible *J. curcas* seeds confirms the fact that variation in edibility is due to this single trait (He et al., 2011).

Studies have clearly shown that the non-toxic nature is a dominant maternal characteristic and accidental outcrossing with toxic *J. curcas* does not affect the phorbol ester content of seeds borne on non-toxic *Jatropha* plants (Sujatha et al., 2005). The genetic distinctness of the edible varieties compared to the toxic varieties has been confirmed by molecular marker analysis of *Jatropha* germplasm (Basha et al., 2009; Sudheer Pamidimarri et al., 2009). He et al. (2011) postulated that the genetic existence of two distinct classes of seed based on phorbol ester content, one high and the other at least 1000 times lower, strongly suggests that phorbol ester content is controlled by a single genetic locus.

The chemical composition of the *Jatropha* seeds and seed kernel meal and the nutritional quality of toxic has been reported and compared with non-toxic provenances (Makkar et al., 2011; Martínez-Herrera et al., 2006). As can be seen from **Table 1,** the oil content in seeds and the fatty acid composition are similar. Seed kernel meal crude protein and ash contents did not differ significantly between accessions. The composition of antinutrients shows clearly that differences between edible and non-edible varieties occur only with regard to the presence or absence of phorbol esters (Francis et al., 2013).

2.5. *Jatropha* proteins

2.5.1. Storage proteins

The seed storage proteins (SSP), are non-enzymatic and have the sole purpose of providing proteins (nitrogen and sulphur source) required during germination and establishment of a new plant (Mandal and K. Mandal, 2000). The systematic terminology used to define classes of seed storage proteins is solubility-based (Osborne, 1924) . Thus, the SSP soluble in H_2O are called albumins, while those soluble in dilute saline, aqueous alcohol, or dilute alkali or acid are globulins, prolamins, or glutelins, respectively. However Fukushima (1991) proposes a new classification based on the genetic structure, having as criteria the presence or absence of introns in the gene, the homology in primary structure, the presence of repetitive structures that influence the secondary structure, its biosynthetic route and the form of accumulation within the seed.

While albumins are found in all seeds, prolamins and glutelins are abundant in monocot seeds and globulins are prevalent in dicot seeds (Jorrin-Novo, 2014). All storage protein fractions are mixtures of components that exhibit polymorphism both within single genotypes and among genotypes of the same species according to species (Shewry et al., 1995).

Table 1. Composition (% in DM) of kernels (with oil) and defatted kernel meal from toxic and non-toxic genotypes of *Jatropha curcas*; and antinutritional factors in unheated kernel meals. (Adapted from: (Makkar et al., 2011)).

	Jatropha curcas [a]	
	Toxic	Non-Toxic
Kernel		
Crude protein	26.6±1.12	26.8 ±1.25
Oil	57.4 ±0.50	57.5 ±0.69
Ash	4.0 ±0.67	4.5 ±0.56
Defatted kernel meal		
Crude protein	57.4 ±0.50	62.4 ±2.65
Ash	57.4 ±0.50	9.1 ±1.04
Component		
Phorbol esters (mg/g kernel) [b]	2.79	ND
Total phenols (% tannic acid equivalent)	0.36	0.22
Tannins (% tannic acid equivalent)	0.04	0.02
Condensed tannins (% leucocyanidin equivalent)	ND	ND
Phytates (%dry matter)	9.40	8.90
Saponins (%diosgenin equivalent)	2.60	3.40
Trypsin inhibitor (mg trypsin inhibited/g sample)	21.31	26.54
Lectin activity (1/mg of meal that produced haemagglutination/ml of assay medium)	51-102	51-102
Glucosinolates	ND	ND
Cyanogenes	ND	ND
Amylase inhibitor	ND	ND

ND: Not detected.

[a] Compiled data from previous studies.

[b] As phorbol-12-myristate-13-acetate equivalent.

In *Jatropha curcas*, the storage proteins extracted from defatted kernel meals of both toxic and non-toxic genotypes revealed that the total protein content is ~89%, although the proportions of the protein fractions varies according the fat extraction method employed, as well as the fractionation procedure. In general, glutelins and globulins are the main fractions whereas prolamins and non-extracted proteins were present in minor quantities (Peralta-Flores et al., 2012).

The levels of all essential amino acids except lysine are comparable with, or higher than, the FAO reference protein for a growing child of 2–5 years of age **Table 2.** A comparison between the amino acid composition of *Jatropha* meals and soybean meal reveal an almost similar pattern for all essential amino acids, except lysine and sulphur amino acids, which were lower and higher, respectively, in *Jatropha* meals. The levels of essential amino acids in the *Jatroph*a meals are higher than or similar to those in the castor bean meal (Makkar et al., 2011).

2.5.2. Miscellaneous proteins

Other proteins were also reported from the seed oil and latex of *Jatropha* plants. Curcin, lectin, β-glucanase, esterases (JEA and JEB) and lipases (JL) were isolated from the seed oil of *J. curcas* (Staubmann et al., 1999; Stirpe et al., 1976; Wei et al., 2005). Aquaporins and betaine aldehyde dehydrogenase are proteins found in the seed oil which function as drought resistant agents (Zhang et al., 2008), while Nath and Dutta (1991) reported the isolation of curcain, a protease from the latex. All proteins mentioned are known to act as functional proteins (Devappa et al., 2010).

2.5.3. Protein isolates and hydrolysates

The use of *Jatropha* seed protein for non-food applications and techno-functional properties has been reported (Gofferjé et al., 2015; Hamarneh et al., 2010; Lestari et al., 2011; Xiao et al., 2015). However, few studies have been conducted to determine the potential of *Jatropha* protein isolate as a food ingredient (Saetae et al., 2011) or the inclusion of detoxified kernel meal in human or animal nutrition (Kumar et al., 2010b).

Protein isolates are the concentrated forms of plant proteins, generally prepared by solubilizing proteins and removing non-protein ingredients. Saetae et al. (2011) obtained a protein isolate from *Jatropha* seed cake by an alkaline extraction and followed by an isoelectric precipitation. The protein isolate had small amounts of phorbol esters, however, were higher than that of the non-toxic variety; phytic acid and saponins were also detected. This protein isolate had unique functional properties such as water binding capacity, emulsion activity, and emulsion stability, which are important factors in food systems.

Saetae and Suntornsuk (2012) used various protein precipitation methods as aqueous acetone, aqueous ethanol and ammonium sulphate to obtain *Jatropha* protein isolates. Different functional properties of the proteins were achieved, aqueous ethanol protein provided the maximum water holding capacity and the higher emulsion activity and stability, while the obtained from ammonium sulphate gave the highest foam capacity and the protein from acetone had the highest foam stability.

Protein hydrolysates are the products that are obtained after the hydrolysis of proteins and this can be achieved by enzymes, acid or alkali. This broad definition encompasses all the products of protein hydrolysis – peptides, amino acids and minerals present in the protein and acid/alkali used to adjust pH (Pasupuleti et al., 2010).

Table 2. Amino acid composition (g/16 g nitrogen) of kernel meals from toxic and non-toxic genotypes of *Jatropha curcas*, soybean meal, and FAO reference protein. (Adapted from: (Makkar et al., 2011)).

Amino acids	*Jatropha curcas* [c]		Soybean meal	FAO reference protein (2-5-year-old children)
	Toxic	Non-Toxic		
Essential				
Methionine	1.56-1.91	1.38-1.76	1.32	2.50 [a]
Cystine	1.77-2.24	1.58-1.81	1.38	
Valine	4.35-5.19	3.79-5.30	4.50	3.50
Isoleucine	3.93-4.53	3.08-4.85	4.16	2.80
Leucine	6.55-6.94	5.92-7.50	7.58	6.60
Phenylalanine	4.08-4.34	3.93-4.89	5.16	6.30 [b]
Tyrosine	2.45-2.99	2.62-3.78	3.35	
Histidine	2.81-3.30	2.65-3.08	3.06	1.90
Lysine	3.63-4.28	3.40-3.49	6.18	5.80
Threonine	3.33-3.96	3.15-3.59	3.78	3.40
Tryptophan	1.31	ND	1.6	1.10
Non-essential				
Serine	4.67-4.80	4.59-4.91	5.18	-
Arginine	11.8-12.2	11.4-12.90	7.64	-
Glutamic acid	14.68-16.7	15.91-16.50	19.92	-
Aspartic acid	9.49-11.8	9.92-11.7	14.14	-
Proline	4.13-4.96	3.80-4.21	5.99	-
Glycine	4.40-4.92	4.18-4.61	4.52	-
Alanine	4.36-5.21	4.26-4.94	4.54	-

ND: Not detected.

[a] Methionine plus cystine.

[b] Phenylalanine plus tyrosine.

[c] Complied data from previous studies.

Protein hydrolysates were produced by treatment of a non-toxic *Jatropha curcas* L. genotype with alcalase as well as the digestive enzymes pepsin and pancreatin (Gallegos Tintoré et al., 2015). This hydrolysis system produced low-molecular-weight hydrolysates with antihypertensive activity inhibitory (Segura-Campos et al., 2013), antioxidant and antithrombotic activities and commercial potential as ''health-enhancing ingredients'' in the production of functional foods. Alike, Gallegos Tintoré et al. (2015) proved the presence of naturally occurring antioxidant and chelating peptides in non-toxic *J. curcas* seeds through a cell culture system.

León-López et al. (2013) optimized a protein isolation process from the seed cake of an edible provenance of *J. curcas* by an alkaline extraction followed by isoelectric precipitation method, via a sequentially integrated approach. They evaluate the influence of four different factors (solubilisation pH, extraction temperature, NaCl addition, and pH for precipitation) on the protein and antinutritional compounds content of the isolate. The *J. curcas* isolate was produced with a higher protein content (93.21%) than previously reported.

Moreover, Valdez-Flores et al. (2016) employed a response surface methodology (RSM) to optimize an extrusion cooking process, before applying an enzymatic hydrolysis in *Jatropha* protein hydrolysates (JPH) which potentiated antioxidant capacity (AOXC) and antihypertensive activity (ACEI). This excellent bioactivity could potentially be used for the control of degenerative diseases such as hypertension and those generated by oxidative cellular stress.

2.5.4. Detoxification effect on conformational and functional properties of proteins

The main challenge for detoxification of *Jatropha* residues has been the removal/inactivation of Phorbol Esters (PEs) from them. Detoxification treatments used on *Jatropha* products to remove, degrade or inactivate PEs fall in three main categories: chemical treatments, biological treatments and physical treatments. The chemical treatments involve the use of many aqueous alkalis and organic solvents, alone or in combination, resulting in substantial lower PEs in the treated material. In some studies, the PEs in the treated materials were undetectable. Biological treatments have used fungi and other microorganisms in submerged or solid-state fermentation systems. Some microbial treatments alone resulted in products in which PEs were not detectable or were present at very low levels.

The comparison of the different methods is hampered by the fact that in many studies in which PEs were undetectable after treatment, the exact analytical procedure and the limit of detection have not been reported. Therefore, it seems necessary to include the outcome of feeding trials in the final assessment of the efficacy of detoxification methods. Such feeding trials are also needed, as the nature and chemical composition of the degradation products of PEs remains unknown and in order to assess if the treatments also reduce the presence of anti-nutritional constituents (Chain, 2015; Makkar, 2016).

Xiao et al. (2015) studied the effect of detoxification on functional properties, structure, and thermal properties of *Jatropha curcas* meal, using a treatment of hydrolysis with enzymes (cellulase plus pectinase) followed by washing with ethanol (65%). The detoxification treatment improved the protein solubility, water and fat absorption capacity, emulsifying activity and stability, foam capacity and stability of the *Jatropha* protein meal.

2.5.5. Influence of protein extraction technique on techno-functional properties

Gofferjé et al. (2015) compared an aqueous and an enzyme-assisted process for protein extraction from screw-pressed (SPJR) and aqueous de-oiled *Jatropha* residue (ADJR) and evaluated different methods for protein recovery. The functionalities of the aqueous-extracted proteins obtained from ADJR and from SPJR were rather similar. However, proteins from ADJR appear to have better emulsifying properties and gelation behaviour, while proteins from SPJR showed better oil binding capacity. The recovery method facilitates the adaption of protein properties to the desired functionalities within certain ranges.

2.6. Feeding value of *Jatropha curcas* proteins

The use *J. curcas* residues or protein isolate as animal feed is possible only after deactivation or removal of phorbol esters. Heat treatment does not completely remove phorbol esters but could inactivate trypsin inhibitors and lectins. The untreated residue as well those obtained after heat treatment containing residual phorbol esters cause toxicity. The treatments that reduce phorbol esters below 3 mg/kg treated residue (as 12-O-tetradecanoylphorbol-13-acettate equivalent) could produce feeds that may be considered safe for animal feeding. Addition of phytase enzymes (to alleviate adverse effects of phytate) and an essential amino acid, lysine, enhances productivity responses when the treated products containing <3 mg phorbol esters/kg were fed to pig, carp, trout and shrimp at half replacement of conventional protein sources (on a crude protein basis) in the diet (Makkar, 2016).

Another possibility is to use a nontoxic variety of *Jatropha curcas*, this condition is advantageous since the seed cake can be used without any prior treatment for animal feed without a health risk to livestock. *J. curcas* seed cake (JSC) has a potential to complement and partially substitute for corn and soybean meal (SBM) as a protein and energy source in ruminant diets. Félix-Bernal et al. (2014) evaluated the feeding value of a seed cake product obtained from the Mexican *J. curcas* nontoxic genotype, as a partial replacement for corn and soybean meal in a finishing diet fed to lambs.

Changes in dietary energy level, and in energy source (fat and NDF vs. starch) in the diet as a result of corn and SBM replacement with JSC affected deposition of body fat. At high levels of inclusion, it tended to increase protein content, but decreased carcass fat content and hot carcass weight were decreased. Based on dry matter intake and performance observed in the study, JSC is a suitable substitute at moderate levels for a portion of the dry-rolled corn and soybean meal in finishing diets. The absence of abnormal blood parameters with increased JSC supplementation levels indicates that liver and pancreatic functions were not altered in lambs.

Heat-labile antinutrients, protease inhibitors, and lectins are easy to inactivate by moist heating, and phytase could be incorporated into the diet for degradation of phytate.

Detoxified *J. curcas kernel* meal (DJKM) contain approximately 9–10% phytate, which can be eliminated by adding 1500 phytase unit of feed (FTU)per kilogram of diet. *J. curcas* protein isolate can replace 50, 62.5, and 75% of fish meal protein, respectively, in fish diets without compromising their growth performance and nutrient utilization (Kumar et al., 2011).

In addition, detoxified *J. curca*s kernel meal could also replace 50% of fish meal protein without adversely affecting growth and nutrient utilization in shrimp. No mortalities, unaffected haematological values, and no adverse histopathological alterations in stomach, intestine, and liver of fish suggested that they were in normal health (Kumar et al., 2010a; Kumar et al., 2011; Nepal et al., 2018).

It has also been fed to turkeys with no significant difference in feed intake and weight gain compared with the soy bean meal-containing diet (Boguhn et al., 2010). In pigs, the average weight gain and feed-to-gain ratio were similar for DJKM-fed groups and the SBM-based control group. In addition, the serum and haematological parameters did not differ among the groups, and values were within the normal range (PES less than 8.25 mg/kg) (Wang et al., 2011). Overall, the DJKM can replace SBM protein in fish, shrimp, turkey, and pig diets by as much as 50% (Makkar et al., 2012).

2.7. Conclusions

Jatropha is a multipurpose plant with many attributes and considerable potential due to various features, such as its high-level adaptability to environmental factors, applicability of seed oil for biofuel production, and generation of productive value-added co-products or by products. Its seeds constitute a source of proteins that are nutritionally promising and could alleviate protein malnutrition.

Marketing of the *Jatropha* kernel meal as an animal feed ingredient could considerably increase value of the *Jatropha* culture system and the global economy of *Jatropha* could be improved if non-toxic varieties are used or if the oil cake could be detoxified. The extraction and isolation of these seed proteins could be the first step to integrating them as food ingredients or additives, therefore, studies of the functional properties can provide valuable information on the potential effectiveness of them during production and processing of food products.

In order to evaluate the effectiveness of protein detoxification treatments, studies that have not detected PEs in the treated products should evaluate the limit of detection of the method used for measuring them and state their levels in the treated material accordingly. Furthermore, feeding studies with the treated material, added in sufficient amounts in the diet to replace a substantial amount of proteins from the conventional feed, and evaluation of the animal productivity responses, blood biochemistry and tissue histopathology must be conducted to ascertain the innocuous nature of the treated *Jatropha products*.

2.8. References

Achten, W.M.J., Maes, W.H., Aerts, R., Verchot, L., Trabucco, A., Mathijs, E., Singh, V.P., Muys, B., 2010. Jatropha: From global hype to local opportunity. Journal of Arid Environments 74, 164-165.

Achten, W.M.J., Verchot, L., Franken, Y.J., Mathijs, E., Singh, V.P., Aerts, R., Muys, B., 2008. Jatropha bio-diesel production and use. Biomass and Bioenergy 32, 1063-1084.

Ali, R., A. Farag, H., A. Amin, N., Farag, I., 2015. Phosphate Rock Catalyst for Biodiesel Production from Waste Frying Oil. Jokull 65(3):233-244.

Basha, S.D., Francis, G., Makkar, H.P.S., Becker, K., Sujatha, M., 2009. A comparative study of biochemical traits and molecular markers for assessment of genetic relationships between Jatropha curcas L. germplasm from different countries. Plant Science 176, 812-823.

Castro Gonzáles, N.F., 2016. International experiences with the cultivation of Jatropha curcas for biodiesel production. Energy 112, 1245-1258.

Contran, N., Chessa, L., Lubino, M., Bellavite, D., Roggero, P.P., Enne, G., 2013. State-of-the-art of the Jatropha curcas productive chain: From sowing to biodiesel and by-products. Industrial Crops and Products 42, 202-215.

Devappa, R.K., Makkar, H.P.S., Becker, K., 2010. Nutritional, Biochemical, and Pharmaceutical Potential of Proteins and Peptides from Jatropha: Review. Journal of Agricultural and Food Chemistry 58, 6543-6555.

Edrisi, S.A., Dubey, R.K., Tripathi, V., Bakshi, M., Srivastava, P., Jamil, S., Singh, H.B., Singh, N., Abhilash, P.C., 2015. Jatropha curcas L.: A crucified plant waiting for resurgence. Renewable and Sustainable Energy Reviews 41, 855-862.

Félix-Bernal, J.A., Angulo-Escalante, M.A., Estrada-Angulo, A., Heredia, J.B., Muy-Rangel, D., López-Soto, M.A., Barreras, A., Plascencia, A., 2014. Feeding value of nontoxic Jatropha curcas seed cake for partially replacing dry-rolled corn and soybean meal in lambs fed finishing diets. Animal Feed Science and Technology 198, 107-116.

Francis, G., Oliver, J., Sujatha, M., 2013. Non-toxic jatropha plants as a potential multipurpose multi-use oilseed crop. Industrial Crops and Products 42, 397-401.

Fukushima, D., 1991. Structures of plant storage proteins and their functions. Food Reviews International 7, 353-381.

Gallegos Tintoré, S., Torres-Fuentes, C., Solorza-Feria, J., Alaiz, M., Girón Calle, J., Martinez-Ayala, A., Chel Guerrero, L., Vioque, J., 2015. Antioxidant and Chelating Activity of Nontoxic Jatropha curcas L. Protein Hydrolysates Produced by In Vitro Digestion Using Pepsin and Pancreatin . Journal of Chemistry, 1-9.

Gofferjé, G., Zöttl, A., Stäbler, A., Herfellner, T., Schweiggert-Weisz, U., Flöter, E., 2015. Influence of Protein Extraction Techniques of Different De-oiled Residues from Jatropha curcas L. on Protein Recovery and Techno-functional Properties. Waste and Biomass Valorization 6, 225-235.

Hamarneh, A.I., Heeres, H.J., Broekhuis, A.A., Picchioni, F., 2010. Extraction of Jatropha curcas proteins and application in polyketone-based wood adhesives. International Journal of Adhesion and Adhesives 30, 615-625.

He, W., King, A.J., Khan, M.A., Cuevas, J.A., Ramiaramanana, D., Graham, I.A., 2011. Analysis of seed phorbol-ester and curcin content together with genetic diversity in multiple provenances of Jatropha curcas L. from Madagascar and Mexico. Plant Physiology and Biochemistry 49, 1183-1190.

Heller, Heller, J., Engels, J., 1996. Physic nut. Jatropha curcas L. International Plant Genetic Resources Institute.

Jongschaap, R.E.E., Corré, W.J., Bindraban, P.S., Brandenburg, W., 2007. Claims and facts on Jatropha curcas L. Global Jatropha curcas evaluation, breeding and propagation programme. Plant Research International. B.V.WageningenStichting Het GroeneWoudt, Laren, Report. 2007;158.

Jorrin-Novo, J., 2014. Plant Proteomics Methods and Protocols. Methods in Molecular Biology. ed. Springer.

Kamel, D.A., Farag, H.A., Amin, N.K., Zatout, A.A., Ali, R.M., 2018. Smart utilization of jatropha (Jatropha curcas Linnaeus) seeds for biodiesel production: Optimization and mechanism. Industrial Crops and Products 111, 407-413.

King, A.J., He, W., Cuevas, J.A., Freudenberger, M., Ramiaramanana, D., Graham, I.A., 2009. Potential of Jatropha curcas as a source of renewable oil and animal feed. Journal of Experimental Botany 60, 2897-2905.

Koh, M.Y., Mohd. Ghazi, T.I., 2011. A review of biodiesel production from Jatropha curcas L. oil. Renewable and Sustainable Energy Reviews 15, 2240-2251.

Kumar, A., Sharma, S., 2008. An evaluation of multipurpose oil seed crop for industrial uses (Jatropha curcas L.): A review. Industrial Crops and Products 28, 1-10.

Kumar, V., Makkar, H.P.S., Amselgruber, W., Becker, K., 2010a. Physiological, haematological and histopathological responses in common carp (Cyprinus carpio L.)

fingerlings fed with differently detoxified Jatropha curcas kernel meal. Food and Chemical Toxicology 48, 2063-2072.

Kumar, V., Makkar, H.P.S., Becker, K., 2010b. Dietary inclusion of detoxified Jatropha curcas kernel meal: effects on growth performance and metabolic efficiency in common carp, Cyprinus carpio L. Fish Physiology and Biochemistry 36, 1159-1170.

Kumar, V., Makkar, H.P.S., Becker, K., 2011. Detoxified Jatropha curcas kernel meal as a dietary protein source: growth performance, nutrient utilization and digestive enzymes in common carp (Cyprinus carpio L.) fingerlings. Aquaculture Nutrition 17, 313-326.

León-López, L., Dávila-Ortiz, G., Jimenez Martinez, C., Hernandez, H., 2013. Sequentially Integrated Optimization of the Conditions to Obtain a High-Protein and Low-Antinutritional Factors Protein Isolate from Edible Jatropha curcas Seed Cake. ISRN Biotechnology, vol. 2013, Article ID 197201, 7 pages, 2013.

Lestari, D., Mulder, W.J., Sanders, J.P.M., 2011. Jatropha seed protein functional properties for technical applications. Biochemical Engineering Journal 53, 297-304.

Maes, W.H., Trabucco, A., Achten, W.M.J., Muys, B., 2009. Climatic growing conditions of Jatropha curcas L. Biomass and Bioenergy 33, 1481-1485.

Makkar, H.P.S., 2016. State-of-the-art on detoxification of Jatropha curcas products aimed for use as animal and fish feed: A review. Animal Feed Science and Technology 222, 87-99.

Makkar, H.P.S., Aderibigbe, A.O., Becker, K., 1998. Comparative evaluation of non-toxic and toxic varieties of Jatropha curcas for chemical composition, digestibility, protein degradability and toxic factors. Food Chemistry 62, 207-215.

Makkar, H.P.S., Becker, K., Sporer, F., Wink, M., 1997. Studies on Nutritive Potential and Toxic Constituents of Different Provenances of Jatropha curcas. Journal of Agricultural and Food Chemistry 45, 3152-3157.

Makkar, H.P.S., Kumar, V., Oyeleye, O.O., Akinleye, A.O., Angulo-Escalante, M.A., Becker, K., 2011. Jatropha platyphylla, a new non-toxic Jatropha species: Physical properties and chemical constituents including toxic and antinutritional factors of seeds. Food Chemistry 125, 63-71.

Mandal, S., K. Mandal, R., 2000. Seed storage proteins and approaches for improvement of their nutritional quality by genetic engineering. Current Science, 79(5), 576-589.

Martínez-Herrera, J., Siddhuraju, P., Francis, G., Dávila-Ortíz, G., Becker, K., 2006. Chemical composition, toxic/antimetabolic constituents, and effects of different treatments on their levels, in four provenances of Jatropha curcas L. from Mexico. Food Chemistry 96, 80-89.

Meher, L.C., Churamani, C.P., Arif, M., Ahmed, Z., Naik, S.N., 2013. Jatropha curcas as a renewable source for bio-fuels—A review. Renewable and Sustainable Energy Reviews 26, 397-407.

Nath, L.K., Dutta, S.K., 1991. Extraction and Purification of Curcain, a Protease from the Latex of Jatropha curcas Linn. Journal of Pharmacy and Pharmacology 43, 111-114.

Nepal, S., Kumar, V., Makkar, H.P.S., Stadtlander, T., Romano, N., Becker, K., 2018. Comparative nutritional value of Jatropha curcas protein isolate and soy protein isolate in common carp. Fish Physiology and Biochemistry 44, 143-162.

Osborne, T.B., 1924. The vegetable proteins. By Thomas B. Osborne. Pp. xiii+154. 2nd edition. Monographs on Biochemistry. London: Longmans green and Co. 1924. Pric 9s. net. Journal of the Society of Chemical Industry 43, 440-440.

Parawira, W., 2010. Biodiesel production from Jatropha curcas: A review. Scientific Research and Essays Vol. 5(14), pp. 1796-1808.

Pasupuleti, V., Holmes, C., Demain, A., 2010. Applications of Protein Hydrolysates in Biotechnology in: Pasupuleti, V. K., Demain, A.L. (Eds.), Protein Hydrolysates in Biotechnology. Springer Netherlands, pp 1-9.

Peralta-Flores, L., Gallegos-Tintoré, S., Solorza-Feria, J., Dávila-Ortíz, G., Chel-Guerrero, L., Martinez-Ayala, A., 2012. Biochemical evaluation of protein fractions from physic nut (Jatropha curcas L.). Grasas y Aceites, [S.l.], v. 63, n. 3, p. 253-259.

Sabandar, C.W., Ahmat, N., Jaafar, F.M., Sahidin, I., 2013. Medicinal property, phytochemistry and pharmacology of several Jatropha species (Euphorbiaceae): A review. Phytochemistry 85, 7-29.

Saetae, D., Kleekayai, T., Jayasena, V., Suntornsuk, W., 2011. Functional properties of protein isolate obtained from physic nut (Jatropha curcas L.) seed cake. Food Science and Biotechnology 20, 29-37.

Saetae, D., Suntornsuk, W., 2012. Functional properties of proteins isolated from phorbol ester-free physic nut (Jatropha curcas L.) seed cake. Food Science and Biotechnology 21, 1179-1185.

Saikia, S.P., Gogoi, A., Mudoi, K.D., Goswami, A., Bora, D., 2012. Assessment of the Potential of Jatropha curcas for Energy Production and Other Uses, in: Carels, N., Sujatha, M., Bahadur, B. (Eds.), Jatropha, Challenges for a New Energy Crop: Volume 1: Farming, Economics and Biofuel. Springer New York, New York, NY, pp. 299-310.

Sanders, J., Scott, E., Weusthuis, R., Mooibroek, H., 2007. Bio-Refinery as the Bio-Inspired Process to Bulk Chemicals. Macromol. Biosci. 2007, 7 (2) 105– 117.

Segura-Campos, M.R., Peralta-Gonz, #xe1, lez, F., Castellanos-Ruelas, A., Chel-Guerrero, L.A., Betancur-Ancona, D.A., 2013. Effect of Jatropha curcas Peptide Fractions on the Angiotensin I-Converting Enzyme Inhibitory Activity. BioMed Research International 2013, 8.

Semwal, S., Arora, A.K., Badoni, R.P., Tuli, D.K., 2011. Biodiesel production using heterogeneous catalysts. Bioresource Technology 102, 2151-2161.

Shewry, P.R., Napier, J.A., Tatham, A.S., 1995. Seed storage proteins: structures and biosynthesis. The Plant Cell 7, 945-956.

Staubmann, R., Ncube, I., Gübitz, G.M., Steiner, W., Read, J.S., 1999. Esterase and lipase activity in Jatropha curcas L. Seeds. Journal of Biotechnology 75, 117-126.

Stirpe, F., Pession, A., Lorenzoni, E., Strocchi, P., Montanaro, L., Sperti, S., 1976. Studies on the proteins from seeds of Croton tiglium and of Jatropha curcas. Toxic properties and inhibition of protein synthesis in vitro. Biochemical Journal. 156(1) pp.1-6.

Sudheer Pamidimarri, D.V.N., Singh, S., Mastan, S.G., Patel, J., Reddy, M.P., 2009. Molecular characterization and identification of markers for toxic and non-toxic varieties of Jatropha curcas L. using RAPD, AFLP and SSR markers. Molecular Biology Reports 36, 1357-1364.

Sujatha, M., Makkar, H.P.S., Becker, K., 2005. Shoot Bud Proliferation from Axillary Nodes and Leaf Sections of Non-toxic Jatropha curcas L. Plant Growth Regulation 47, 83-90.

Valdés Rodríguez, O., Vázquez, A., Muñoz Gamboa, C., 2014. Drivers and Consequences of the First Jatropha curcas Plantations in Mexico. Sustainability 6, 3732.

Valdez-Flores, M., Germán-Báez, L.J., Gutiérrez-Dorado, R., Medina-Godoy, S., Norzagaray-Valenzuela, C., Hernández-Verdugo, S., Reyes-Moreno, C., Valdez-Ortiz, A., 2016. Improving bioactivities of Jatropha curcas protein hydrolysates by optimizing with response surface methodology the extrusion cooking process. Industrial Crops and Products 85, 353-360.

Vera-Castillo, Y.B., Cuevas, J.A., Valenzuela-Zapata, A.G., Urbano, B., González-Andrés, F., 2014. Biodiversity and indigenous management of the endangered non-toxic germplasm of Jatropha curcas L. in the Totonacapan (Mexico), and the implications for its conservation. Genetic Resources and Crop Evolution 61, 1263-1278.

Wang, H., Chen, Y., Zhao, Y.n., Liu, H., Liu, J., Makkar, H.P.S., Becker, K., 2011. Effects of replacing soybean meal by detoxified Jatropha curcas kernel meal in the diet of growing pigs on their growth, serum biochemical parameters and visceral organs. Animal Feed Science and Technology 170, 141-146.

Wang, M.-W., Hao, X., Chen, K., 2007. Biological screening of natural products and drug innovation in China. Philosophical Transactions of the Royal Society B: Biological Sciences 362, 1093-1105.

Wei, Q., Liao, Y., Chen, Y., Wang, S.H., Xu, Y., Tang, L., Chen, F., Eloff, J.N., 2005. Isolation, characterisation and antifungal activity of β-1,3-glucanase from seeds of Jatropha curcas. South African Journal of Botany 71, 95-99.

Xiao, J., Zhang, H., Niu, L., 2015. Effect of Detoxification on Conformational and Functional Properties of Jatropha curcas Proteins. International Journal of Food Properties 18, 1524-1534.

Zhang, F.-L., Niu, B., Wang, Y.-C., Chen, F., Wang, S.-H., Xu, Y., Jiang, L.-D., Gao, S., Wu, J., Tang, L., Jia, Y.-J., 2008. A novel betaine aldehyde dehydrogenase gene from Jatropha curcas, encoding an enzyme implicated in adaptation to environmental stress. Plant Science 174, 510-518.

Chapter 3

Proteomic analysis of non-toxic *Jatropha curcas* byproduct cake: Fractionation and identification of the major components.

This chapter has been published as: León-Villanueva A., Huerta-Ocampo J.A., Barrera-Pacheco A., Medina-Godoy S., Barba de la Rosa A., Proteomic analysis of non-toxic *Jatropha curcas* byproduct cake: Fractionation and identification of the major components, Industrial Crops & Products, 111(2048)694-704.

Abstract

Jatropha curcas non-toxic genotypes have been reported in Mexico and the press-cake, after oil extraction, represents a potential of new source of protein for food and feed uses. However, the characterization of the press-cake proteins is still unknown. The aim of this work was to carry out the molecular characterization of *J. curcas* seed storage proteins. Proteins in press-cake were pre-fractionated according to the classical Osborne procedure. Main protein fraction in *J. curcas* cake was represented by glutelins, the electrophoretic analysis showed that glutelins and globulins have the same profile, indicating that oil extraction process could have effect on globulins agglomeration. Protein fractions were analysed by two-dimensional gel electrophoresis and mass spectrometry, results provide a new dataset of protein species or proteoforms that are accumulated in *J. curcas* endosperm. The identification of toxic proteins such as curcin in the non-toxic variety could represent that this protein has important roles in seeds. Regulatory proteins such as proteasome subunits and 14-3-3 were identified. A group of different heat shock and stress defence protein species was detected. Proteases related with inhibitory activity against DPPIV were also detected; this could support the potential use of *J. curcas* cake as nutraceutical food.

Keywords: *Jatropha curcas*; LC-MS/MS; mass spectrometry; non-toxic; proteome; seed storage proteins.

3.1. Introduction

Jatropha curcas L. is a stem-succulent tree native to America, which as *Ricinus* and *Manihot* belongs to the family *Euphorbiaceae*. *J. curcas* was spread by Portuguese sailors to Africa and Asia (Fairless, 2007; Maghuly and Laimer, 2013). This subtropical plant can grow through different climatic and soil conditions, it has several uses in different agricultural systems such as barriers against wind and soil erosion as well as a source of firewood (Dias et al., 2012; Maghuly and Laimer, 2013). *J. curcas* main attraction is due to its high potential as plant for biodiesel production (Maghuly and Laimer, 2013). *J. curcas* seeds contains 30-45% toxic oil, with a high amount of oleic and linoleic acids which make it more suitable for fuel purposes, as compared with other vegetable oils, due to its high rate fuel consumption and its higher oxidation stability (Fairless, 2007; Gübitz et al., 1999; Openshaw, 2000; Pramanik, 2003). *J. curcas* oil has therefore been used for long time as a raw material for paints and soap production as well as for lamp and lubricating oils (Kumar and Sharma, 2008). Besides, different parts of *J. curcas* plant contain a range of interesting metabolites and bioactive compounds, which is taking great attention for research as medicinal plant (Sabandar et al., 2013).

The mature seeds of *J. curcas* have a tick endosperm representing more than 90% of their weight in which is embedded a small embryo (Liu et al., 2009). The seeds are rich on protein (25-30%) and oil (55-62%), values that depends on the region agroclimatic characteristics. After oil extraction from dehulled kernels, the oil is converted into biodiesel and the resulting press-cake is a rich source of protein (60-63%) that could be an excellent protein source. The proteins present in the press-cake are rich of essential amino acids even with higher values (except lysine) than those reported by the Food and Agriculture Organization reference protein (Haas and Mittelbach, 2000; Martinez-Herrera et al., 2012). However, due to the highly toxic and anti-nutritional compounds presents in *J. curcas* seeds, make the press-cake and oil unsuitable for the use as feedstuff or for human consumption (Maghuly and Laimer, 2013).

In Mexico, *J. curcas* L. grows wild in semitropical and tropical climates, however only Mexico has reported a nontoxic *J. curcas* genotype (He et al., 2011; Perea-Domínguez et al., 2017). The seeds of this nontoxic genotype are traditionally used to prepare a range of traditional dishes in Veracruz, Puebla, and Hidalgo States of Mexico (Makkar and Becker, 2009; Martinez-Herrera, 2012).

The exploitation of *J. curcas* press-cakes has been limited by the little knowledge about some aspects of the biochemistry of these seeds such as the type and functionality of proteins concentrated in the press-cake. Therefore, it is necessary to generate information on these aspects that would help to design innovative biotechnological approaches for the use of the high amount of proteins present in press-cake for future applications in the food industry.

The availability of the *J. curcas* genome and transcriptome data reported in the public databases (Costa et al., 2010; King et al., 2011) have allowed the increase of studies toward the seed proteome characterization. Shah et al. (2015) have used the label-free quantitative proteome analysis in order to analyse the proteins presents in the whole *J. curcas* seed endosperm in searching for information on phorbol esters biosynthesis, mechanism which still is not elucidated. Pinheiro et al. (2013) and Soares et al. (2014) focused in the proteomic analysis of the inner integument from developing seeds, while Shah et al. (2016) carried out the proteome analysis on gerontoplasts isolated from the inner integument of developing seeds. Liu et al. (2015) carried out the proteomics analysis of oil body associated protein species by using gels-based proteomic technique, whereas Liu et al. (2013) used the comparative proteomic approach to profile the protein changes during seed development.

However, to date there is no information about the physical properties of *J. curcas* seed storage proteins presents in press-cakes. It is well known that the functional and molecular characterization of protein-enriched fractions is the first step in designing strategies that allow them to be integrated as additives or food ingredients (Rezig et al., 2013). In this sense, Osborne´s protein classification (Osborne, 1908), which is based on protein solubility characteristics, is the most common method for seed storage protein characterization and seems to be an excellent pre-fractionation step towards proteome characterization. For these reasons the aim of the present work was to carry out the characterization of proteins present in press-cake obtained after oil extraction of non-toxic *J. curcas*.

Gel-based proteomics and LC-MS/MS were used in order to obtain a new description of *J. curcas* press-cake proteins; such valuable information will help in the way to design technologies for development of new food products.

3.2. Material and methods

3.2.1. *J. curcas* press-cake preparation

Non-toxic *Jatropha curcas* seeds (Puebla's ecotype, voucher specimen numbered 53203) were obtained from ripe fruits from plants cultivated in "La Esmeralda" Experimental Field of the Agricultures Association of West Sinaloa River (AARSP, Guasave, Mexico). *J. curcas* seed press cake was produced after oil pressing from dehulled seeds (kernels) using a screw-press. Two press cake samples (500 g each) were collected and air-dried under ambient conditions. The combined air-dried press cakes were milled to a small particle size (40-mesh) using a Cyclotec 1093 mill (FOSS Tecator, Hilleroed, Denmark) and then defatted with n-hexane (1:10, w/v) for 4 h with agitation and air-dried in a fume hood after decantation of the hexane. Dry meals were kept in plastic bags at 4°C until used.

3.2.2. Meal protein sequential fractionation based on Osborne´s solubility

Protein fractions were sequentially extracted according to a modified Osborne´s procedure (Ribeiro et al., 2004). Briefly, the defatted meal was extracted with water containing 10 mM CaCl2 and 10 mM MgCl2 (1:10, w/v) pH 8.0, with constant stirring for 4 h at 4°C. The slurry was centrifuged at 30,000g for 1 h at 4°C. The supernatant was recovered and extensively dialyzed (12.4 kDa MW cut-off) against distilled water. After dialysis, the suspension was centrifuged at 15,000g for 15 min at 4°C and supernatant (albumins) was collected and freeze-dried.

The resulting pellet from albumins extraction was resuspended with 100 mM Tris-HCl, pH 7.5, containing 10% NaCl (w/v), 10 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM ethylene glycol-bis (2-aminoethylether)-N, N, N', N'-tetraacetic acid (EGTA) (1:10, w/v). The suspension was stirred for 4 h at 4°C. The insoluble proteins were removed by centrifugation at 30,000g for 1 h at 4°C. The precipitate (globulins fraction) was dialyzed, centrifuged and freeze-dried.

The insoluble pellet resulting from globulins extraction was resuspended in 75% aqueous ethanol (1:10, w/v) and centrifuged (30,000g at 4°C for 15 min). The supernatant was dialyzed against distilled water. The supernatant (prolamins) was recovered and freeze-dried. The insoluble pellet from the previous extraction was resuspended (1:10, w/v) with 50 mM sodium borate buffer, pH 10, containing 1% (v/v) β-mercaptoethanol, and 1% (w/v) sodium dodecyl sulphate (SDS). The suspension was stirred at room temperature for 2 h and centrifuged at 30,000g for 15 min at 20°C. After dialysis against distilled water, the fraction (glutelins) was freeze-dried. All samples were kept at -70°C until use. A scheme of the whole extraction procedure is presented in Supplementary Fig. S1.

3.2.3. Protein sample preparation

Freeze-dried protein fractions (five grams) were mixed with 50 mL of its respective extraction solution and 1% (w/v) of polyvinylpolypyrrolidone (PVPP). The suspensions were mixed in a vortex for 1 min and sonicated for 2.5 min at 20 kHz with 35% of amplitude (GE-505, Ultrasonic Processor, Sonics & Materials, Inc., Newtown, CT, USA). Samples were maintained at 4°C during suspension steps and centrifuged at 15,000g for 15 min at 4°C (Beckman Avanti J26-XP, Beckman Coulter, Brea, CA, USA). Supernatants were transferred to new tubes and proteins were precipitated by adding four volumes of ice-cold acetone and incubated overnight at -20°C. After 15 min of centrifugation at 15,000g for 15 min at 4°C, the supernatant was decanted and discarded, with the residual pellet being washed twice with ice-cold acetone and allowed to dry at room temperature. The resulting dried pellets were suspended in rehydration buffer (8 M urea, 2% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 0.56% (w/v) dithiothreitol (DTT), 0.002% bromophenol blue) mixed in vortex for 30 s and sonicated for 80 s. The suspensions were centrifuged under the previous conditions and the supernatants were recovered. Protein

concentration was determined by using protein assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) used as standard.

3.2.4. Two-dimensional gel electrophoresis (2-DE) and gel image analysis

Proteins (900 µg) were suspended in 450 µL of rehydration buffer, containing 0.5% (v/v) IPG buffer (Bio-Rad) in the range of 5-8 (albumins) and 3-10 for globulins and glutelins fractions. Proteins were loaded onto 24 cm linear immobilized pH gradient (IPG, Bio-Rad) strips 5-8 (albumins) or 3-10 (globulins and glutelins). Passive rehydration was carried out at room temperature for 14-16 h. The IEF was conducted at 50 mA per IPG strip and 20°C in an Ettan IPGphor 3 system (GE Healthcare, Piscataway, NJ, USA) under the following conditions: (I) 150 V gradient for 2 h (II) 200 V gradient for 2 h, (III) 400 V gradient for 2 h, (IV) 1500 V gradient for 2 h, (V) 4500 V gradient for 3 h, (VI) 10,000 V gradient for 3 h; and (VII) holding at 10,000 V for 10 h. After IEF, the IPG strips were stored at -20°C or immediately equilibrated for 15 min in equilibration buffer [50 mM Tris–HCl pH 8.8, 6 M Urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue, 65 mM DTT]. Strips were placed directly onto 13% polyacrylamide-SDS slab gels and the second dimension was performed using an EttanTM DALT-six Electrophoresis Unit (GE Healthcare), using SDS electrophoresis buffer [25 mM Tris pH 8.8, 192 mM glycine, and 0.1% (w/v) SDS] and resolved at 20 mA/gel until the dye (bromophenol blue) reached the bottom of the gels. Three different extractions were prepared for gel replicates using the same method. After SDS-PAGE gels were stained with PhastGelTM Blue R-350 (GE Healthcare) and scanned at 100 µm resolution using a Pharos FX Plus Molecular Imager (Bio-Rad). Image analysis was performed with PDQuest 2-D Analysis Software v8.0 (Bio-Rad). The molecular masses of proteins in gels were determined by co-electrophoresis of molecular weight standards (BenchMark Protein Ladder, Invitrogen. Carlsbad, CA, USA), while isoelectric point (p*I*) was determined by migration of protein spots on the linear IPG strips.

3.2.5. Liquid chromatography-tandem mass spectrometry analyses (LC-MS/MS)

Protein spots were carefully excised from the 2-DE gels and distained, reduced with 10 mM DTT in 25 mM ammonium bicarbonate followed by protein alkylation with 55 mM iodoacetamide. Protein digestion was carried out overnight at 37°C with sequencing grade trypsin (Promega, Madison, WI, USA). Nanoscale LC separation of tryptic peptides was performed with a nanoACQUITY UPLC System (Waters, Milford, MA, USA) and LC-MS/MS analysis was carried out in a SYNAPT-HDMS Q-TOF (Waters) as previously reported (Huerta-Ocampo et al., 2014) with brief modifications: Accurate mass data were collected in an alternating Data Dependent Acquisiton mode (DDA). In low energy mode, data were collected at constant collision energy of 3 eV. In elevated-energy mode, the collision energy was ramped from 15 to 45 eV during 3 s of integration.

3.2.6. Protein identification

MS/MS spectra datasets were used to generate PKL files (.pkl) using ProteinLynx Global Server v2.4 (Waters). Proteins were then identified using PKL files and the MASCOT search engine v2.3 (Matrix Science, London, UK). Searches were conducted using the *Viridiplantae* subset of the NCBInr protein database (2391213 sequences, December 2013) and an in-house database containing the *Jatropha curcas* nucleotide coding sequences available at the NCBI database (77340 sequences, December 2013). Trypsin was used as the specific protease and one missed cleavage was allowed. The mass tolerance for precursor and fragment ions was set to 100 ppm and 0.1 Da respectively and peptide charges were set at +2, +3, and +4. Carbamidomethylation of cysteine was set as fixed modification and oxidation of methionine was set as variable modification. Identifications were considered successful when significant MASCOT scores (>33 for *J. curcas* nucleotide in-house and >51 for *Viridiplantae* protein database) were obtained, indicating the identity or extensive homology at $p<0.01$ and the presence of at least two peptides were considered necessary for reliable identification. Identified proteins were classified into different categories to Gene Ontology (http://www.geneontology.org/). A biological pathway diagram showing the identified enzymes involved in a metabolic network was generated using the visualization and analysis tool PathVisio v3.2.4 (Kutmon et al., 2015).

3.3. Results

3.3.1 Protein extraction

Although Osborne´s classification based on solubility criteria is an ambiguous system, due that all depends in solutions used for extractions, is the most useful method for seed storage protein characterization and is a very useful method for protein pre-fractionation towards seeds storage proteins proteomics analysis (Jorrin-Novo, 2014). Hence, the proteins in press-cake *J. curcas* were subjected to sequential extraction. It was observed that globulins fraction corresponds only to 20.17%, while glutelins represented the main fraction with 42.03% (Supplementary Table S1), which is different to all legumes where the main fraction is globulins. The representative electrophoretic profile in one-dimension gel (SDS-PAGE) of different *J. curcas* press-cake protein fractions is shown in **Figure 4A**.

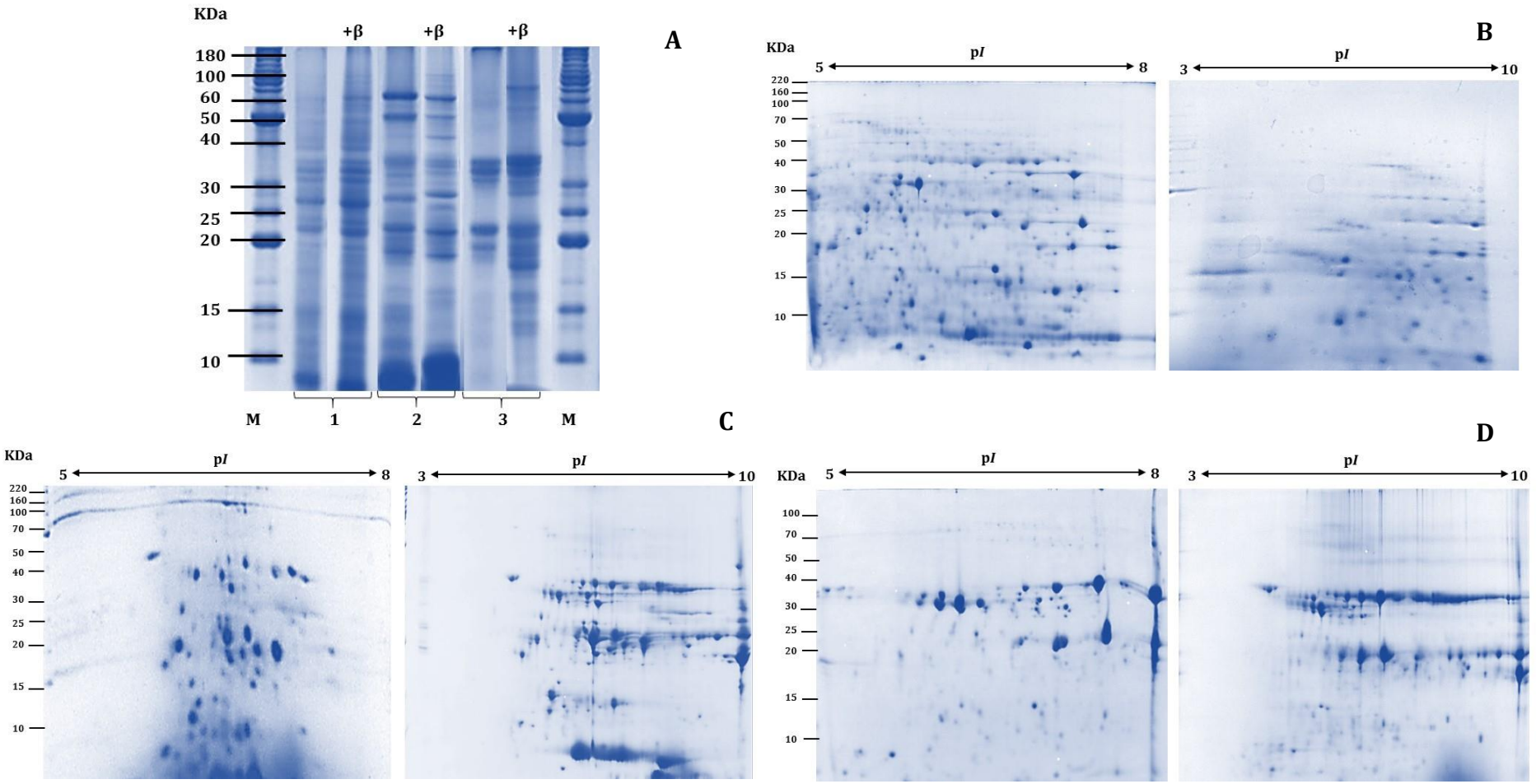

Figure 4. A) One-dimension electrophoresis profile of *Jatropha curcas* seed storage proteins. Lines M= molecular weight markers, Lines 1= albumins, Lines 2=globulins, Lines 3= glutelins. Representative 2-DE gels of *J. curcas* seed storage proteins for **B)** albumins; **C)** globulins; **D)** glutelins.

3.2.2-DE maps of seed protein fractions

The water-soluble protein fraction (albumins) was separated by 2-DE in the range of linear pH 5-8, while the salt soluble proteins (globulins) and glutelins were resolved in the range of linear pH 3-10. In the albumins fraction 434 spots were separated in 2-DE gels (**Figure 4B**, Supplementary Figure S2), while 310 spots for globulins (**Figure 4C**, Supplementary Figure S3) and 175 spots for glutelins (**Figure 4D**, Supplementary Figure S3) were resolved. From 84.1 to 96.0% of the protein spots from the three seed storage protein fractions were successfully identified by LC-MS/MS analysis and homology database search (**Table 3**). All identified proteins were classified into its functional classes. In albumins fraction were identified more than 100 protein species associated with well-known storage reservoir (**Figure 5**). Enzymes involved in glycolysis, TCA cycle. Enzymes related with sugars, nitrogen, and lipid metabolisms were also represented, as well proteins related with oxide-reduction function (SOD, GST). Proteasome subunits, 14-3-3 proteins, and enzymes related with defence and detoxification (LEA, glyoxalase, cyclophilin, and lactoylglutathione lyase) were also identified. Several heat shock proteins and proteases were identified (SERPIN-ZX-like, cysteine proteinase, leucine aminopeptidase). Interestingly in albumins fraction were detected enzymes related with the THF pathway and purine metabolism that have not been reported before (Table 2, Supplementary File S2).

Globulins and glutelins fractions showed 24 and 7 protein species, respectively (**Figure 5**) with several proteoforms representing each protein species. Globulins fraction was composed mainly by legumin B-like, 2S albumin-like and germin-like protein, curcin 1 and enzymes related to energy production such as ATP synthase and luminal-binding protein (Table 3 and Supplementary File S3). Glutelins, although the most abundant fraction in *J. curcas* press-cake was composed mainly of high molecular weight globulins (**Table 4**, Supplementary File S4). In Supplementary File S5 is shown the list of theoretical and experimental molecular weight/isoelectric point corresponding to all identified proteins.

Table 3. Protein identification of *Jatropha curcas* seed storage protein fractions (albumins, globulins and glutelins) by LC–MS/MS and homology database search.

Fraction	Spots	Identified (%)	Non-identified (%)	Unique
Albumins	434	365 (84.1)	69 (15.9)	100
Globulins	310	296 (95.5)	14 (4.5)	24
Glutelins	175	168 (96.0)	7 (5.0)	7

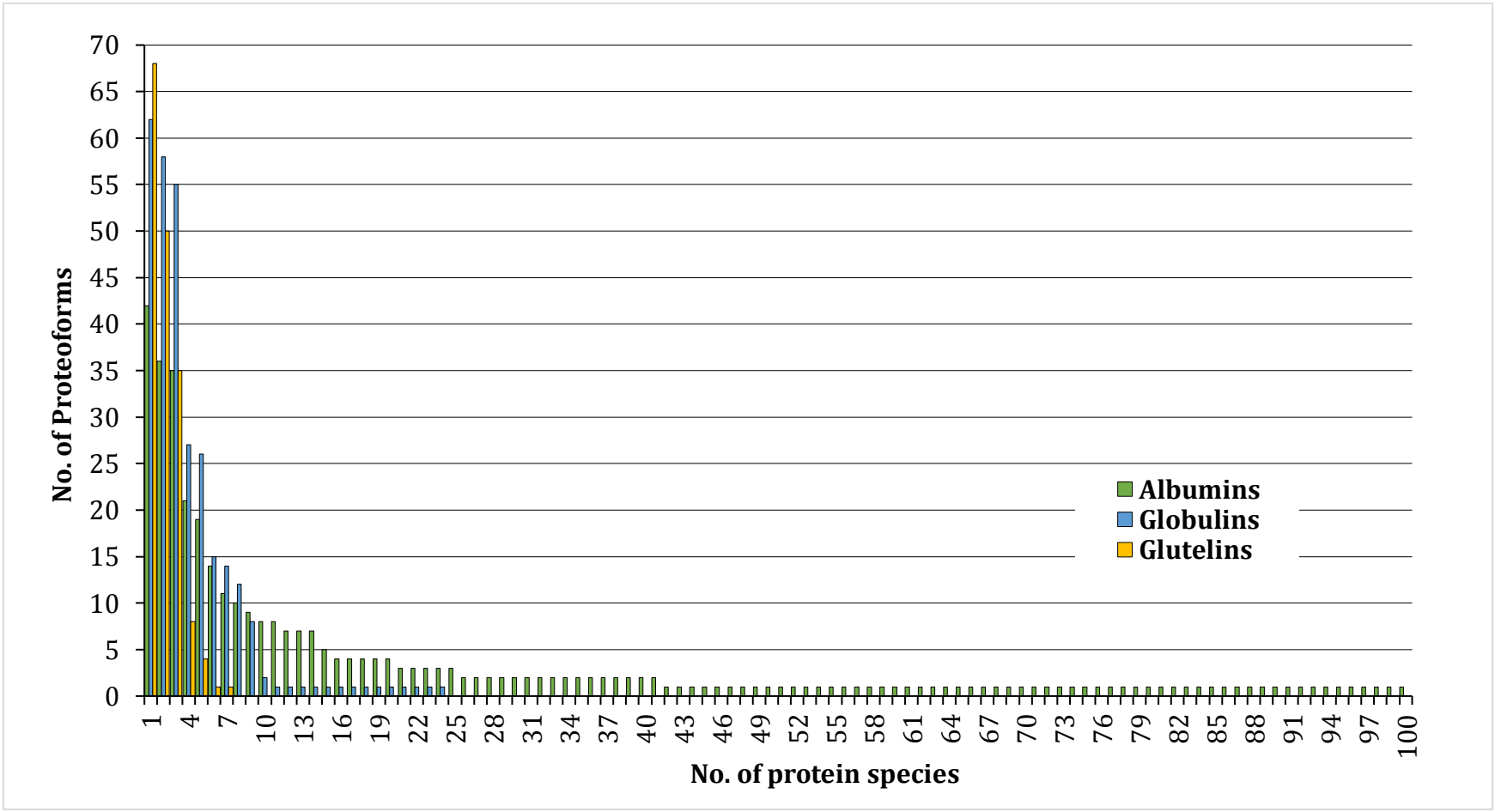

Figure 5. Diagram indicating the number of proteoforms from different protein species detected in *Jatropha* seed storage proteins (albumins, globulins, glutelins).

3.4. Discussion

3.4.1. *J. curcas* seeds protein fractionation

It is recognized that the total set of protein species and their corresponding proteoforms (frequently abundant) that constitute the cell´s proteome could not be captured in just one experiment (Romero-Rodríguez et al., 2014). Therefore, the use of fractionation techniques is necessary in order to obtain a more complete proteome characterization. Osborne´s method has been long used for seed protein fractionation and is still one of the preferred procedures for protein pre-fractionation before proteomics profiling of seeds.

The high lipid content in seeds turns *J. curcas* into a high value crop, however the press-cake obtained from non-toxic *J. curcas* has the potential to be used as source of food. In this sense, seed protein storage proteins are the basis for seed protein characterization; hence the seed storage protein fractions in *J. curcas* non-toxic seeds were characterized.

Most of enzymatic proteins identified were found in albumins fraction (water soluble-proteins). The network of metabolic activities of identified enzymes is represented in **Figure 6**. Globulins and glutelins were represented by nutrient reservoir proteins (**Table 5 and 6**).

3.4.2. Protein species in albumins fraction

3.4.2.1. Protein species related to nutrient reservoir

Several proteoforms of legumin B and legumin A were detected (45 and 36, spots, respectively). 11S globulin was detected in 21 spots and globulin-1 S in 9 spots (**Table 4**). Globulin 1 (GLB1) is one of the most abundant proteins accumulated in maize seed tissues and has been designated as an excellent indicator for the scrutiny of genetic variation (Hilton and Gaut, 1998). Two isoforms of vicilin-like antimicrobial peptides 2-2 (AMPs) were detected in 88 spots while 38 spots corresponded to 2S-albumins (Table 2). Antimicrobial peptides have been frequently isolated from seeds among other plant tissues (Franco et al., 2006; Lipkin et al., 2005; Pelegrini et al., 2006 and 2008). It has been reported that *J. curcas* is a good source of cyclic peptides with diverse biological functions such as antimalarial, antiplatelet, antiproliferative, and cytotoxic (Pinto et al., 2015). Xiao et al. (2011) reported the JCpep7 peptide that displayed antimicrobial activity against Gram-negative pathogens like Shigella dysenteriae, Pseudomonas aeruginosa and Salmonella typhimurium. These bioactive peptides found in *J. curcas* could contribute in the development of antimicrobial strategies based in the use of novel natural sources (Mandal et al., 2009).

One LEA protein (Em-like) was detected in 2 spots. LEAs (Late embryogenesis abundant proteins) are widely recognized as associated with desiccation tolerance in

Table 4. Identification by LC/MS–MS of albumins fraction proteins extracted from non-toxic *J. curcas* meal.

Protein name[a]	Spots[b]	*J. curcas* number[c]	NCBI accession number[d]	Theor. kDa/p*I*[e]	Query Cover. /E-value[f]	Ident[g]
Nutrient Reservoir						
Legumin B-like precursor	42	Jcr4S00279.60	NP_001295688.1	57.5/6.75	100/0	100
Legumin B-like precursor	3	Jcr4S00279.80	NP_001295689.1	50.7/9.46	100/0	93
Legumin A-like	36	Jcr4S01636.60	XP_012085316.1	55.1/6.38	100/0	100
11S globulin seed storage protein 2-like	21	Jcr4S01636.40	XP_012085320.1	54.0/ 8.77	95/0	100
Globulin-1 S allele	2	Jcr4S03153.60	XP_012079069.1	78.2/5.38	66/0	100
Vicilin-like antimicrobial peptides 2-2	19	Jcr4S00603.20	XP_012064865.1	56.8/6.04	$94/2^{e-128}$	93
Vicilin-like antimicrobial peptides 2-2	1	Jcr4S03723.20	XP_012092200.1	50.7/6.04	100/0	86
2S albumin	35	Jcr4S00619.70	XP_012070746.1	16.2/5.53	$100/2^{e-96}$	100
2S albumin-like	1	Jcr4S00619.40	XP_012071216.1	16.2/8.38	$100/2^{e-97}$	100
2S albumin-like	1	Jcr4S00619.60	XP_012071217.1	16.6/6.81	$100/1^{e-81}$	100
2S albumin precursor, putative	1	Jcr4S00619.50	XP_012070745.1	16.4/6.88	$100/1^{e-100}$	100
Cupin 2	1	Jcr4S15278.20	XP_012079069.1	70.1/5.61	68/0	100
Em-like protein, protein SLE2	2	Jcr4S06850.20	XP_012087069.1	9.8/5.50	$100/2^{e-60}$	100
Curcin, 1Ribosome-inactivating protein cucurmosin-like precursor,	8	Jcr4S01069.20	NP_001295744.1	50.9/8.90	100/0	100
Glycolysis, TCA cycle						
Glyceraldehyde-3-P DH, cytosolic	14	Jcr4S00100.140	XP_012079890.1	44.5/7.05	$82/9^{e-140}$	99
Fructose-bisphospahte aldolase cytoplasmic	11	Jcr4S14120.10	XP_012066441.1	29.4/7.75	100/0	100
Enolase	8	Jcr4S00171.20	XP_012090397.1	52.3/5.71	100/0	100
Enolase-like	1	Jcr4S01799.10	XP_012090393.1	46.9/8.0	100/0	100

Enolase 1, chloroplastic	1	Jcr4S01892.10	XP_012092922.1	44.0/5.62	100/0	100
Triosephosphate isomerase, chloroplastic	1	Jcr4S02839.20	XP_012084383.1	41.6/7.60	100/0	100
Triosephosphate isomerase, cytosolic	1	Jcr4S19239.10	XP_012075954.1	36.5/8.07	100/0	100
2,3-bisphosphoglycerate-independent phosphoglycerate mutase	7	Jcr4S00101.20	XP_012090969.1	61.5/ 5.86	100/0	100
Phosphoglucomutase	1	Jcr4S02212.70	XP_012093262.1	32.8/6.23	94/0	97
Phosphoglucomutase, putative	2	Jcr4S15954.10	XP_012093260.1	35.9/ 8.84	89/2^{e-180}	100
Aldehyde dehydrogenase family 2 member B4, mitochondrial-like	2	Jcr4S03546.10	XP_012080093.1	59.5/8.26	94/0	100
Pyruvate dehydrogenase E1 component subunit beta-1, mitochondrial isoform X1	4	Jcr4S00168.90	XP_012088753.1	40.6/5.64	100/0	100
Cytosolic malate dehydrogenase	8	Jcr4S00279.20	XP_012069835.1	36.0/6.40	100/0	100
Malate dehydrogenase, mitochondrial	7	Jcr4S03295.10	XP_012078553.1	37.0/ 8.54	100/0	100
Aconitate hydratase, cytoplasmic	1	Jcr4S06787.10	XP_012089852.1	11.6/8.38	95/0	81
Citrate synthase, mitochondrial	1	Jcr4S00215.140	XP_012092602.1	49.4/ 6.33	100/0	100
Citrate synthase, mitochondrial	1	Jcr4S01687.10	XP_012092625.1	82.2/6.03	100/0	97
Carbohydrate metabolism						
UTP--glucose-1-phosphate uridylyltransferase	4	Jcr4S00684.10	XP_012074723.1	58.1/6.74	100/0	100
Sorbitol Dehydrogenase family protein	2	Jcr4S01523.120	XP_012090335.1	42.7/5.97	100/0	95
Beta-galactosidase 8	1	Jcr4S02941.10	XP_012064843.1	89.0/7.94	100/0	88
Beta-xylosidase/alpha-L-arabinofuranosidase 1	1	Jcr4S03968.30	XP_012080785.1	84.4/6.63	100/0	100
Aldose 1-epimerase-like	1	Jcr4S03178.30	XP_012086275.1	37.0/5.64	100/0	100
Lipid metabolism						
Acyl-CoA-binding protein, partial	1	Jcr4S00983.40	XP_012070513.1	9.4/10.0	81/3^{e-36}	88
Hydroxyacyl-ACP Dehydrase	1	Jcr4S03305.10	XP_012086368.1	9.0/8.93	100/3^{e-49}	100
Oil body-associated 2B-like (LOC105649726)	1	Jcr4S00805.80	XP_012091855.1	28.2/5.91	100/0	100

Amino acids and purine metabolism						
Ureidoglycolate hydrolase (LOC105635368)	2	Jcr4S03735.10	XP_012073838.1	21.9/5.54	100/6^{e-146}	100
Alanine aminotransferase 2-like	1	Jcr4S00131.20	XP_012068795.1	55.6/5.83	100/0	100
Nitrogen regulatory protein P-II	1	Jcr4S00680.140	XP_012082957.1	23.4/ 9.73	100/2^{e-130}	92
Reactive Intermediate Deaminase A, chloroplastic	1	Jcr4S00736.130	XP_012070823.1	22.2/8.99	100/3^{e-123}	91
Beta-ureidopropionase	1	Jcr4S00584.60	XP_012081364.1	49.3/5.86	100/0	100
Detoxification and plant resistance						100
4-hydroxy-4-methyl-2-oxoglutarate aldolase 2 (putative)	1	Jcr4S00591.70	XP_012085762.1	17.9/5.68	100/1^{e-115}	99
Dienelactone hydrolase family protein	1	Jcr4S03008.10	XP_002321135.1	28.5/ 5.77	82/3e-^{124}	81
Lactoylglutathione lyase	1	Jcr4S01524.20	XP_012064721.1	26.0/8.50	100/5^{e-142}	100
Cyclophilin	2	Jcr4S01173.10	XP_012084373.1	18.3/8.68	100/4^{e-112}	100
Lactoylglutathione lyase, chloroplast isoform X1	2	Jcr4S00034.190	XP_012076751.1	34.2/ 8.91	100/0	100
Superoxide dismutase [Mn], mitochondrial-like	4	Jcr4S00073.110	NP_001295624.1	26.41/6.71	100/0	100
Cu/Zn superoxide dismutase	1	Jcr4S02704.30	NP_001295663.1	25.1/6.08	92/1^{e-130}	100
Cu/Zn superoxide dismutase family protein	1	Jcr4S00546.10	XP_012089157.1	20.7/6.74	100/4^{e-86}	77
Growth Regulation						
Thiamine thiazole synthase, chloroplastic	2	Jcr4S00619.120	XP_012070751.1	37.8/ 5.53	90/0	100
Phosphatidylethanolamine binding protein	1	Jcr4S05660.30	XP_012089808.1	18.4/5.14	100/5^{e-123}	100
Luminal-binding protein 5	2	Jcr4S22943.10	XP_012074649.1	73.6/5.11	96/0	100
Luminal-binding protein 5-like	1	Jcr4S00151.70	XP_012086670.1	69.9/5.14	100/0	100
Proteases						
Cysteine proteinase RD19a-like	1	Jcr4S00377.90	XP_012071959.1	41.5/5.81	100/0	100
Serpin-ZX-like	1	Jcr4S00079.140	XP_012071719.1	42.4/5.94	100/0	100
Leucine aminopeptidase 1-like	3	Jcr4S00343.100	XP_012071507.1	74.1/8.48	100/0	100

Oxide-Reduction						
Glutaredoxin GRX	10	Jcr4S06568.10	ADB02895.1	11.4/6.71	89/2e^{-64}	100
Glutaredoxin	3	Jcr4S01536.70	XP_012080039.1	12.2/8.17	86/0	80
Thioredoxin H-type	1	Jcr4S04538.10	NP_001295646.1	21.1/8.54	68/5^{e-66}	100
Thioredoxin reductase NTRB-like	1	Jcr4S09888.10	XP_012087579.1	35.3/9.35	97/2^{e-160}	81
Protein disulfide-isomerase precursor	4	Jcr4S06094.20	NP_001295629.1	56.8/4.85	100/0	99
1-Cys peroxiredoxin	1	Jcr4S01479.40	XP_012067545.1	14.9/7.03	100/0	91
Peroxiredoxin	4	Jcr4S07807.20	XP_012067545.1	24.3/6.08	100/5^{e-158}	100
Peroxidase 12-like	2	Jcr4S06050.30	XP_012073952.1	14.5/4.58	95/0	100
Ferritin, plant	1	Jcr4S03468.40	KDP22838.1	28.1/5.51	91/1^{e-132}	100
Glutathione reductase, cytosolic	1	Jcr4S00410.70	XP_012071166.1	53.0/5.63	100/0	100
Glutathione S-transferase DHAR2-like	1	Jcr4S05806.20	NP_001295678.1	22.5/5.75	100/5^{e-146}	100
Glutathione S-transferase parC (Hypothetical protein JCGZ_06274)	1	Jcr4S12402.20	KDP37218.1	25.7/6.02	100/2^{e-162}	100
Glutathione-S-transferase theta, GST	1	Jcr4S03348.10	XP_012092523.1	24.3/6.24	100/2^{e-150}	99
Glutathione-S-transferase theta, partial	1	Jcr4S00141.30	XP_012082716.1	24.2/6.24	100/2^{e-131}	98
Peroxiredoxin	1	Jcr4S00501.60	XP_012076732.1	17.6/5.15	100/2^{e-94}	100
NAD(P)-dependent oxidoreductase (uncharacterized protein At5g02240)	1	Jcr4S05357.40	XP_012074957.1	23.9/5.94	100/0	100
Heat Shock						
Heat shock protein 83-like	7	Jcr4S00535.40	XP_012086831.1	78.9/4.98	98/0	100
Chaperonin CPN60-2, mitocondrial	4	Jcr4S02414.50	XP_012077667.1	58.0/5.9	100/0	91
FAM10 family protein At4g22670	3	Jcr4S00409.160	XP_012087117.1	52.9/5.21	100/0	100
Heat shock protein	2	Jcr4S08787.20	XP_012089450.1	65.2/5.26	100/0	91
Small heat shock protein	2	Jcr4S00024.10	ADT65203.1	15.3/7.79	84/3^{e-54}	99
Small heat shock protein	2	Jcr4S00071.90	NP_001295605.1	58.5/7.06	58/8^{e-100}	98
15.7 kDa heat shock protein, peroxisomal	1	Jcr4S02140.20	XP_012086420.1	15.9/6.85	100/7^{e-99}	100
17.8 kDa class I heat shock protein-like	1	Jcr4S14146.30	XP_012088215.1	18.4/6.78	100/1^{e-87}	100
Chaperonin	1	Jcr4S02314.20	XP_012077092.1	26.8/8.76	100/9^{e-176}	100

Heat shock 70 kDa protein 15-like	1	Jcr4S00447.50	XP_012073334.1	92.7/5.24	100/0	100
Heat shock cognate 70 kDa protein 2-like	1	Jcr4S03314.10	XP_012089411.1	71.4/5.14	100/0	100
Nucleoside diphosphate kinase B	3	Jcr4S02985.40	NP_001295742.1	16.3/6.32	100/2^{e-104}	100
Heat shock 70 kDa protein, mitochondrial	2	Jcr4S02598.30	XP_012074575.1	73.3/5.66	98/0	100
Ribosome, Proteasome						
Elongation factor 1-beta, putative	1	Jcr4S00111.50	XP_012079853.1	26.1/4.49	96/4^{e-102}	100
Regulator of ribonuclease activity	1	Jcr4S00285.100	XP_012081861.1	17.7/5.33	100/7^{e-115}	100
Ribonuclease 3 family protein	1	Jcr4S01880.100	XP_012080098.1	17.5/9.75	86/1^{e-77}	99
Proteasome subunit alpha type	1	Jcr4S01752.50	XP_012077064.1	27.4/5.92	100/0	100
Proteasome subunit alpha type 3 family protein	1	Jcr4S03336.20	XP_012075968.1	21.5/8.82	100/6^{e-142}	99
Proteasome subunit alpha type-2-A	1	Jcr4S02802.40	XP_012087375.1	24/5.30	100/1^{e-171}	100
Proteasome subunit alpha type-6-like	1	Jcr4S00843.120	XP_012079628.1	27.4/ 5.81	100/0	100
Proteasome subunit beta type	1	Jcr4S01070.40	XP_012079483.1	27.2/6.13	100/2^{e-137}	82
Proteasome subunit beta type	1	Jcr4S00057.260	XP_012083566.1	24.9/ 6.95	100/1^{e-159}	100
Small ubiquitin-related modifier 2-like	1	Jcr4U30955.10	XP_012080638.1	11.6/4.95	100/3^{e-69}	100
14-3-3 protein 7	1	Jcr4S02291.60	XP_012067879.1	27.8/4.92	100/9^{e-169}	99
Non characterized						
WD-40 like beta propeller (Uncharacterized protein LOC105638961)	2	Jcr4S00283.70	XP_012078271.1	77.6/6.15	100/0	100
DPPIV_N (uncharacterized protein LOC105642694 isoform X2)	1	Jcr4S01894.20	XP_012082992.1	56.1/5.61	100/0	97
Pentatricopeptide repeat-containing protein At4g14850	1	Jcr4S00033.30	XP_012069644.1	87.8/5.92	100/0	100

[a]Protein names, [b]Number of spots (Supplementary Fig. S2) where the same protein was identified. [c]Accession numbers according to *Jatropha curcas* genome database (release 4.5, May 2014) were obtained after homology database identification for every single spot analyzed by LC–MS/MS. dNCBInr accession numbers, [e]Theoretical mass (kDa) and pI of identified proteins, [f]Query coverage, E-value and [g]% of identity were obtained after blastx alignment of *J. curcas* genome nucleotide sequences against de *Viridiplante* subset of the NCBInr protein database.

seeds. Shih et al. (2010) identified an Em-like protein in rice, which was not been identified previously in cereals, by other hand Vicient et al. (2010) reported two Em genes in Arabidopsis.

He et al. (2011) described that non-toxic *J. curcas* does not contain phorbol esters, nevertheless contains curcins. In agreement with this statement, four faint spots were detected in the *J. curcas* 2-DE map, which are not enough to cause the toxic effects. Lin et al. (2010) have indicated that curcin hemagglutinating activity is presented at more than 7.8 mg/L and LD50 for oral semi-lethal dose was reported to be 104.73 mg/kg. Curcins type-1 are proteins that specifically are accumulated in the endosperm (Gu et al., 2015; Lin et al., 2003a) and are classified as Type I ribosome-inactivating proteins (RIP). Zhang et al. (2017) reported a novel RIP from *J. curcas*, however it is not well documented its function, but it may be part of the response to biotic and abiotic stresses (Qin et al., 2009). Curcins have also related with antitumor activity (Lin et al., 2003b, Luo et al., 2006).

3.4.2.2. Protein species involved in sugar metabolism

Glycolisis is well represented in *J. curcas* albumins fraction, glyceraldheyde-3P-DH was detected in 14 spots, two isoforms of phosphoglucomutase and triose phosphate isomerase and three isoforms of enolase were identified. The pyruvate dehydrogenase E1 component subunit beta-1, part of the complex that carried out the conversion of pyruvate to Acetyl-CoA, was detected in four spots. Aldehyde dehydrogenase, phosphoglycerate mutase, and enzymes related to the TCA cycle (aconitase, two isoforms of citrate synthase and two isoforms of malate dehydrogenase) were identified (Table 2).

Uridyltransferase (UTP-glucose-1-phosphate uridyltransferase) or glucose-1-phosphate uridylytransferase (detected in 2 spots) is an enzyme involved in carbohydrate metabolism with an important role in glycogenesis and cell wall synthesis (Dai et al., 2006). In relation to the sugars metabolism, beta-galactosidase 8 and beta-xylosidase/alpha-L-arabinofuranosidase 1 were detected in *J. curcas*. Aldose-1-epimarase, which catalyses the first step in galactose metabolism was also detected in one spot. Two proteoforms of sorbitol dehydrogenase enzyme, which converts sorbitol into fructose, were also detected.

3.4.2.2. Protein species related fatty acid metabolism

Proteins related to fatty acid metabolism were identified in albumins fraction (Table 2). Among them, the hydroxyacyl-ACP-dehydrase, enzyme involved in fatty acid synthesis is the key enzyme of the fatty acid synthesis (FAS) system (Lung et al., 2016) was identified in one spot. One proteoform of the acyl-Co-A-binding protein was identified in *J. curcas* albumins fraction (Table 2), this is an enzyme that binds medium- and long-chain acyl CoA esters with very high affinity. Acyl-Co-A-binding protein may function as maintenance, protection, and transport of the Acyl-CoA pool (Yurchenko and Weselake, 2011).

In *J. curcas* seeds the triacylglycerol's (TAGs) are accumulated in oil bodies, which consist of a central core of neutral lipids, delimited by a monolayer of phospho- and glico-lipids including sterols and proteins associated to the oil bodies surface. One proteoform of the oil body-associated 2B-like protein in albumins fraction was identified. In maize (*Zea mays* L.), the oil body associated protein 1 gene (obap1) was found mainly expressed in scutellum during maturation (López-Ribera et al., 2014).

3.4.2.3. Protein species related to amino acids and purine metabolism

Nitrogen in seeds does not only come from root nitrogen uptake during the later growing period, but also from the redistribution from the vegetative organs, in addition, the recycling and redistribution of nitrogen in plants is important for the environmental stress response (Zhang et al., 2010). Four proteoforms related to amino acids and purine metabolism were identified in albumins fraction: Ureidoglycolate hydrolase, Alanine aminotransferase 2-like, Nitrogen regulatory protein P-II, and Reactive Intermediate Deaminase A, chloroplastic. Ureidoglycolate (two proteoforms) in an intermediate of purine catabolism catalyses the final step of ureide degradation in which inorganic nitrogen is re-assimilated (Li et al., 2015; Werner et al., 2013). The beta-propionase enzyme, detected in *J. curcas* albumins fraction (one spot), acts on carbon-nitrogen bonds, specifically in linear amides and participates in pyrimidine, beta-alanine metabolism, and pantothenate biosynthesis (Shen et al., 2014).

Alanine Amino transferase, an enzyme belonging to the L-alanine degradation and the nitrogen regulatory protein P-II, a constituent of theadenylation cascade involved in the regulation of glutamine synthetase (GS)activity were detected in one isoform (Table 2). In nitrogen-limiting conditions, P-II is uridylylated establishing a complex that allows the deadenylation of GS, thus activating the enzyme. On the contrary, under nitrogen surplus, this complex is deuridylated promoting adenylation and the consequent inactivation of GS (Brown et al., 1971; Jonsson and Nordlund, 2007). In plants, the function of reactive intermediate deaminase A is still unclear, however recent reports allowed to establish its contribution in the biosynthesis of branched-chain amino acids (Niehaus et al., 2014), one proteoform was detected in *J. curcas* press-cake albumins fraction.

3.4.2.4. Protein species involved in detoxification and plant resistance

4-hydroxy-4-methyl-2-oxoglutarate aldolase (4HMO), dienelactone hydrolase, lactoylglutathione lyase, and cyclophilin were detected in J. curcas albumins fraction. The enzyme 4HMO cleaves 4-carboxy-4-hydroxy-2-oxoglutarate giving as a product pyruvate. Participates in benzoate degradation via hydroxylation, which links aromatic catabolism to central cellular metabolism (Tack et al., 1972). Dienelactone hydrolases play a crucial role in the bacterial degradation of chloroaromatic compounds. Many representatives of this group of xenobiotic compounds are converted to chlorosubstituted catechols by the initial enzymes of aromatic catabolism (Schlöman et al., 1990). Two isoforms of lactoylglutathione lyase were detected, this is an enzyme that catalyses the isomerization of hemithioacetal adducts, which are cytotoxic compounds

generated under abiotc stresses, including metal toxicity (Nahar et al., 2017). Cyclophilin (two isoforms) belongs to the immunophilin superfamily with peptidyl-prolyl cis-trans isomerase (PPIase activity). Catalyse the interconversion of the cis- and trans-rotamers of the peptidyl-prolyl amide bond of peptides. The interaction of soybean cyclophylin GmCYP1 with the isoflavonoid regulator GmMYB176 and 14-3-3 proteins suggest its role in defence in soybean (Mainali, 2017). Interestingly, 14-3-3 proteins were also detected in *J. curcas*.

3.4.2.6. Protein species related to plant growth regulation

Thiamine thiazole synthase (TH1, two proteoforms) plays a central in thiamine biosynthesis, but it is also important in abiotic stress responses and mitochondrial DNA damage tolerance (Li et al., 2016). It has also been associated with heat tolerance in rice (Chen et al., 2011) and is involved in ABA-regulated stomatal movement and in the plant´s drought response (Li et al., 2016). Phosphatidylethanolamine binding protein (one isoform) includes various functions such as lipid binding, neuronal development, control of the switch between shoot growth and flower structures and the regulation of signalling such as the MPA kinase and the NF-kappaB pathways (Vallée et al., 2003; Yeung et al., 2001). Luminal-binding protein detected in three proteoforms, is involves in storage of calcium pool inside the endoplasmic reticulum lumen, accumulation of this protein confers resistance to drought (Valente et al., 2009). In soybean root tips the induction of the luminal-binding protein 5 was observed during flood conditions (Komatsu et al., 2012).

3.4.2.6. Proteases and oxidoreductases

Cysteine protease RD19a-like, leucine aminopeptidase (three proteoforms), and serine proteinase inhibitor (SERPIN-ZX-like) were detected in *J. curcas* albumins fraction. Cysteine proteases RD19a-like (one proteoform) or cathepsin F-like proteases are members of the Papain-like cysteine protease, a large class of proteolytic enzymes associated with several plant processes (Richau et al., 2012). Serine protease inhibitor (Serpin, one proteoform) is a family of proteases inhibitors widely distributed. Members of the group of leucine aminopeptidase (three proteoforms), are implicated in transcriptional regulation (Asqui et al., 2017).

Glutathione-S-transferases (GSTs), detected in *J. curcas* albumins (four proteoforms), are a family of phase II detoxification enzymes. The acquired resistance to chemotherapy, herbicides, insecticides and microbial antibiotics has been attributed to the presence of GST (Townsed and Tew, 2003). Glutaredoxin (13 isoforms) have similar functions of thioredoxin (TRX), both are proteins involved in cell protection against oxidative stress damage (Sánchez-Riego et al., 2016). Protein disulphide isomerase (four proteoforms) participates throughout the maturation of extracellular proteins adding disulphide bonds to stabilize it or to covalently join it to other proteins (Wang and Tsou, 1993). Glutathione reductase (GR, spot 23) or glutathione-disulphide reductase (GSR) catalyses the reduction of glutathione disulphide (GSSG) to the

sulfhydryl form glutathione (GSH), which is a critical molecule in resisting oxidative stress and the maintenance of the cell reduced environment (Lüersen et al., 2013).

Iron is an essential nutrient for all cells, but its excess is harmful to cells, and so iron homeostasis must be controlled. Ferritins play important roles in sequestering or releasing iron as needed (Borg et al., 2012) and are exclusively targeted to plastids and mitochondria (Borg et al., 2012; Briat et al., 2010; Zancani et al., 2004). In this work, a ferritin was identified in albumins fraction (one proteoform). A recent report indicates that ferritins from wheat (*Triticum aestivum* L.) play important roles in enhancing tolerance in stresses associated with ROS (Zang et al., 2017).

3.4.2.8. Heat shock protein, ribosome, energy, and proteasome

A set of several proteoforms of heat shock proteins, small heat shock proteins, and chaperonins were identified in albumins fraction (Table 2). The nucleoside diphosphate kinase B (NDPKs, three isoforms) is an enzyme that catalyses, in a reversible manner, the production of different nucleoside diphosphates (NDP) and triphosphates (NTP) (Kihara et al., 2011). The regulator of ribonuclease (one proteoform) activity acts as a regulator of the endonuclease RNase E and inhibiting the RNA processing. Ribonuclease 3 is a family of ribonucleases that recognizes dsRNA and cleaves it to transform them into mature RNAs, this enzyme was detected in one proteoform. Ubiquitin-fold modifier conjugating (UBL, one isoform) is covalently linked to target proteins but its physiological functions are still not known (Daniel and Liebau, 2014).

3.4.3. Protein species in globulins and glutelins fraction

Globulins fractions were represented by legumin A and B, 2S albumins, vicilin-like antimicrobial peptides, Germin-like protein 5-1, and curcin 1. Germins (GER) and germin-like proteins (GLPs), together with sucrose-binding proteins and seed globulins, are part of protein superfamily called cupin. Large numbers of GERs and GLPs have been functionally characterized from diverse plant species, their accumulation is related to biotic and abiotic stress response (Ilyas et al., 2016). Despite their solubility properties, globulins and glutelins are structurally similar, even evidences indicate that both protein share a common gene origin (Okita et al., 1989). Our results indicate that similar proteins were identified in both fractions (**Table 5 and 6**).

Table 5. Identification by LC-MS/MS of globulin fraction proteins extracted from non-toxic *Jatropha curcas* meal.

Protein name[a]	Spots[b]	*J. curcas* Number[c]	NCBI accession number[d]	Theor. kDa/*pI*[e]	Query coverage / E-value[f]	Ident[g]
Nutrient reservoir						
Legumin B-like	64	Jcr4S00279.60	NP_001295688.1	57.5/6.75	100/0	100
Legumin B-like	27	Jcr4S00279.80	KDP40339.1	50.7/9.46	100/0	93
Legumin A-like	58	Jcr4S01636.60	XP_012085316.1	65.4/9.15	79/0	87
Legumin A-like	16	Jcr4S01636.70	XP_012085315.1	52.9/5.40	100/0	100
11S globulin seed storage protein	55	Jcr4S01636.40	XP_012085320.1	54.0/8.77	95/0	100
2S albumin-like	26	Jcr4S00619.70	XP_012070746.1	16.3/5.53	100/$2e^{-96}$	100
2S albumin-like	10	Jcr4S00619.90	XP_012071219.1	20.4/8.30	98/$9e^{-64}$	100
2S albumin-like	2	Jcr4S00619.60	XP_012071219.1	16.6/6.81	100/3^{e}-^{80}	
2S albumin-like	1	Jcr4S00619.80	XP_012070747.1	16.6/6.34	85/$5e^{-84}$	100
Vicilin-like antimicrobial peptides 2-2	14	Jcr4S00603.20	XP_012064865.1	57.0/6.04	100/0	100
Vicilin-like antimicrobial peptides 2-2	1	Jcr4S03723.20	XP_012092200.1	50.7/6.04	100 / 0	86
Germin-like protein 5-1	1	Jcr4S00027.200	XP_012088803.1	23.5/6.90	88 / $6e^{-122}$	99
Curcin 1	9	Jcr4S01069.20	ADN39429.1	51.0/8.90	60 / 0	100
Metabolism						
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	1	Jcr4S00100.140	XP_012079890.1	44.5/7.05	82/$1e^{-139}$	99
Malate dehydrogenase	1	Jcr4S00279.20	XP_012069835.1	69.9/5.14	95/0	94
Malate dehydrogenase, mitochondrial	1	Jcr4S03295.10	XP_012078553.1	37.0/8.54	96/0	99
Oxide-reduction						
Protein disulfide-isomerase precursor	2	Jcr4S06094.20	NP_001295629.1	56.9/4.85	100/0	100

Superoxide dismutase [Mn], mitocondrial-like	1	Jcr4S00073.110	NP_001295624.1	30.4/6.79	100/1e^{-163}	87
Glutaredoxin GRX	1	Jcr4S06568.10	NP_001295635.1	11.47/6.71	89/2e^{-64}	100
Ribosomal, energy						
60S acidic ribosomal protein P3-like	1	Jcr4S16124.30	XP_012075150.1	12.2/4.24	100 / 1e^{-29}	100
ATP synthase subunit beta, mitochondrial	1	Jcr4S01269.80	XP_012075690.1	61.1/6.30	89 / 0	96
ATP synthase subunit beta, mitocondrial like	1	Jcr4S00914.20	XP_012066038.1	59.7/6.06	94 / 0	100
AtpB (chloroplast)	1	Jcr4S00559.20	YP_002720119.1	50.0/4.91	97 / 0	99
Luminal-binding protein 5	1	Jcr4S22943.10	XP_012074649.1	73.7/5.11	100 / 0	100
Luminal-binding protein 5-like	1	Jcr4S00151.70	XP_012086670.1	69.9/5.14	95 / 0	94
Non identified	13	---	---	---	---	---

[a]Protein names, [b]Number of spots (Supplementary Figure S3) where the same protein was identified.[c]Accession numbers according to *Jatropha curcas* genome database (release 4.5, May 2014) were obtained after homology database identification for every single spot analyzed by LC-MS/MS. [d]NCBInr accession numbers, [e]Theoretical mass (kDa) and pI of identified proteins, [f]Query coverage, E-value and [g]Percentage of identity were obtained after blastx alignment of *J. curcas* genome nucleotide sequences against de *Viridiplante* subset of the NCBInr protein database.

Table 6. Identification by LC–MS/MS of glutelin fraction proteins extracted from non-toxic *J. curcas* meal.

Protein name[a]	Spots[b]	*J. curcas* number[c]	NCBI accession number[d]	Theor. kDa/p*I*[e]	Query Cover. /E-value[f]	Ident[g]
Legumin B-like precursor	69	Jcr4S00279.60	NP_001295688.1	57.5/6.75	100/0	99
Vicilin-like antimicrobial peptides 2-2	3	Jcr4S00603.20	XP_012064865.1	56.8/6.04	100/0	100
2S albumin-like	1	Jcr4S00619.70	XP_012070746.1	16.2/5.53	100/2^{e-96}	100
Legumin A-like	8	Jcr4S01636.70	XP_012085315.1	52.9/5.40	100/0	100
Legumin A-like	51	Jcr4S01636.60	XP_012085316.1	65.4/9.15	79/0	87
11S globulin seed storage protein 2- like	35	Jcr4S01636.40	XP_012085320.1	54.0/8.77	95/0	100
Not identified	7	--	--	--	--	--

[a]Protein names, [b]Number of spots (Supplementary Figure S3) where the same protein was identified.[c]Accession numbers according to *Jatropha curcas* genome database (release 4.5, May 2014) were obtained after homology database identification for every single spot analyzed by LC-MS/MS. [d]NCBInr accession numbers, [e]Theoretical mass (kDa) and pI of identified proteins, [f]Query coverage, E-value and [g]Percentage of identity were obtained after blastx alignment of *J. curcas* genome nucleotide sequences against de *Viridiplante* subset of the NCBInr protein database.

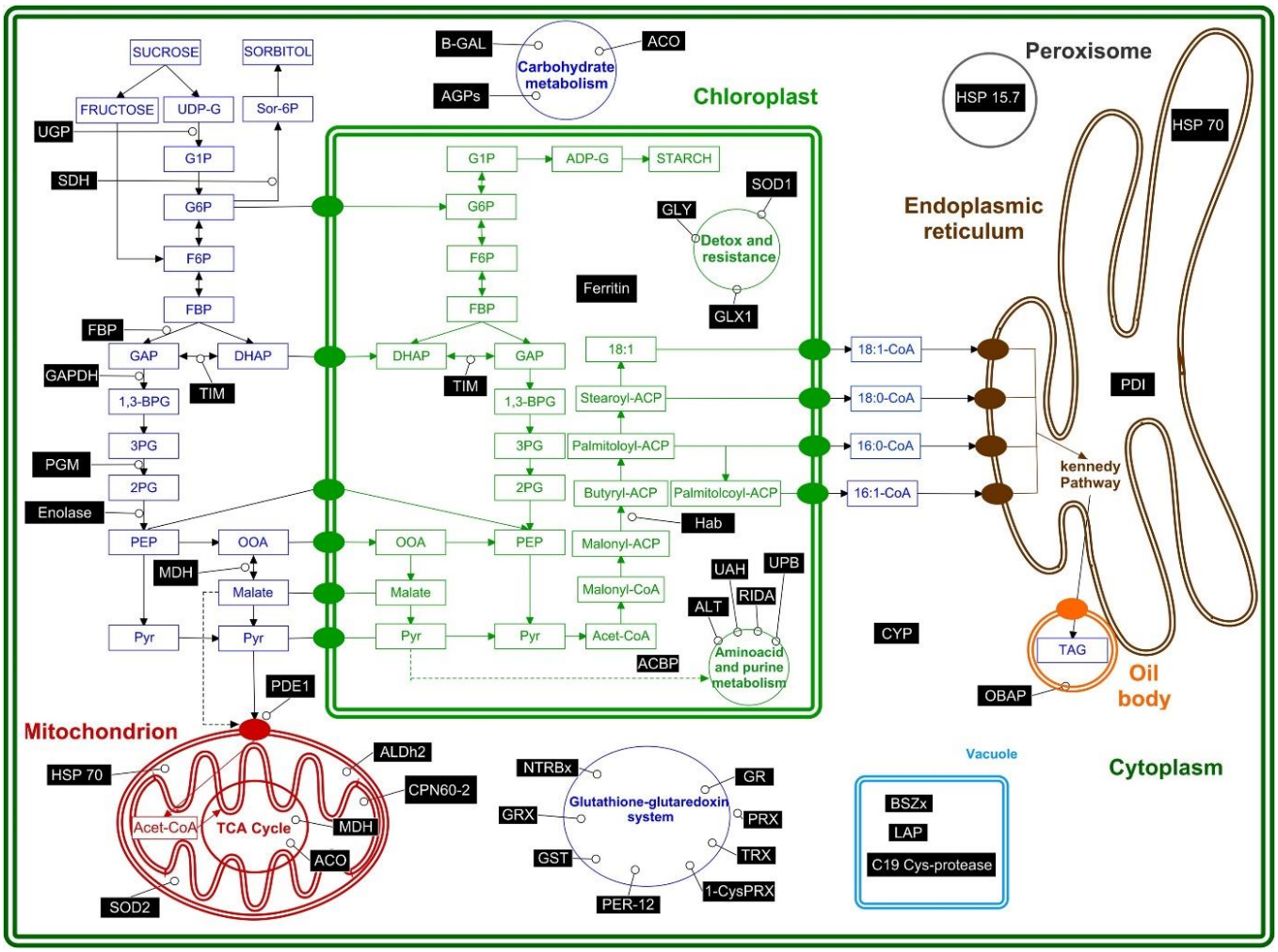

Figure 6. Metabolic network of non-toxic *Jatropha curcas* seeds. The identified protein spots are indicated in black spots (Table 3.2). Diagram was generated using the visualization and analysis tool PathVisio 3.2.4.

3.5. Conclusions

In the present study, the protein composition of seed storage proteins of non-toxic *J. curcas* press-cake after oil extraction was investigated using proteomics tools. Results have shown that albumins or water-soluble proteins are rich on different protein species related with carbohydrate metabolism. Other proteins involved in lipid, nitrogen, and purine metabolism were also identified. Proteins related with detoxification of xenobiotics were detected. Interestingly, the 14-3-3 proteins and NDPK, which are components of signaling pathways as well as cyclophillin that is one of protein targets of 14-3-3 were detected in *J. curcas* water soluble fraction. Although toxic protein such as curcin was detected, the amount present in the non-toxic *J. curcas* seeds may be not enough to cause toxic effects. This study contributes to the information of *J. curcas* seed proteome.

3.6. Acknowledgments

Thanks to CONACYT-MEXICO GRANT 56787 (Laboratory for Nanoscience and Nanotechnology Research-LINAN), INFRA-2013-01 No. 204373 and CONACYT-MEXICO GRANT CB-2008-103601.

3.7. References

Asqui, S.L, Vercammen, D., Serrano, I., Valls, M., Rivas, S., Van Breusegem, F., Conlon, F.L., Dangl, J.L., Coll, N.S., 2017. AtSERPIN1 is an inhibitor of the metascapase AtMC1-mediated cell death and autocatalytic processing in planta. New Phytol. doi: 10.1111/nph.14446.

Borg, S., Brinch-Pedersen, H., Tauris, B., Madsen, L.H., Darbani, B., Noeparvar, S., Holm, P.B., 2012. Wheat ferritins: Improving the iron content of the wheat grain. J. Cereal Sci. 56, 204–213.

Briat, J.F., Duc, C., Ravet, K., Gaymard, F., 2010. Ferritins and iron storage in plants. Biochim. Biophys. Acta. 1800, 806–814.

Brown, M.S., Segal, A., Stadtman, E.R., 1971. Modulation of glutamine synthetase adenylylation and deadenylylation is mediated by metabolic transformation of the PII-regulatory protein. Proc. Nat. Acad. Sci. USA 68, 2949-2953.

Chen, X., Zhang, W., Zhang, B., Zhou, J., Wang, Y., Yang, Q., Ke, Y., He, H., 2011. Phosphoproteins regulated by heat stress in rice leaves. Proteome Sci. 9, 37.

Costa, G.G., Cardoso, K.C., Del Bem, L.E., Lima, A.C., Cunha, M.A.S., de Campos-Leite, L.; Vicentini, R., Papes, F., Moreira, R.C., Yunes, J.A., Campos, F.A.P., Da Silva, M.J., 2010. Transcriptome analysis of the oil-rich seed of the bioenergy crop *Jatropha curcas* L. BMC Genomics 11, 462.

Dai, N., Petreikov, M., Portnoy, V., Katzir, N., Pharr, D., Schaffer, A.A., 2006. Cloning and expression analysis of a UDP-galactose/glucose pyorphosphorylase from melon fruit provides evidence for the major metabolic pathway of galactose metabolism in raffinose oligosaccharide metabolizing plants. Plant Physiol. 142, 294-304.

Daniel, J., Liebau, E., 2014. The Ufm1 cascade. Cells 3, 627-638.

Dias, L.A., Missio, R.F., Dias, D.C., 2012. Antiquity, botany, origin and domestication of *Jatropha curcas* (*Euphorbiaceae*), a plant species with potential for biodiesel production. Genet. Mol. Res. 11, 2719–2728.

Fairless, D., 2007. Biofuel: the little shrub that could–maybe. Nature 449, 652–655. doi: 10.2038/449652a.

Franco, O.L., Murad, A.M., Leite, J.R., Mendes, P.A.M., Prates, M.V., Bloch, JrC., 2006. Identification of a cowpea g-thionin with bactericidal activity. FEBS J. 273, 3489–3497.

Gu, K., Tian, D., Mao, H., Wu, L., Yin, Z., 2015. Development of marker-free transgenic *Jatropha curcas* producing curcin-deficient seeds through endosperm-specific RNAi-mediated gnee silencing. BMC Plant Biol. 15, 242.

Gübitz, G.M., Mittelbach, M., Trabi, M., 1999. Exploitation of the tropical oil seed plant *Jatropha curcas* L. Bioresour. Technol. 67, 73–82.

Haas, W., Mittelbach, M., 2000. Detoxification experiments with the seed oil from *Jatropha curcas* L. Ind. Crops Prod. 12, 111–118.

He, W., King, A.J., Khan, M.W., Cuevas, J.A., Ramiaramanana, D., Graham, I.A., 2011. Analysis of seed phorbol-ester and curcin content together with genetic diversity in multiple provenances of *Jatropha curcas* L. from Madagascar and Mexico. Plant Physiol. Biochem. 49, 1183-1190.

Hilton, H., Gaut, B.S., 1998. Speciation and domestication in maize and its wild relatives: Evidence from the Globulin-1 Gene. Genetics 150, 863-872.

Huerta-Ocampo, J.A., Barrera-Pacheco, Mendoza-Hernández, C.S., Espitia-Rangel, E., Mock, H.P., Barba de la Rosa, A.P., 2014. Salt stress-induced alterations in the root proteome of *Amaranthus cruentus* L. J. Proteome Res.13, 3607-3627.

Ilyas, M., Rasheed, A., Mahmood, T., 2016. Functional characterization of germin and germin-like protein genes in various plant species using transgenic approaches. Biotechnol. Lett. 38, 1405-1421.

Jonsson, A., Nordlund, S., 2007. In vitro studies of the uridylylation of the three PII protein paralogs from *Rhodospirillum rubrum*: the transferase activity of R. Rubrum GlnD is regulated by a-ketoglutarate and divalent cations but not by glutamine. J. Bacteriol. 189, 3471-3478.

Jorrin-Novo, J.V., 2014. Plant Proteomics: Methods and protocols. In Methods in Molecular Biology, vol. 1072, Chapter 1, pp 3-13. Springer. DOI 10.1007/978-1-62703-631-3-1.

Kihara, A., Saburi, W., Wakuta, Sh., Kim, M.-H., Hamada, Sh., Ito, H., Imai, R., Matsui, H., 2011. Physiological and biochemical characterization of three nucleoside diphospahte kinase isozymes from rice (*Oryza sativa* L.). Biosci. Bitoechnol. Biochem. 75, 1740-1745.

King, A.J., Li, Y., Graham, I.A., 2011. Profiling the developing Jatropha *curcas* L. Seed transcriptome by pyrosequencing. Bioenerg Res. 4, 211-221.

Komatsu, S., Kuki, R., Nanjo, Y., Hiraga, S., Furukawa, K., 2012. Comprehensive analysis of endoplasmic reticulum-enriched fraction in root tips of soybean under flooding stress using proteomics techniques. J. Proteomics 77, S31-S60,

Kumar, A., Sharma, S., 2008. An evaluation of multipurpose oil seed crop for industrial uses (*Jatropha curcas* L.): A review. Ind. Crops Prod. 28, 1–10.

Kutmon, M., van Iersel, M.P., Bohler, A., Kelder T, Nunes, N., Pico, A.R., Evelo, C.T., 2015. PathVisio 3: An Extendable Pathway Analysis Toolbox PLos Computational Biology 11(2):e1004085. doi: 10.1371/journal.pcbi.1004085.

López-Ribera, I., La Paz, J.L., Repiso, C., García, N., Miguel M., Hernández, M.L., Martínez-Rivas, J.M., Vicient, C.M., 2014. The evolutionary conserved oil body associated protein OBAP1 participates in the regulation of oil body size. Plant Physiol. 164, 1237-1249.

Lüersen, K., Stegehake, D., Daniel, J., Drscher, M., Arjonina, I., Ajonina, C., Hertel, P., Woltersdorf, Ch., Liebau, E., 2013. The glutathione reductase GSR-1 determines stress tolerance and longevity in *Caenorhabditis elegans*. PloS One, 8(4):e60731. doi: 10.1371/journal.pone.0060731.

Li, Ch.-L., Wang, M., Wu, X.-M., Chen, D.-H., Lv, H.-J., Shen, J.-L., Qiao, Z., Zhang, W., 2016. THI1, a thiamine thiazole synthase, interacts with Ca2+- dependent protein kinase CPK33 and modulates the S-type anion channels and stomatal closure in Arabidopsis. Plant Physiol. 170, 1090-1104.

Li, J., Qin, R.-Y., Li, H., Xu, R.-F., Yang, Y.-Ch., Ni, D.-H., Li, H.-M., Wei, P.-Ch., Yang, J.-B., 2015. Low-temperature-induced expression of rice ureidoglycoalte amidohydrolase is mediated by a C-repeat/dehydration-responsive element that specifically interacts with rice C-repeat-binding factor 3. Front. Plant Sci. 6.1011. Doi:10.333989/fpls.2015.01011.

Lin, J., Chen, Y., Xu, Y., Yan, F., Tang, L., Chen, F., 2003a. Cloning and expression of curcin, a ribosome-inactivating protein from the seeds of *Jatropha curcas*. Acta Bot Sin. 45, 858–863.

Lin, J., Yan, F., Tang, L., Chen, F., 2003b. Antitumor effects of curcin from seeds of *Jatropha curcas*. Acta Pharmacol Sin. 24, 241–246.

Lin, J., Zhou, X., Wang, J., Jiang, P., Tang, K., 2010. Purification and characterization of curcin, a toxic lectin from the seed of *Jatropha curcas*. Prep Biochem Biotechnol. 40, 107-118.

Lipkin, A., Anisimova, V., Nikonorova, A., Babakov, A., Krause, E., Bienert, M., Grishin, E., Egorov, T., 2005. An antimicrobial peptide Ar-AMP from amaranth (*Amaranthus retroflexus* L.) seeds. Phytochem. 66, 2426-2431.

Liu, H., Liu, Y.J., Yang, M.F., Shen, S.H., 2009. A comparative analysis of embryo and endosperm proteome from seeds of *Jatropha curcas*. J Integr Plant Biol. 51,850–857.

Liu, H., Wang, C., Chen, F., Shen, S., 2015. Proteomic analysis of oil bodies in mature *Jatropha curcas* seeds with different lipid content. J. Proteomics 113,403-414.

Liu, H., Wang, C., Komatsu, S., He, M., Liu, G., Sheri, S., 2013. Proteomic analysis of the seed development in *Jatropha curcas*: From carbon flux to the lipid accumulation. J. Proteomics 91, 23-40.

Lung, S.-Ch., Chye, M.-L. 2016., Acyl-Co-A-binding proteins (ACBPs) in plant development. 2016. Subcell. Biochem. 86, 363-404.

Luo, M.J., Yang, X.Y., Liu, W.X., Xu, Y., Huang, P., Yan, F., Chen, F., 2006. Expression, purification and anti-tumor activity of curcin. Acta Biochim. Biophys. Sin. 38, 663–668.

Maghuly, F., Laimer, M., 2013. *Jatropha curcas*, a biofuel crop: Functional genomics for understanding metabolic pathways and genetic improvement. Biotechnol. J. 8, 1172-1182.

Mainali, H.R., Anguraj-Vadivel, A.K., Li, X., Gijzen, M., Dhaubhadel, S., 2017. Soybean cyclophiln GmCYP1 interacts with an isoflavonoid regulator GmMYB176. Sci. Rep. 7:39550. doi: 10.1038/srep39550.

Makkar, H.P.S., Becker, K., 2009. *Jatropha curcas*, a promising crop for the generation of biodiesel and value-added coproducts. Eur. Lipid Sci. Technol. 111, 773-787.

Mandal, S.M., Dey, S., Mandal, M., Sarkar, S., Maria-Neto, S., Franco, O.L., 2009. Identification and structural insights of three novel antimicrobial peptides isolated from green coconut water. Peptides 30, 633-637.

Martínez-Herrera, J., Jimenez-Martínez, Ch., Martínez-Ayala, A., Garduño-Siciliano, L., Mora-Escobedo, R., Davila-Ortíz, G., Chamorro-Cevallos, G., Makkar, H., Becker, K., 2012. Evaluation of the nutritional quality of nontoxic kernel flour from *Jatropha curcas* L. in rats. J. Food Qual. 35, 152-158.

Nahar, K., Hasanuzzaman, M., Suzuki, T., Fujita, M., 2017. Polyamines-induced aluminum tolerance in mung bean: A study on antioxidant defense and methyulglyoxal detoxificasiton systems: Ecotoxicol. 26, 58-73.

Niehaus, T.D., Nguyen, T.N.D., Gidda, S.K., ElBadawi-Sidhu, M., Lambrecht, J.A., McCarty, D.R., Downs, D.M., Cooper, A.J.L., Fiehn, O., Mullen, R.T., Hanson, A.D., 2014. Arabidopsis and maize RidA proteins preempt reactive enamine/imine damage to branched-chain amino acid biosynthesis in plastids. Plant Cell 26, 3010-3022.

Okita, T.W., Hwang, Y.S., Hnilo, J., Kim, W.T., Aryan, A.P., Larson, R., Krishnan, H.B., 1989. Structure and expression of the rice glutelin multigene family. J. Biol. Chem. 264, 12573-12581.

Openshaw, K., 2000. A review of *Jatropha curcas*: An oil plant of unfulfilled promise. Biomass Bioenergy 19, 1–15.

Osborne, T.B., 1908. Our present knowledge of plant proteins. Science 28, 417–427.

Pelegrini, P.B., Murad, A.M., Silva, L.P., dos Santos, R.C.P., Costa, F.T., Tagliari, P.D., Bloch, C.Jr., Noronha, E.F., Miller, R.N., Franco, O.L., 2008. Identification of a novel storage glycine-rich peptide from guava (*Psidium guajava*) seeds with activity against Gram-negative bacteria. Peptides 29, 1271–1279.

Pelegrini, P.B., Noronha, E.F., Muniz, M.A., Vasconcelos, I.M., Chiarello, M.D., Oliveira, J.T., Franco, O.L., 2006. An antifungal peptide from passion fruit (*Passiflora edulis*) seeds with similarities to 2S albumin proteins. Biochem. Biophys. Acta 1764, 1141–1146.

Perea-Domínguez, X.P., Espinosa-Alonso, L.G., Hosseinian, F., HadiNezhad, M., Valdez-Morales, M., Medina-Godoy, S., 2017. Phenolic profile and antioxidant activity form non-toxic Mexican *Jatropha curcas* L. shell methanolic extracts. Natural Prod. Res. 31, 610-614.

Pinheiro, C.B., Shah, M., Soares, E.L., Nogueira, F.C.S., Carvalho, P.C., Junqueira, M., Araújo, G.D.T., Soares, A.A., Domont, G.B., Cmapos, F.A.P., 2013. Proteome analysis of plastids from developing seeds of *Jatropha curcas* L. J. Proteome Res.12, 5137-5145.

Pinto, M.E., Batista, J.M.Jr., Koehbach, J., Gaur, P., Sharma, A., Nakabashi, M., Cilli, E.M., Gissel, G.M., Verli, H., Gruber, C.W., Bianch, E.W., Tavares, J.F., da Silva, M.S., Garcia, C.R., Bolsani, V.S., 2015. Ribifolin, and orbitide from *Jatropha ribifolia*, and its potential antimalarial activity. J. Nat. Prod. 27, 374-380.

Pramanik, A, 2003. Properties and use of *Jatropha curcas* oil and diesel fuel blends in compression ignition engine. Renew Energy 28, 239–248.

Qin, X., Zheng, X., Shao, C., Gao, J., Jiang, L., Zhu, X., Yan, F., Tang, L., Xu, Y., Chen, F., 2009. Stress-induced curcin-L promoter in leaves of *Jatropha curcas* L. and characterization in transgenic tobacco. Planta 230, 387–395.

Rezig, L., Chibani, F., Chouaibi, M., Dalgalarrondo, M., Hessini, K., Guéguen, J., Hamdi, S., 2013. Pumpkin (*Cucurbita maxima*) seed proteins: sequential extraction processing and fraction characterization. J. Agric. Food Chem. 61, 7715-7721.

Ribeiro, A.C., Teixeira, A.R., Ferreira, R.B., 2004. Characterization of globulins from common vetch (*Vicia sativa* L.). J. Agric. Food Chem. 52, 4913-4920.

Richau, K.H., Kaschani, F., Verdoes, M., Pansuriya, T.C., Niessen, S., Stüber, K., Colby, T., Overkleeft, H.S., Bogyo, M., Van der Hoorn, R.A.L., 2012. Subclassification and biochemical analysis of plant papain-like cysteine proteases display subfamily-specific characteristics. Plant Physiol. 158, 1583- 1599.

Romero-Rodríguez, M.C., Maldonado-Alconada, A.M., Valledor, L., Jorrin-Novo, J.V., 2014. Back to Osborne, sequential protein extraction and LC-MS analysis for the characterization of the Holm Oak seed proteome. In: Plant Proteomics: Methods and Protocols, Methods in Molecular Biology, vol. 1072, Jesus V. Jorrin-Novo et al. (eds.). Springer Science+Business Media, LLC. Chapter 27, pp. 379-389, DOI 10.1007/978-1-62703-631-3_27.

Sánchez-Riego, A.M., Mata-Cabana, A., Galmozzi, C.V., Florencio, F.J., 2016. NADPH-Thioredoxin reductase C mediates the response to oxidative stress and

thermotolerance in the Cyanobacterium Anabaena sp. PCC7120. Front. Microbiol. 18, 7:1283. doi:10.3389/fmicb.2016.01283.

Sabandar, C.W., Ahmat, N., Jaafar, F.M., Sahidin, I., 2013. Medicinal property, phytochemistry and pharmacology of several *Jatropha* species (*Euphorbiaceae*): A review. Phytochem. 85, 7–29.

Schlöman, M., Fischer, P., Schmidt, E., 1990. Enzymatic formation, stability, and spontaneous reactions of 4-fluoromuconolactone, a metabolite of the bacterial degradation of 4-fluorobenzoate. J. Bacteriol. 172, 5119-5129.

Shah, M., Soares, E.L., Carvalho, P.C., Soares, A.A., Domont, G.B., Nogueira, F.C.S., Campos F.A.P., 2015. Proteomic analysis of the endosperm ontogeny of *Jatropha curcas* L. seeds. J. Proteome Res. 14, 2557-2568.

Shah, M., Soares, E.L., Lima, M.L.B., Pinheiro, C.B., Soares, A.A., Domont, G.B., Nogueira, F.C.S., Campos, F.A.P., 2016. Deep proteome analysis of gerntoplasts from the inner integument of developing seeds of *Jatropha curcas*. J. Proteomics 143, 346-352.

Shen, Y.-H., Chen, Y.-H., Liu, H.-Y., Chiang, F.-Y., Wang, Y.-Ch., Hou, L.-Y., Lin, J.-Sh., L, Ch.-Ch., Lin, H.-H., Lai, H.-M., Jeng, Sh.-T., 2014. Expression of a gene encoding β-ureidopropionase is critical for pollen germination in tomatoes. Physiol. Plantarum 150, 425-435.

Shih, M.-D., Huang, L.-T., Wei, F.J., Wu, M.-T., Hoekstra, F.A., Hsing, Y.l., 2010. OsLEA1a, a new Em-like protein of cereal plants. Plant Cell Physiol. 51, 2132-2144.

Soares, E.L., Shah, M., Soares, A.A., Costa, J.H., Carvalho, P., Domont, G.B., Nogueira, F.C.S., Campos, F.A.P., 2014. Proteome analysis of the inner integument from developing *Jatropha curcas* L. seeds. J. Proteome Res. 13, 3562-3570.

Tack, B.F., Chapman, P.J., Dagley, S., 1972. Purification and properties of 4- hydroxy-4-methyl-2-oxoglutarate aldolase. J. Biol. Chem. 247, 6444-6449.

Townsend, D.M., Tew, K.D., 2003. The role of gluthathione-s-transferase in anti-cancer drug resistance. Oncogene 22, 7369-7375.

Valente, M.A., Faria, J.A., Soares-Ramos, J.R., Reis, P.A., Pinheiro, G.L., Piovesan, N.D., Morais, A.T., Menezes, C.C., Cano, M.A., Fietto, L.G., Loureiro, M.E., Aragao, F.J., Fontes, E.P., 2009. The ER luminal binding protein (BiP) mediates an increase in drought tolerance in soybean and delays drought- induced leaf senescence in soybean and tobacco. J. Exp. Bot. 60, 533-546.

Vallée, B.S., Coadou, G., Labbé, H., Sy, D., Vovelle, F., Schoentgen, F., 2003. Peptides corresponding to the N- and C-terminal parts of PEBP are well- structured in solution: New insights into their possible interaction with partners in vivo. J. Peptide Res. 61, 47-57.

Vicient, C.M., Hull, G., Guilleminot, J., Devic, M., Delseny, M., 2000. Differential expression of the Arabidopsis genes coding for Em-like proteins. J. Exp. Bot. 51, 1211-1220.

Wang, C.C., Tsou, C.L., 1993. Protein disulfide isomerase is both an enzyme and a chaperone. FASEB J. 7, 1515-1517.

Werner, A.K., Medina-Escobar, N., Zulawski, M., Sparkes, I.A., Cao, F.Q., Witte,C.-P., 2013. The ureide-degrading reactions of purine ring catabolism employ three amido hydrolases and one amino hydrolase in Arabidopsis, soybean, and rice. Plant Physiol. 163, 672–681. doi:10.1104/pp.11 3.224261.

Xiao, J., Zhang, H., Niu, L., Wang, X., 2011. Efficient screening of a novel antimicrobial peptide from *Jatropha curcas* by cell membrane affinity chromatography. J. Agric. Food Chem. 59, 1145-1151.

Yeung, K.C., Rose, D.W., Dhillon, A.S., Yaros, D., Gustafsson, M., Chatterjee, D., McFerran, B., Wyche, J., Kolch, W., Sediviy, J.M., 2001. Raf Kinase inhibitor protein interacts with NF-B-inducing kinase and TAK1 and inhibits NF-B activation. Mol. Cell. Biol. 21, 7207-7217.

Yurchenko, O.P., Weselake, R.J., 2011. Involvement of low molecular mass soluble acyl-CoA-binding protein in seed oil biosynthesis. N. Biotechnol. 28, 97–109.

Zancani, M., Peresson, C., Biroccio, A., Federici, G., Urbani, A., Murgia, I., Soave, C., Micali, F., Vianello, A., Macri, F., 2004. Evidence for the presence of ferritin in plant mitochondria. Eur. J. Biochem. 271, 3657–3664.

Zang, Y., Geng, X., Wang, F., Liu, Z., Zhang, L., Zhao, Y., Tian, X, Ni, Zh., Yao, Y., Xin, M., Hu, Zh., Sun, Q., Peng, H., 2017. Overexpression of wheat ferritin gene TaFER-5B enhances tolerance to heat stress and other abiotic stresses associated with the ROS scavenging. BMC Plant Biol. 17:14, doi: 10.1186/s12870-016-0958-2.

Zhang, Y., Yang, Q., Li, Ch., Ding, M., Lv, X., Tao, Ch., Yu, H., Chen, F., Xu, Y., 2017. Curcin C, a novel type I ribosome-inactivating protein from the post- germination cotyledons of *Jatropha curcas*. Amino acids, 2456-8, doi: 10.1007/s00726-017-2456-8.

Chapter 4

Chemical and functional characterization of major protein fractions extracted from non-toxic *Jatropha curcas* byproduct meals.

This chapter has been submitted to the Journal of American Oil Chemists Society as: León-Villanueva A., Espinosa-Alonso L. G., Valdez-Morales M., Barba de la Rosa A. P., Medina-Godoy S., Chemical and functional characterization of major protein fractions extracted from non-toxic *Jatropha curcas* byproduct meals. JAOCS-18-0012.

Abstract

After oil extraction from energetic seed, deffated meal byproduct became a potential source of proteins. Defatted meal (JCDM) and the protein fractions obtained from *Jatropha curcas* non-toxic seeds were analyzed for its physicochemical and functional properties. Glutelins and globulins were the predominant protein fractions obtained from JCDM (42.03 AND 20.17g $100g^{-1}$ of protein, respectively). Leucine, phenylalanine+tyrosine, and histidine content in JCDM and all fractions meet the recommendation for children according to FAO/WHO (1985). Sulfur-containing amino acids (cysteine and methionine) were over FAO/WHO recommendation except in JCDM and prolamin fraction. The solubility profiles showed minimum values at pH 5–6. The high emulsifying and foaming properties of all the proteins were displayed at alkaline conditions (pH 9.0).

Keywords: Seed storage protein, *Jatropha curcas*, Globulins, Glutelins, Functional properties.

4.1. Introduction

During the last two decades, the increased interest in *Jatropha curcas* L., a plant of the family *Euphorbiaceae*, is due to the high oil content (40–60%) of its seeds, as its use as an alternative biodiesel source. The biodiesel production generates two byproducts of the oil extraction namely the protein-rich press cake and the seed coat, which also offer opportunities for adding value to the crop. In the future, large-scale *Jatropha* cultivations projected will result in the availability of high amounts of pressed seed cake or kernel meal, which are rich in proteins of high quality (Marrufo-Estrada et al., 2013). Some research has been conducted to extract protein from *Jatropha* meal in combination with a detoxification process to produce protein concentrates and protein isolates (Devappa and Swamylingappa, 2008; Makkar et al., 2008) to assess the possibility of its use in food formulations or animal diets.

Jatropha seed protein utilization for non-food applications and techno-functional properties have been reported (Gofferjé et al., 2015; Hamarneh et al., 2010; Lestari et al., 2011; Xiao et al., 2015). However, few studies have been conducted to determine the potential of *Jatropha* protein isolate as a food ingredient (Saetae et al., 2011) or the inclusion of detoxified kernel meal in human or animal nutrition (Kumar et al., 2010).

Although almost all the *Jatropha* species so far studied have been found to be toxic, edible or non-toxic provenances have been reported, to the best of our knowledge only in Mexico; characterized by free or negligible amounts of phorbol esters content (Makkar and Becker, 2009). This would allow that seed meal from edible varieties could be used as an economic source of protein feed and food resource. It has also been proven that protein isolates from these non-toxic genotypes are a good substrate for the production of protein hydrolysates as a source of bioactive peptides with beneficial biological activity (Marrufo-Estrada et al., 2013).

Extraction and isolation of seed proteins is the first step to integrating them as food ingredients or additives, therefore, studies of the functional properties of new protein sources can provide valuable information on the potential effectiveness of them during production and processing of food products. There is a lack of information in the literature on the functional properties of proteins from non-toxic *Jatropha curcas* seed cake. This study aimed to investigate the extraction and characterization of the proteins contained in *Jatropha curcas* defatted meal (JCDM) and test their functional properties such as solubility, water/oil holding capacity, emulsifying activity and emulsion stability, foaming capacity and foam stability over a wide range of conditions that are relevant to food systems.

4.2. Materials and methods

4.2.1. Starting materials

Ecotype Puebla non-toxic *Jatropha curcas* seeds (voucher specimen numbered 53203) -considered as non-toxic material-(Makkar et al., 1997; Martinez-Herrera et al., 2012; Martínez-Herrera et al., 2006), were obtained from ripe fruits from plants cultivated in La Esmeralda experimental field of the Agricultures Association of West Sinaloa River (AARSP, Guasave, Sinaloa, Mexico). A commercial soy protein isolate was use as a reference (Cat. No. YP928A, America Alimentos, Jalisco, Mexico). All reagents were analytical grade and were purchased from J.T. Baker (Phillipsburg, NJ, USA), Sigma (Sigma-Aldrich Chemical Co., St Louis, MO, USA), Invitrogen (Invitrogen Corp, Carlsbad, CA) and Bio-Rad (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Pure corn oil was purchased from a local supermarket and was used to prepare oil-in-water emulsions.

4.2.2. Sample preparation

4.2.2.1 Press cake and *Jatropha curcas* meal

Non-toxic *Jatropha curcas* seed press cake (PC) was produced after oil pressing from de-hulled seeds (kernels) using a screw-press. Press cake was collected and air-dried under ambient conditions and milled to a small particle size (40-mesh) using a Cyclotec 1093 mill (FOSS Tecator, Sweden), this become *Jatropha curcas* meal (JCM).

4.2.2.2 *Jatropha curcas* defatted meal

Jatropha curcas defatted meal (JCDM) was produced from *Jatropha curcas* meal (JCM) by further oil extraction with n-hexane (34 mL g^{-1}) for 4 h with agitation and air-dried in a fume hood after decantation of the hexane. Dry JCM and JCDM were kept in plastic bags at 4°C until used.

4.2.3. Proximate composition analysis

The moisture, fat, protein (%N x 6.25), crude fiber and ash contents of JCM and JCDM were analyzed using the standard AOAC (2005) method numbers of 930.15, 920.39, 990.03, 978.10, 942.05, respectively. Nitrogen free materials (NFM) were calculated by difference. Fat, crude fiber, ash, protein and NFM results were expressed in dry matter. All the measurements were made in triplicate and the mean values reported.

4.2.4. Protein sequential fractionation based on solubility criteria

Protein fractions were sequentially extracted according to a modified Osborne procedure (Ribeiro et al., 2004) as follows. For the water-soluble proteins extraction, suspensions of JCDM/distilled water (34 mL g^{-1}) containing 0.01 M CaCl2 and 0.01 M MgCl2 were stirred for 4 h at 4 °C and centrifuged at 30,000xg for 1 h at 4 °C. After dialyzing extensively (3 kDa MW cut-off, Sigma-Aldrich) against distilled water for 24 h, the supernatant was centrifuged again, and then freeze-dried to produce the albumin fraction. To produce the globulin fraction, the pellet obtained by the first centrifugation was re-suspended in a solution of 0.1 M Tris-HCl buffer pH 7.5, containing 10% NaCl (w

v^{-1}), 0.01 M EDTA and 0.01 M EGTA, (34 mL g^{-1}), and the resulting suspension stirred for 4h at 4 °C. After centrifugation under the same conditions as described earlier, the supernatant was dialyzed and centrifuged again, the corresponding precipitate was then freeze-dried.

The insoluble pellet resulting from the salt extraction was then treated with 75% ethanol (5 mL g-1), stirred for 2 h at 4 °C and centrifuged (30,000xg, 15 min, 4 °C). The supernatant was dialyzed against distilled water and freeze-dried to obtain the prolamin fraction. The insoluble pellet from the previous extraction was treated with 0.05 M sodium borate buffer, pH 10, containing 1% β-mercaptoethanol and 1% SDS (5 mL g-1 of dry mass). The suspension was stirred for 2 h at room temperature and centrifuged (30,000xg, 15 min, 20 °C). After dialysis against distilled water, the fraction (glutelins) was freeze-dried.

For total soluble protein content (control), the protein samples were dispersed in 0.1 N NaOH with magnetic stirring and the centrifuged under the same conditions.

4.2.5. Determination of amino acid profile

The JCDM and the extracted protein fractions were hydrolyzed in order to determine the amino acid profile. The protein hydrolysis was carried out using 6 M HCl at 110 °C for 24 h. For cysteine analysis, the samples were oxidized using performic acid followed by gas phase hydrolysis at 110 °C for 24 h. For tryptophan analysis, 5 M NaOH was used for liquid hydrolysis of the samples at 110 °C for 24 h. After hydrolysis, all amino acids were determined by an amino acid analyzer (Waters Acquity UPLC) using an AccQTag Ultra chemistry column. Samples were analyzed in duplicate and results are reported as an average.

4.2.6. SDS-PAGE analysis of protein fractions

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a discontinuous buffering system with 12% separating gel and 4% stacking gel (Laemmli, 1970) in a Mini-Protean III Apparatus (Bio-Rad, Hercules, CA, USA). Aliquots of freeze-dried protein fractions (1 mg mL^{-1}) were solubilized in loading buffer. For reducing condition, β-mercaptoethanol was added (0.05 mL mL^{-1} final concentration) to the samples before heating them at 100 °C for 5 min followed by centrifugation at 10,000xg for 10 min. For each sample, 10 µL of supernatant were loaded onto each lane. A protein molecular weight ladder (wide range 10-220 kDa BenchmarkTM Unstained Protein Ladder, Invitrogen Corp, Carlsbad, CA) was also run on the gel, to allow molecular weight determination. The electrophoresis was performed at constant current of 15 mA in stacking gel and 25 mA in separating gel until the tracking dye reached the bottom of the gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 and distained until background become clear, and protein bands were visible. Gel was scanned using a ChemiDocTM XRS+ Molecular imager® and analyzed using GelAnalyzer 2010a software (Bio-Rad, Hercules, CA, USA), to estimate the molecular weight of protein bands.

4.2.7 Functional properties

4.2.7.1. Determination of solubility

The solubility of the samples was determined as a function of pH, using the method of Aluko and Yada (1995), with some modifications. Briefly, protein (0.01 g mL^{-1}) dispersions were prepared in 0.01 M Na2HPO4. The pH of the solutions was carefully adjusted from pH 2.0 to 10.0 with either 0.1 N HCl or 0.1 N NaOH. After 1 h of stirring at 25 °C, the dispersions were centrifuged at 4,000xg for 20 min. The protein content in the supernatants was determined by the Bradford method using bovine serum albumin (BSA) as the standard. The protein solubility was determining by dividing the protein content in the supernatant by the total protein content in the control used.

4.2.7.2. Determination of water holding and oil absorption capacities

The water holding capacity (WHC) and oil absorption capacity (OAC) were determined using the method of Tomotake et al. (2002) with minor modifications. One gram of protein was weighed into pre-weighed centrifuge tube and 10 ml of Milli-Q water was added. The mixture was vortexed for 1 min and allowed to stand for 30 min at ambient temperature and then centrifuged at 5000xg for 30 min. The supernatant was decanted, carefully, and the tube with sediment was weighed again. WHC was calculated as the amount of water held by one gram of sample. The OAC was determined using similar procedure using 0.5 g of protein and 5 ml pure corn oil. The OAC was expressed as the amount of oil per gram of sample.

4.2.7.3. Emulsifying Properties

Emulsifying activity index (EAI) and emulsifying stability index (ESI) were determined by the turbidimetric method of Pearce and Kinsella (1978) with some modifications, 3 mL of protein solution (1.0 mg mL-1) was dispersed in 0.05 M Tris-HCl buffer (pH 7.5). A total of 1 mL of pure corn oil was then added to this dispersion and the pH was adjusted to 2, 3, 5, 7 or 9 using 0.1 M HCl or 0.1M NaOH. The emulsion was obtained by homogenizing the mixture in a T10 basic Ultra Turrax with shaft S10N-8G (IKA, Wilmington, NC, USA) for 1 min at a speed of 25,000 rpm. A total of 50 µL of the emulsion was pipetted from bottom into 5 ml of SDS solution (0.001 g mL-1), immediately (0 min) and 10 min after homogenization. The absorbance of the dilute emulsion was recorded at 500 nm wavelength at specific intervals using a MultiskanTM Go spectrophotometer (Thermo Scientific, USA). The turbidity of emulsion was calculated using Eq. (1)

$$T = 2.303 \times \frac{A}{L} \times D \quad (1)$$

where T is the turbidity of emulsion in m-1, A is the absorbance (dimensionless), D is the dilution factor (dimensionless) and L is the light path length in m. The emulsifying

activity index (EAI, expressed in $m^2\ g^{-1}$) of proteins and emulsion stability index (ESI, expressed as %) were calculated using Eqs. (2) and (3), respectively:

$$\mathrm{EAI} = \frac{2 \times T_0}{\phi \times C \times 1000} \tag{2}$$

where T_0 is the turbidity of the fresh emulsion in m^{-1}, ϕ is the oil volume fraction (dimensionless), C is the concentration of protein in the dispersion (mg mL^{-1})

$$\mathrm{ESI}\ \%_{30\,\mathrm{min}} = \frac{A_{500\mathrm{nm},\ 30\ \mathrm{min}}}{A_{500\mathrm{nm}}} \times 100\% \tag{3}$$

where $A_{500\mathrm{nm}}$ = absorbance of the fresh emulsion and $A_{500\ \mathrm{nm},\ 30\ \mathrm{min}}$ = absorbance of the emulsion after 30 min.

2.8.4. Foaming properties

Foaming properties tests were performed based on the method of Lestari et al. (2011) whipping a 0.5% protein solution at room temperature in a pH range from 2.0 to 9.0. Foam expansion (FE) and foam stability volume (FVS) were then calculated using equations 4 and 5 as follows:

$$\mathrm{FE}\% = \frac{Vf_0}{Vl_i} \times 100\% \tag{4}$$

$$\mathrm{FVS}\%_{30\,\mathrm{min}} = \frac{Vf_{30\,\mathrm{min}}}{Vf_0} \times 100\% \tag{5}$$

where V_{li}=initial liquid volume; V_{f0}= foam volume created and V_{f30min}= foam volume at 30 min.

4.2.8. Statistical analyses

ANOVA was used to compare the means. The means were obtained from triplicate determinations for all the above-mentioned variables. The EAI and ESI were measured on three different days, producing on each day three different emulsions of the same sample, and taking three aliquots (replicates) of each emulsion. The results are given as the mean ± standard deviation. The significant difference between two means was calculated using the Tukey method at 95% confidence level ($p < 0.05$).

4.3. Results and discussion

4.3.1. Chemical characterization

4.3.1.1. Proximate composition

Proximate composition of whole and defatted meal from non-toxic *J. curcas* is shown in **Table 7.** The chemical composition analysis results did not differ from those reported by Makkar et al. (2011), and Peralta-Flores et al. (2012). In the whole meal, protein and fat (253.8 and 546.1 g kg^{-1}, respectively were the principal components; while for defatted meal were protein and NFM (571.3 and 232.7 g kg^{-1}, respectively).

The protein content of *Jatropha curcas* meal is similar to other sources of the same *Euphorbiaceae* family such *Ditaxis heterantha* and *Ricinodendrum heudelotii* (Espino-Sevilla et al., 2013; Tchiegang et al., 2006). Saetae et al. (Saetae et al., 2011) reported a high protein (235 g kg^{-1}) and fat content (148 g kg^{-1}) for seed cake which was lower than that reported in seed meals.

Comparing with other sources used in food industry, non-toxic *Jatropha curcas* protein content was found to be higher than amaranth (150- 180 g kg^{-1}) and sunflower proteins (200 g kg^{-1}) (Barba de la Rosa et al., 1992; Pérez et al., 2005) but lower than soybean (365-432 g kg^{-1}) (Sharma et al., 2013). After hexane extraction, the oil content of meal was reduced from to 546.1 g kg^{-1} to 53 g kg^{-1}.

Although protein content in seed cake or meals could often differ because of differences in cultivars, growth conditions, climate and soil, as well as oil extraction methods, non-toxic *Jatropha curcas* may serve as a potential non-conventional source of plant protein.

Table 7. Proximate composition (% in dry matter) of *Jatropha curcas* meal (JCM) and *Jatropha curcas* defatted meal (JCDM) from non-toxic genotype.

Component (%)	*Jatropha curcas* meal	
	Whole	Defatted
Moisture	4.58 ± 0.05	7.81 ± 0.23
Fat	54.61 ± 0.17	5.30 ± 0.22
Protein	25.38 ± 0.20	57.13 ± 0.14
NFM	10.48 ± 0.56	23.27 ± 0.21
Fiber	5.30 ± 0.10	4.42 ± 0.19
Ash	4.23 ± 0.24	9.89 ± 0.15

Means ± standard deviations (*n*=3).

4.3.1.2. Seed storage protein fractionation

The Osborne (1924) solubility-based protein fractionation data (Table S1) indicated that glutelin (NaOH-soluble) and globulin (NaCl-soluble) proteins were the predominant fractions in non-toxic Jatropha curcas seed, accounting for 420.3 g kg^{-1} and 201.7 g kg^{-1}, respectively. While, albumin fraction (water-soluble proteins) represented the 123.5 g kg^{-1} and prolamin fraction (ethanol-soluble) the 62.1 g kg^{-1}. In other words, the main storage proteins in *Jatropha curcas* seeds were glutelins and globulins accounted for the 622 g kg^{-1} of total protein. This profile is similar with that found by Selje-Assmann et al. (2007) using as raw material heat-treated defatted flour from both toxic and non-toxic varieties of *Jatropha curcas* from Mexico.

Previous works conducted in *Euphorbiaceaes* have reported a protein content of 250 g kg^{-1} for albumin in *Plukenetia volubilis* seed (Sathe et al., 2002) and 488 g kg^{-1} for glutelin in *Detaxis heterantha* (Espino-Sevilla et al., 2013).

As confirmed here by SDS–PAGE analysis (**Figure 7**), the Osborne method shows some inaccuracies as not all the proteins strictly fulfil the solubility criteria (Ribeiro et al., 2004). A recent report of our group, use this storage protein fractionation for identification of the major component using a two-dimensional gel electrophoresis and mass spectrometry approach (León-Villanueva et al., 2018). It was reported that 11s globulin, legumin A and B proteins were detected in albumin fraction. It was also reported globulin and glutelin fractions share typical legumin and vicilin like proteins, due to a common gene origin.

4.3.2. Amino acid composition

The amino acid composition of non-toxic *Jatropha curcas* defatted meal and the four protein fractions are shown in **Table 8**, these results are presented alongside the FAO/WHO (2007) recommended mode of the essential amino acid for (2-5 years old) child and adult. In general, the amino acid composition is comparable to that in the toxic and non-toxic genotypes of *J. curcas* and *J. platyphylla* (Aluko et al. 1995).

It is noteworthy that the levels of all the essential amino acids in defatted meal were higher than the FAO/WHO requirements concerning adults, while only sulphur-containing amino acids, lysine and tryptophan were slightly lower regarding child recommendation. Glutamic and aspartic acid were the most abundant acidic amino acids accounting for 182.5 and 117.5 g kg-1 protein, respectively, which is consistent with most storage proteins (Carbonaro et al., 1997). Although a very similar amino acid pattern was found among the four protein fractions and defatted meal, the globulin fraction contained the highest amount of sulphur-containing amino acids while prolamin contained the highest amount of aromatic amino acids such as phenylalanine and tyrosine.

As a nutritional parameter, according only to FAO/WHO child requirements, due to its low level, tryptophan could be considered as the first limiting amino acid for all *J. curcas* proteins, followed by the sulphur-containing amino acids and, as in most cereals, by lysine.

Table 8. Amino acids composition of JCDM and protein fractions (g 100 g^{-1} protein) from non-toxic *Jatropha curcas* genotype[a].

Amino acid		Protein fraction				FAO/WHO[b]	
	Defatted meal	Albumin	Globulin	Glutelin	Prolamin	Child	Adult
Essential							
Methionine + Cysteine	1.36	3.02	3.60	3.12	1.25	2.50	1.70
Valine	5.20	4.88	2.99	5.12	5.30	3.50	1.30
Isoleucine	4.02	3.20	3.06	2.67	4.52	2.80	1.30
Leucine	7.62	8.46	6.84	7.59	8.20	6.60	1.90
Phenylalanine + Tyrosine	6.98	6.78	6.69	6.58	7.30	6.30	1.90
Histidine	2.62	2.08	2.60	3.01	2.54	1.90	1.60
Lysine	3.00	5.94	5.99	2.34	6.40	5.80	1.60
Threonine	4.74	3.00	3.23	3.20	6.20	3.40	0.90
Tryptophan	0.68	0.99	0.70	0.82	0.50	1.10	0.50
Non essential							
Ser	6.90	5.14	6.50	5.72	6.85		
Arginine	13.79	9.48	13.72	12.75	9.37		
Glutamic acid	18.25	19.92	21.53	19.56	13.55		
Aspartic acid	11.75	12.66	9.80	11.96	9.05		
Proline	2.03	3.53	3.78	4.57	4.73		
Glycine	5.59	4.92	4.47	5.52	7.81		
Alanine	5.47	6.00	4.50	5.47	6.43		

[a] The values reported represent the average of three determinations.

[b] FAO/WHO reference protein, 2007.

ND: Not determined.

4.3.3. Electrophoresis patterns

The different Osborne protein fractions were analyzed by SDS–PAGE reducing conditions (**Figure 7**). A clear cross-contamination between them could be detected. Indeed, some bands of the albumin fraction matched with those present in the globulin and glutelin fractions although their relative amounts were different. This result confirms that overlapping is a major problem for the Osborne fractionation, as found for other seeds (Ribeiro et al., 2004), with this occurring due to partial protein solubilities in the extraction solutions.

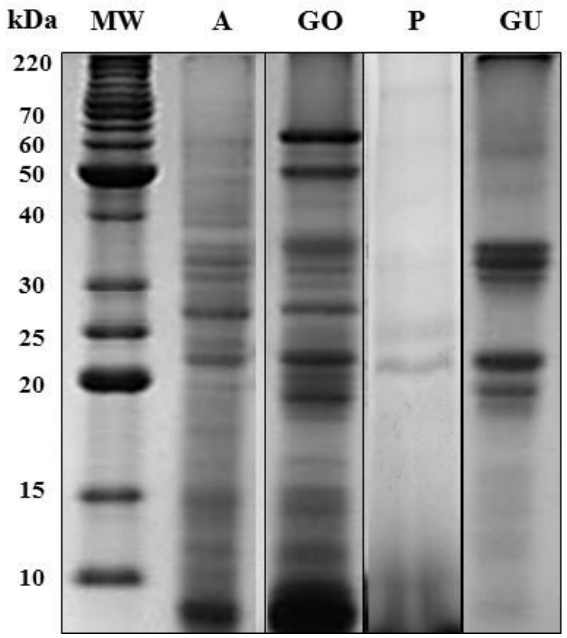

Figure 7. SDS–PAGE band patterns under reducing conditions of non-toxic *Jatropha curcas* proteins: (MW) Molecular weight standards; (A) albumins; (GO) globulins; (P) prolamins; (GU) glutelins.

The albumin and globulin fractions (lanes A and GO) showed many bands in the 13-62 kDa range. Three polypeptides of about 23, 28 and 32 kDa were detected, although in different relative amounts, in both the fractions. In contrast two intense bands at 51 and 64 kDa appeared only in the globulin fraction indicating these proteins were less soluble in water. The prolamin fraction (lane P) showed only few faint bands, which seem to derive from a cross-contamination with the salt-soluble fraction. This bands were barely detectable, confirming the presence of a very low quantity of prolamins in non-toxic *Jatropha curcas* defatted meal.

Finally, the glutelin fraction (lane GU) showed five intense bands at 21, 23, 32, 36 and 38 kDa which matched with some of the albumin or globulin fractions. Using non-reducing conditions (data not shown) did not alter the proteins band profiles significantly, which suggests minimal concentrations on disulfide bonds in the proteins. The polypeptide composition of the different fractions is similar with the results of Peralta-Flores et al., 2012.

4.3.4. Functionality tests

4.3.4.1. Protein solubility (PS)

Solubility is an important prerequisite for a protein in order to be useful as a functional ingredient in foods. Solubility is a physicochemical property of a protein that critically affects texture, color and sensory properties of products including emulsifying, foaming and gel forming properties (Adebiyi and Aluko, 2011). Solubility of proteins depends on amino acid composition, molecular weight and surface characteristics of constituent amino acids and environmental factors such as pH (Lestari et al., 2011).

The PS profiles of non-toxic *Jatropha curcas* defatted meal and protein fractions are exhibited in **Figure 8**. All the samples showed a similar trend as U-shaped curves, the solubility decreased as the pH reached the isoelectric point (between pH 5.0-6.0) where all the samples presented minimum solubility values: albumin (59.7%), globulin (32.9%), glutelin (19.0%) and defatted meal (5.3%). Protein solubility increased above and below the isoelectric point, this may attribute to the loss of electrostatic repulsive forces, leading to the neutral charge of protein molecules (Chavan et al., 2001).

Similar results were also observed in proteins from different plants (Deng et al., 2011; Espino-Sevilla et al., 2013; Lawal et al., 2005). Lestari et al.,2011; reported that the minimum solubility of *Jatropha* press cake and de-oiled press cake occurred at pH 5.0, while Saetae et al. (2011) report a minimum solubility at pH 4.0 for protein isolate from the same source.

The maximum protein solubility of albumin (89.4%) and glutelin (85.1%) was observed at pH 12.0 while globulin (90.6%) and defatted meal (86.5%) was observed at pH 10 and pH 11.0, respectively. Bigelow (1967) suggested that the presence of a low number of hydrophobic residues, elevated charge, electrostatic repulsion, and ionic hydration could promote the solubility of protein, which was in accordance with the findings for the albumin fraction which solubility was greater than those of globulin and glutelin in a broad range of pH, indicating potential applications in aqueous systems, while globulin and glutelin are more suitable to high-alkaline or high-acid environments; so they could be considered as a valuable ingredient for the formulation of acidic fruit juice or protein-fortified beverages.

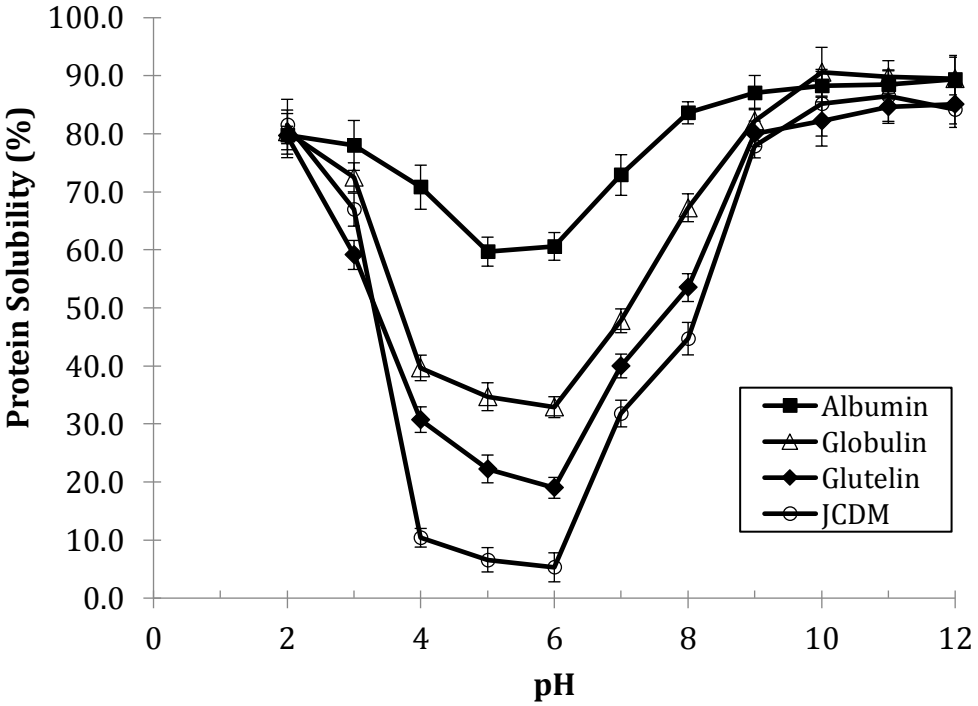

Figure 8. Solubility of non-toxic *Jatropha curcas* defatted meal (JCDM) and protein fractions as a function of pH.

4.3.4.2. Water holding and oil absorption capacities

The water holding capacity (WHC) and the oil absorption capacity (OAC) are described as the ability of a protein to hold an amount of added water or oil. The WHC of proteins is a function of several parameters, including size, shape, steric factors, conformational characteristics, hydrophilic-hydrophobic balance of amino acids in the protein molecules as well as lipids, carbohydrates and tannins associated with them. In the current study the WHC of the proteins was found to be in the following order: Soy protein isolate, SPI (4.17 g g^{-1}) > JCDM (2.22 g g^{-1}) > albumin (2.15 g g^{-1}) > glutelin (1.80 g g^{-1}) > globulin (1.39 g g^{-1}) (Table S2).

The WHC of defatted meal is similar to that observed by Xiao et al. (2015) in detoxified *J. curcas* meal and higher than that reported by Saetae et al. (2011) in protein isolate, and it is also in the range reported for defatted flours of some cereal, dry bean and tree nut seeds commercially sold; but lower than those reported for some oilseed defatted flours or protein isolates (Kaushik et al., 2016; Mundi and Aluko, 2012).

The albumin fraction showed a high WHC compared with cowpea protein isolate (1.68 g g^{-1}) (Mwasaru et al., 1999) and sesame protein isolate (1.5 g g^{-1}) (Khalid et al., 2003) which have been widely studied. This high WHC in the albumins may be attributed to a

more open structure and greater flexibility that enhances interaction with water when compared to the more globular structure like in the globulins.

The oil absorption capacity (OAC) could influence emulsifying capacity, a highly desirable characteristic of proteins and protein products. The OAC is also important for the development of new food products with flavor binding properties and ability to reduce rate of oxidative rancidity since high OAC allows the stabilization of high fat food products.

In this study, the results showed that the OAC of JCDM (3.74 g g^{-1}) was higher than that observed by Xiao et al.(2015) and Saetae et al.(2011), this high OAC could be attributed to a more hydrophobic nature of the proteins, which are involved in oil absorption; and is also reflected in the high AOC values of the globulin (3.03 g g^{-1}), glutelin (2.47 g g^{-1}) and albumin (2.41 g g^{-1}) fractions, all these higher than the AOC of SPI (2.35 g g^{-1}).

4.3.4.3. Emulsifying properties

Emulsifying properties of protein depends on the hydrophilic-lipophilic balance. At the oil-water interphase, the protein orients lipophilic residues to the oil phase and hydrophilic residues to the aqueous phase. The net charge of the lipophilic-hydrophilic interphase determines the emulsifying properties.

Emulsifying activity index (EAI) expresses the interfacial area stabilized per unit weight of protein, characterizing the ability of a protein to absorb to the oil-water interface therefore the capacity to aid formation and stabilization of a newly created emulsion preventing flocculation and coalescence.

The effect of pH on the emulsifying properties in terms of emulsifying activity of DJCM and protein fractions is show in **Figure 9A** and the results were compared with a commercial soy protein isolate. The lowest EAI values of albumin (48.2 m2 g^{-1}), globulin (63.3 m^2g^{-1}), glutelin (67.4 m^2g^{-1}), and JCDM (38.7 m^2g^{-1}) were obtained at near isoelectric point, which is similar to the trends of protein solubility-pH profiles; SPI also showed the lowest EAI value of 63.4 m^2g^{-1} at pH 5. These results were in agreement with the behaviour reported by Lestari et al. (2011) and Saetae et al. (2011) for *Jatropha* press cake, de-oiled press cake and protein isolate, respectively. Deng et al. (2011) and Zhong et al. (2012) found a similar trend for protein isolates from *Ginkgo biloba* and *Caragana koeshinskii* respectively.

At alkaline conditions (pH 9.0), EAI increased and reached the highest value, with values of 193.9 m^2g^{-1} for albumin, 231.0 m^2g^{-1} for globulin, 222.2 m^2g^{-1} for glutelin and 152.0 m^2g^{-1} for JCDM, all higher than SPI (133.6 m^2g^{-1}). Dependence of EAI on pH was expected, as it is known that emulsion capacity of a total protein depends upon hydrophilic-lipophilic balance, which is affected by pH.

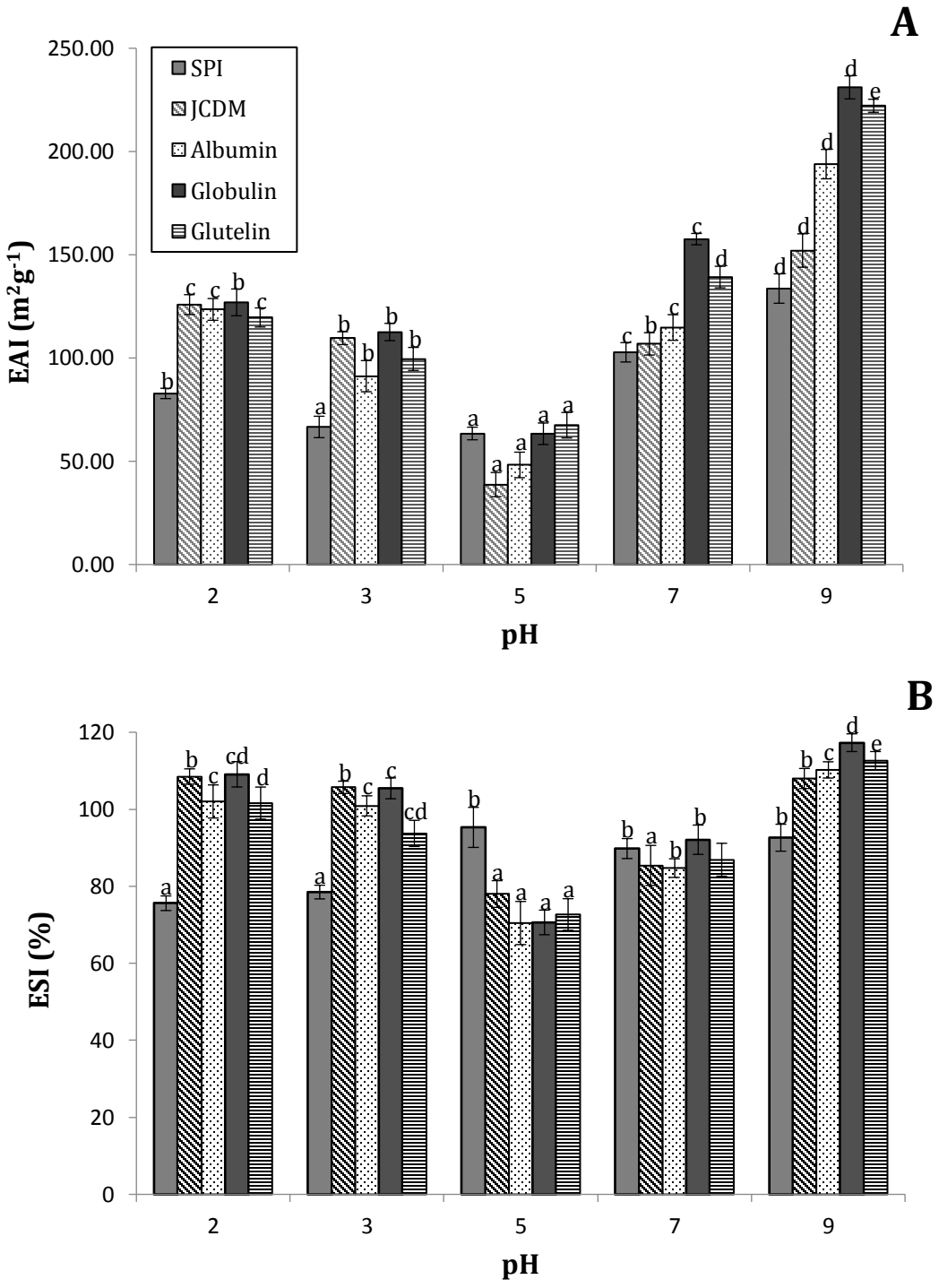

Figure 9. Emulsion activity index (EAI) (A); and emulsion stability index (ESI) (B) of non-toxic *Jatropha curcas* defatted meal (JCDM), protein fractions and soy protein.

Emulsifying stability index (ESI) reflects the ability of the proteins to impart strength to emulsion for resistance to stress, retarding the flocculation and coalescence of the oil globules, and describes the stability in its physicochemical property over a define period of time.

As shown in **Figure 9B**, non-toxic *J. curcas* proteins presented ESI values above 80%, which reflects the high ability to stabilize emulsions. All samples had the higher values isolate (SPI) as a function of pH. Graph represents the mean ± SD (n=3). The bars with different letters are significantly ($p < 0.05$) different, under alkaline conditions (pH 9.0), which is in correspondence with the results reported by Lestari et al. (2011). For its part, Saetae et al. (2011) reported that *Jatropha* protein isolate showed an emulsifying activity index with a similar trend to its emulsion activity with low emulsion stability (12.0-28.8%) under acidic pH condition and it increase (80.0-83.7%) under the basic pH condition. This increment in ESI at high pH might be attributed to the increase in the columbic repulsion between neighbouring droplets, coupled with increased hydration of the charged protein molecules. These factors result in the reduction of interface energy and combination of emulsion droplet, which might account for a higher ESI value (Chavan et al., 2001).

The EAI and ESI values of these proteins indicate that they could serve as potential ingredients in many food formulations, such as salad dressing, sausages, comminuted meats, and mayonnaise, or as alternative emulsifiers for oil/water mixtures.

4.3.4.4. Foaming properties

Foam expansion (FE) is the foam volume formed during a defined period of foaming generation relative to its initial volume, which shows the ability of the protein to form interfacial thin layer between gas bubbles, while foam volume stability (FVS) describes protein ability to prevent bubbles coalescence (Lestari et al., 2011). In addition to adsorption at the air/water interface, the solubility, conformational change and rearrangement at the interface, as well as cohesive viscoelastic film formation via intermolecular interactions are basic requirements for a protein to be a good foaming agent (Bora, 2002).

The effect of pH on FE and FVS of non-toxic *J. curcas* proteins and SPI is shown in **Table 9**. Under acidic and neutral conditions, the FE of the JCDM was low (68–110%) but even at pH 7 (about 96%) was higher than the reported by Lestari et al. (2011) and Xiao et al. (2015). At pH 9, JCDM presented the highest FE (about 385%) of all the proteins evaluated.

Generally, for albumin, globulin, and glutelin the variation of the foaming capacity was pH dependent with the minimum percentage observed at pH 5, which is the point of least protein solubility. The glutelin fraction presented higher values than JCDM and SPI at almost all range of pH but as the rest of the proteins it also had a lower FE value than JCDM at alkaline conditions. Just like in emulsion formation, foaming

expansion at different pH is more likely correlated to conformational state and rheological properties of the protein films. This means that pH will facilitate protein unfolding at the surface, which leads to protein adsorption to the air-water interfacial films.

The values of FVS obtained for JCDM protein were pH dependent showing a correspondence with protein solubility curve (Figure 8), the minimum foaming stability percentage observed was near the isoelectric point and reached the highest value at alkaline conditions (pH 9.0). Similar observations were reported by Lestari et al. (2011) and Saetae et al. (2011); JCDM also showed the ability to maintain a higher FVS than SPI at all the pH range.

As regards to the protein fractions, the higher FVS was found in glutelin followed by globulin; which suggests that in these two fractions the protein–protein interactions were favoured to form strong interfacial membranes at the air–water interface. The exhibited excellent foam stability indicates that these fractions could be used as a substitute to foam forming food proteins.

Table 9. Foam expansion (FE) and foam volume stability (FVS) of proteins from non-toxic *Jatropha curcas* seed: Deffated meal (JCDM), albumin, globulin, glutelin, and soy protein isolate (SPI) at different pH at 20 °C.

Sample	Foaming properties	pH				
		2	3	5	7	9
JCDM	% FE	110.0 ±10.5^{a}	98.0 ± 6.2^{a}	68.0 ± 5.3^{a}	96.7 ±18.2^{a}	385.0 ±12.6^{d}
	% FVS	48.4 ± 3.1^{b}	40.0 ± 4.2^{a}	35.0 ± 5.0^{b}	49.9 ± 4.5^{c}	85.0 ± 6.3^{c}
Albumin	% FE	123.0 ± 6.9^{a}	118.0 ± 6.4^{ab}	109.0 ±10.4^{b}	125.0 ± 4.0^{b}	231.0 ± 5.9^{a}
	% FVS	43.3 ± 4.5^{b}	33.2 ± 3.8^{a}	20.0 ± 2.0^{a}	28.8 ±16.4^{bc}	44.6 ± 6.6^{a}
Globulin	% FE	198.0 ± 3.5^{b}	140.0 ±12.6^{b}	115.0 ± 4.5^{b}	189.0 ± 5.9^{c}	242.0 ± 2.6^{a}
	% FVS	86.0 ± 0.1^{c}	88.8 ± 1.2^{b}	80.0 ± 2.5^{c}	80.0 ± 0.0^{d}	82.5 ± 4.5^{c}
Glutelin	% FE	264.0 ±11.4^{d}	245.0 ±11.8^{c}	120.0 ±18.0^{b}	303.0 ± 6.8^{e}	336.0 ±12.5^{c}
	% FVS	95.0 ± 4.5^{d}	92.1 ± 1.8^{b}	75.1 ± 5.0^{c}	90.8 ± 2.0^{d}	94.6 ± 2.5^{c}
SPI	% FE	236.0 ± 3.7^{c}	220.0 ±15.1^{c}	200.0 ±14.3^{c}	268.0 ±10.7^{d}	307.0 ± 7.4^{b}
	% FVS	26.0 ± 0.2^{a}	31.1 ± 4.7^{a}	45.7 ± 8.0^{b}	19.8 ± 8.2^{a}	65.0 ± 5.0^{b}

Each value in the table is the mean of three determinations ± standard deviation. Means within a column with different superscript lowercase letters are significantly different ($p < 0.05$)

4.4. Conclusions

The physicochemical and functional properties of the defatted meal and major protein fractions from non-toxic *Jatropha curcas* were investigated. The most abundant protein fractions were glutelin and globulin followed by albumin and prolamin. JCDM and its four fractions contained most of the essential amino acids. The subunits of protein fractions were found with molecular weight ranging from 20.0 to 70.0 kDa. In general, JCDM and protein fractions had the best performance at alkaline conditions (above pH 9), due to its high solubility, emulsifying properties and foaming properties. As compared with SPI, JCDM and protein fractions exhibited better oil absorption capacity and emulsifying properties but less water holding capacity. JCDM showed the highest foam capacity at pH 9 while the glutelin fraction showed the best foam volume stability in all the pH range evaluated. The results revealed that JCDM and protein fractions with suitable functional properties could be produced from non-toxic *J. curcas* as a good protein ingredient in food systems. Further studies are needed to devise a procedure to extract these proteins in a food-grade manner and contribute to enhance the financial viability and sustainability of a *Jatropha*-based biodiesel industry.

4.5. Acknowledgments

This work was supported by the National Council of Science and Technology Mexico (CONACYT-MEXICO) Grant 103601. ALV also thanks to CONACYT for fellowship 171851.

4.6. References

Adebiyi, A.P., Aluko, R.E., 2011. Functional properties of protein fractions obtained from commercial yellow field pea (*Pisum sativum* L.) seed protein isolate. Food Chem. 128, 902-908.

Aluko, R.E., Yada, R.Y., 1995. Some Functional Properties of a Cowpea (*Vigna unguiculata*) Globulin Isolate Treated with Transglutaminase. Bioscience, Biotechnology, and Biochemistry 59, 2298-2299.

AOAC, 2005. Official methods of analysis (18th ed.) Association of Official Analytical Chemists, Arlington, VA.

Barba de la Rosa, A.P., Gueguen, J., Paredes-Lopez, O., Viroben, G., 1992. Fractionation procedures, electrophoretic characterization, and amino acid composition of amaranth seed proteins. J. Agric. Food Chem. 40, 931-936.

Bigelow, C.C., 1967. On the average hydrophobicity of proteins and the relation between it and protein structure. J. Theor. Biol. 16, 187-211.

Bora, P.S., 2002. Functional properties of native and succinylated lentil (*Lens culinaris*) globulins. Food Chem. 77, 171-176.

Carbonaro, M., Cappelloni, M., Nicoli, S., Lucarini, M., Carnovale, E., 1997. Solubility−Digestibility Relationship of Legume Proteins. J. Agric. Food Chem. 45, 3387-3394.

Chavan, U.D., McKenzie, D.B., Shahidi, F., 2001. Functional properties of protein isolates from beach pea (*Lathyrus maritimus* L.). Food Chem. 74, 177-187.

Deng, Q., Wang, L., Wei, F., Xie, B., Huang, F., Huang, W., Shi, J., Huang, Q., Tian, B., Xue, S., 2011. Functional properties of protein isolates, globulin and albumin extracted from Ginkgo biloba seeds. Food Chem. 124, 1458-1465.

Devappa, R.K., Swamylingappa, B., 2008. Biochemical and nutritional evaluation of *Jatropha* protein isolate prepared by steam injection heating for reduction of toxic and antinutritional factors. J Sci Food Agric 88, 911-919.

Espino-Sevilla, M.T., Jaramillo-Flores, M.E., Hernández-Gutiérrez, R., Mateos-Díaz, J.C., Espinosa-Andrews, H., Barba de la Rosa, A.P., Rodiles-López, J.O., Villanueva-Rodríguez, S., Lugo-Cervantes, E.C., 2013. Functional properties of *Ditaxis heterantha* proteins. Food Sci. Nutr. 1, 254-265.

FAO/WHO, 2007. Protein and amino acid requirements in human nutrition. Report of a Joint WHO/FAO/UNU Expert Consultation, WHO technical report series no. 935. World Health Organization, Geneva, Switzerland.

Gofferjé, G., Zöttl, A., Stäbler, A., Herfellner, T., Schweiggert-Weisz, U., Flöter, E., 2015. Influence of Protein Extraction Techniques of Different De-oiled Residues from *Jatropha curcas* L. on Protein Recovery and Techno-functional Properties. Waste Biomass Valorization 6, 225-235.

Hamarneh, A.I., Heeres, H.J., Broekhuis, A.A., Picchioni, F., 2010. Extraction of *Jatropha curcas* proteins and application in polyketone-based wood adhesives. Int. J. Adhes. Adhes. 30, 615-625.

Kaushik, P., Dowling, K., McKnight, S., Barrow, C.J., Wang, B., Adhikari, B., 2016. Preparation, characterization and functional properties of flax seed protein isolate. Food Chem. 197, Part A, 212-220.

Khalid, E.K., Babiker, E.E., El Tinay, A.H., 2003. Solubility and functional properties of sesame seed proteins as influenced by pH and/or salt concentration. Food Chem. 82, 361-366.

Kumar, V., Makkar, H.P.S., Becker, K., 2010. Dietary inclusion of detoxified *Jatropha curcas* kernel meal: effects on growth performance and metabolic efficiency in common carp, Cyprinus carpio L. Fish Physiol. Biochem. 36, 1159-1170.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lawal, O.S., Adebowale, K.O., Ogunsanwo, B.M., Sosanwo, O.A., Bankole, S.A., 2005. On the functional properties of globulin and albumin protein fractions and flours of African locust bean (*Parkia biglobossa*). Food Chem. 92, 681-691.

León-Villanueva A., Huerta-Ocampo J.A., Barrera-Pacheco A., Medina-Godoy S. and Barba de la Rosa A.P., 2018. Proteomic analysis of non-toxic *Jatropha curcas* byproduct cake: Fractionation and identification of the major components. Ind Crops Prod 111: 694–704.

Lestari, D., Mulder, W.J., Sanders, J.P.M., 2011. Jatropha seed protein functional properties for technical applications. Biochem. Eng. J. 53, 297-304.

Makkar, H.P.S., Becker, K., 2009. Jatropha curcas, a promising crop for the generation of biodiesel and value-added coproducts. Eur. J. Lipid Sci. Technol. 111, 773-787.

Makkar, H.P.S., Becker, K., Sporer, F., Wink, M., 1997. Studies on Nutritive Potential and Toxic Constituents of Different Provenances of *Jatropha curcas*. J. Agric. Food Chem. 45, 3152-3157.

Makkar, H.P.S., Francis, G., Becker, K., 2008. Protein concentrate from *Jatropha curcas* screw-pressed seed cake and toxic and antinutritional factors in protein concentrate. J. Sci. Food Agric. 88, 1542-1548.

Makkar, H.P.S., Kumar, V., Oyeleye, O.O., Akinleye, A.O., Angulo-Escalante, M.A., Becker, K., 2011. *Jatropha platyphylla*, a new non-toxic *Jatropha* species: Physical properties and chemical constituents including toxic and antinutritional factors of seeds. Food Chem. 125, 63-71.

Marrufo-Estrada, D.M., Segura-Campos, M.R., Chel-Guerrero, L.A., Betancur-Ancona, D.A., 2013. Defatted *Jatropha curcas* flour and protein isolate as materials for protein hydrolysates with biological activity. Food Chem 138, 77-83.

Martinez-Herrera, J., Jimenez Martinez, C., Martinez-Ayala, A., Garduño-Siciliano, L., Mora-Escobedo, R., Dávila-Ortiz, G., Chamorro-Cevallos, G., Makkar, H., Francis, G., Becker, K., 2012. Evaluation of the nutritional quality of nontoxic kernel flour from *Jatropha curcas* L. in rats. J. Food Qual. 35, 152-158.

Martínez-Herrera, J., Siddhuraju, P., Francis, G., Dávila-Ortíz, G., Becker, K., 2006. Chemical composition, toxic/antimetabolic constituents, and effects of different treatments on their levels, in four provenances of *Jatropha curcas* L. from Mexico. Food Chem. 96, 80-89.

Mundi, S., Aluko, R.E., 2012. Physicochemical and functional properties of kidney bean albumin and globulin protein fractions. Food Res. Int. 48, 299-306.

Mwasaru, M.A., Muhammad, K., Bakar, J., Man, Y.B.C., 1999. Effects of isolation technique and conditions on the extractability, physicochemical and functional properties of pigeonpea (*Cajanus cajan*) and cowpea (*Vigna unguiculata*) protein isolates. I. Physicochemical properties. Food Chem. 67, 435-443.

Pearce, K.N., Kinsella, J.E., 1978. Emulsifying properties of proteins: evaluation of a turbidimetric technique. J. Agric. Food Chem. 26, 716-723.

Peralta-Flores, L., Gallegos-Tintoré, S., Solorza-Feria, J., Dávila-Ortíz, G., Chel-Guerrero, L., Martínez-Ayala, A., 2012. Biochemical evaluation of protein fractions from physic nut (*Jatropha curcas* L.). Grasas Aceites 63, 253-259.

Pérez, S.G., Vereijken, J.M., van Koningsveld, G.A., Gruppen, H., Voragen, A.G.J., 2005. Physicochemical Properties of 2S Albumins and the Corresponding Protein Isolate from Sunflower (*Helianthus annuus*). J. Food Sci. 70, C98-C103.

Ribeiro, A.C., Teixeira, A.R., Ferreira, R.B., 2004. Characterization of Globulins from Common Vetch (*Vicia sativa* L.). J. Agric. Food Chem. 52, 4913-4920.

Saetae, D., Kleekayai, T., Jayasena, V., Suntornsuk, W., 2011. Functional properties of protein isolate obtained from physic nut (*Jatropha curcas* L.) seed cake. Food Sci. Biotechnol. 20, 29-37.

Sathe, S.K., Hamaker, B.R., Sze-Tao, K.W.C., Venkatachalam, M., 2002. Isolation, Purification, and Biochemical Characterization of a Novel Water-Soluble Protein from Inca Peanut (*Plukenetia volubilis* L.). J. Agric. Food Chem. 50, 4906-4908.

Selje-Assmann, N., Makkar, H.P.S., Hoffmann, E.M., Francis, G., Becker, K., 2007. Quantitative and qualitative analyses of seed storage proteins from toxic and non-toxic varieties of Jatropha curcas L., International Symposium on Energy and Protein Metabolism. EAAP Publication Vichy, France., p. 625.

Sharma, S., Goyal, R., Barwal, S., 2013. Domestic processing effects on physicochemical, nutritional and anti-nutritional attributes in soybean (*Glycine max* L. Merill). Int. Food Res. J. 20, 3203-3209.

Tchiegang, C., Blandine, L., Kenfack, M., Tenin, D., Ndjouenkeu, R., 2006. Physicochemical and Functional Properties of Defatted Cakes from Two Euphorbiaceae from Cameroon: *Riconodendron Heudelotii* (Bail) and *Tetracarpidium conophorum* (Müll. Arg.). J. Food Technol. 4, 96-100.

Tomotake, H., Shimaoka, I., Kayashita, J., Nakajoh, M., Kato, N., 2002. Physicochemical and Functional Properties of Buckwheat Protein Product. J. Agric. Food Chem. 50, 2125-2129.

Xiao, J., Zhang, H., Niu, L., 2015. Effect of Detoxification on Conformational and Functional Properties of *Jatropha curcas* Proteins. Int. J. Food Prop. 18, 1524-1534.

Zhong, C., Wang, R., Zhou, Z., Jia, S.-R., Tan, Z.-L., Han, P.-P., 2012. Functional Properties of Protein Isolates from *Caragana korshinskii* Kom. Extracted by Three Different Methods. J. Agric. Food Chem. 60, 10337-10342.

Chapter 5 General conclusions and recommendations

SDS–PAGE analysis confirmed that the Osborne method shows some inaccuracies in non-toxic *J. curcas* byproduct cake fractionation, as not all the proteins strictly fulfil the solubility criteria. 11s globulin, legumin A and B proteins were detected in albumin fraction. It was also reported that globulin and glutelin fractions share typical legumin and vicilin like proteins, due to a common gene origin.

Seed storage proteins were mainly represented by the glutelins fraction (42.03%) followed by globulins (20.17%), which is different to all legumes where the main fraction is globulins. These fractions showed 7 and 24 protein species respectively, with several proteoforms representing each protein species. Glutelins, was composed mainly of high molecular weight globulins, and globulins fraction was composed mainly by legumin B-like, 2S albumin-like and germin-like protein, curcin 1 and enzymes related to energy production such as ATP synthase and luminal-binding protein. Most of enzymatic proteins identified were found in albumins fraction and were related to nutrient reservoir; sugar, fatty acid, amino acids and purine metabolism; detoxification, plant resistance and growth regulation.

The chemical composition of the *Jatropha* meal and protein fractions produced from non-toxic *J. curcas* byproduct cake, were similar to previous reports in other non-toxic provenances, the amino acid composition was not different and levels of essential amino acids, except lysine, were higher than that of the FAO/WHO reference protein and comparable to commercial soy isolates.

Defatted meal and proteins fractions showed suitable functional properties to take them in consideration as a good protein ingredient in food systems. In general, they had the best performance at alkaline conditions (above pH 9), due to its high solubility, emulsifying and foaming properties. As compared with soy protein isolate, exhibited better oil absorption capacity and emulsifying properties but less water holding capacity. Defatted meal showed the highest foam capacity at pH 9 while the glutelin fraction the best foam volume stability in all the pH range evaluated.

If *Jatropha curcas* L. commercial interest due its importance as a feedstock in the biodiesel production persists, the generation of high amounts of pressed seed cake or kernel meal will be produced. Marketing these byproducts could considerably increase the value of the jatropha culture system and its global economy could be improved if non-toxic varieties are used or if the oil cake could be detoxified.

There are reports that non-toxic *Jatropha* accessions could be as prolific as the elite toxic accessions as far as early seed productivity is concerned. Kernel meal from these sources could be considered a promising source of vegetal protein. The extraction and isolation of these proteins could be the first step to integrate them as food ingredients or additives.

In Mexico, thousands of hectares in different states are currently being used to cultivate *J. curcas* from imported seeds, these seeds are of the toxic variant. Instead of promoting the investing in large industrial detoxification factories; it would be cheaper, easier and safer to promote the identification, study, propagation and cultivation of non-toxic endemic varieties. Moreover, the standardization of the methods used in the determination of phorbol esters and other phytochemicals, as well as feeding studies with these materials must be conducted to ascertain the innocuous nature of future *Jatropha* products.

6. Supplementary information.

Table 10. Supplementary S1. Proportion of different protein fractions of non-toxic *Jatropha curcas* defatted meal.

Table S1

Protein fraction	Proportion (%)
Albumins	12.35 ± 0.18
Globulins	20.17 ± 0.12
Prolamins	6.21 ± 0.10
Glutelins	42.03 ± 0.25
Insoluble residue	19.25 ± 0.17

% of each fraction in relation to the total protein content in the meal. Means ± standard deviations (*n*=3).

Table 11. Supplementary S2. Water absorption and oil-absorption capacities of *J.curcas* non-toxic defatted meal, protein fractions and soy protein isolate at pH 7.0 [a].

Table S2

Protein	WAC (g/g)	OAC (g/g)
Defatted meal	2.22 ± 0.07^{b}	3.74 ± 0.80^{a}
Albumin	2.15 ± 0.09^{b}	2.41 ± 0.15^{b}
Globulin	1.39 ± 0.16^{d}	3.03 ± 0.12^{ab}
Glutelin	1.80 ± 0.06^{c}	2.47 ± 0.10^{b}
Soy protein isolate	4.17 ± 0.14^{a}	2.35 ± 0.50^{b}

[a]Means ± standard deviations (*n*=3).
Means within a column with different superscript lowercase letters are significantly different ($p < 0.05$)

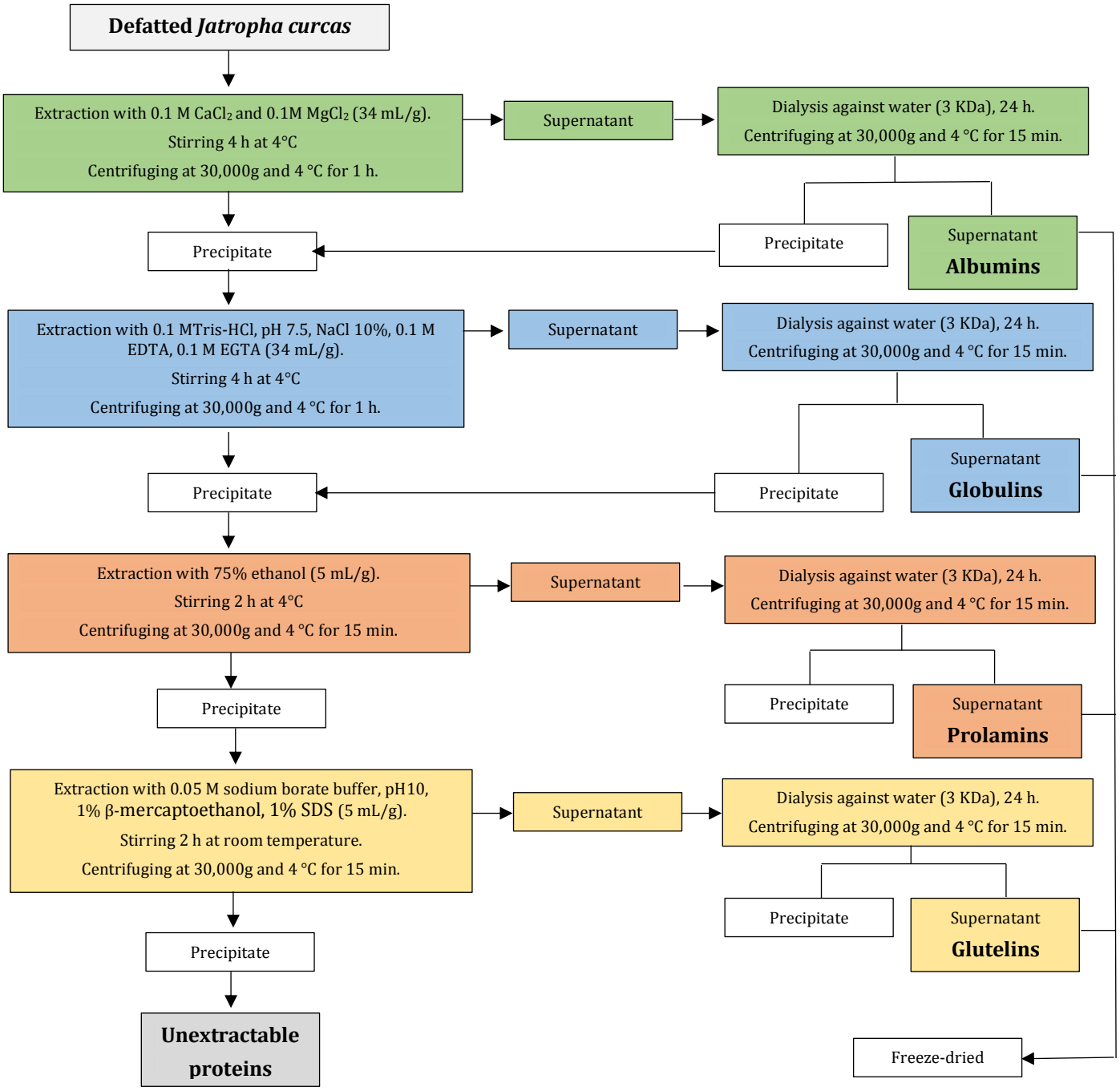

Figure 10. Supplementary S1. Scheme of non-toxic *Jatropha curcas* protein fraction (albumins, globulins, prolamins, and glutelins) extraction according to a modified Osborne´s procedure.

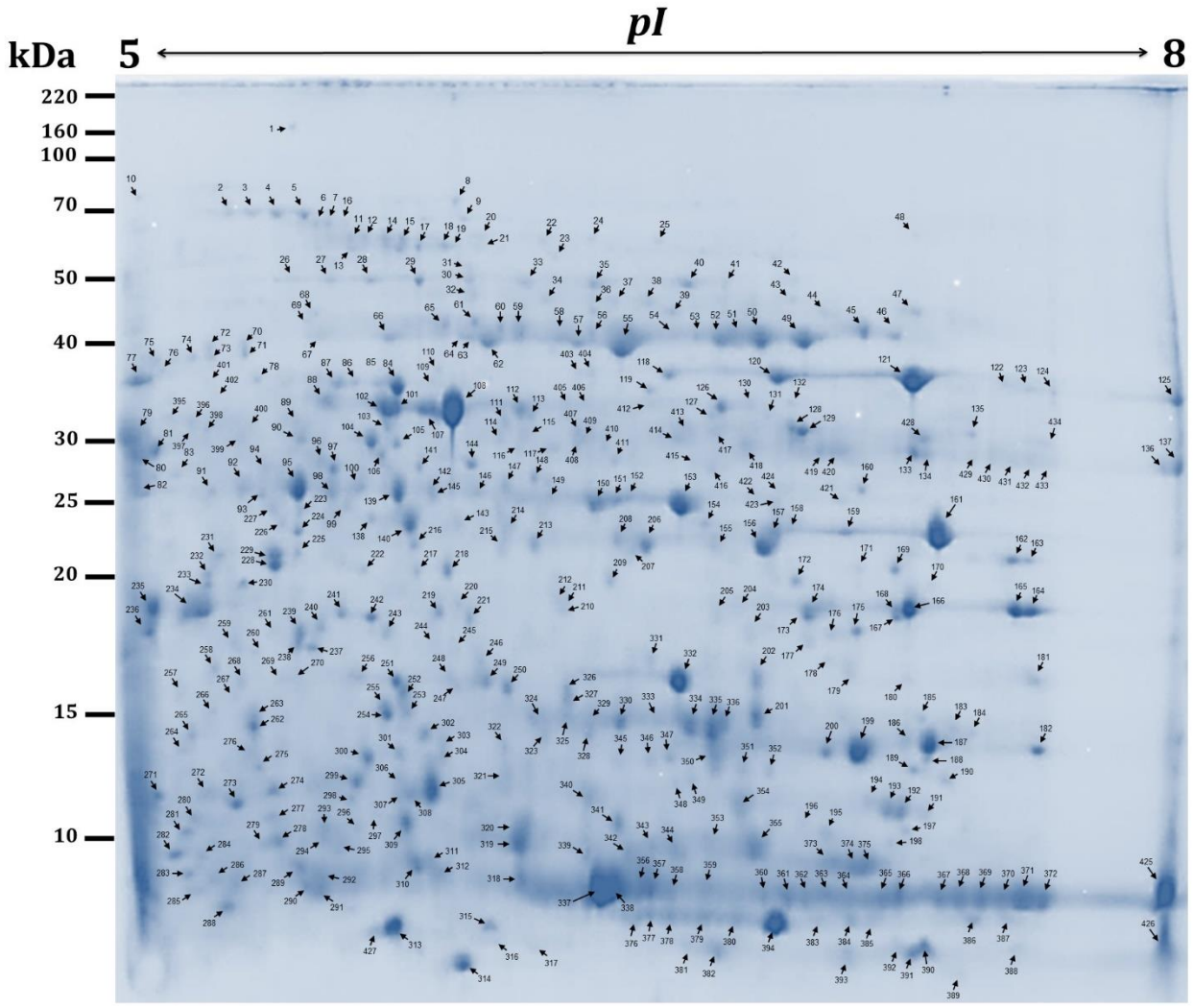

Figure 11. Supplementary S2. Representative 2-DE gel of *Jatropha curcas* albumins fraction. Numbers indicates the spot number assigned for LC-MS/MS analysis reported in Table 4.

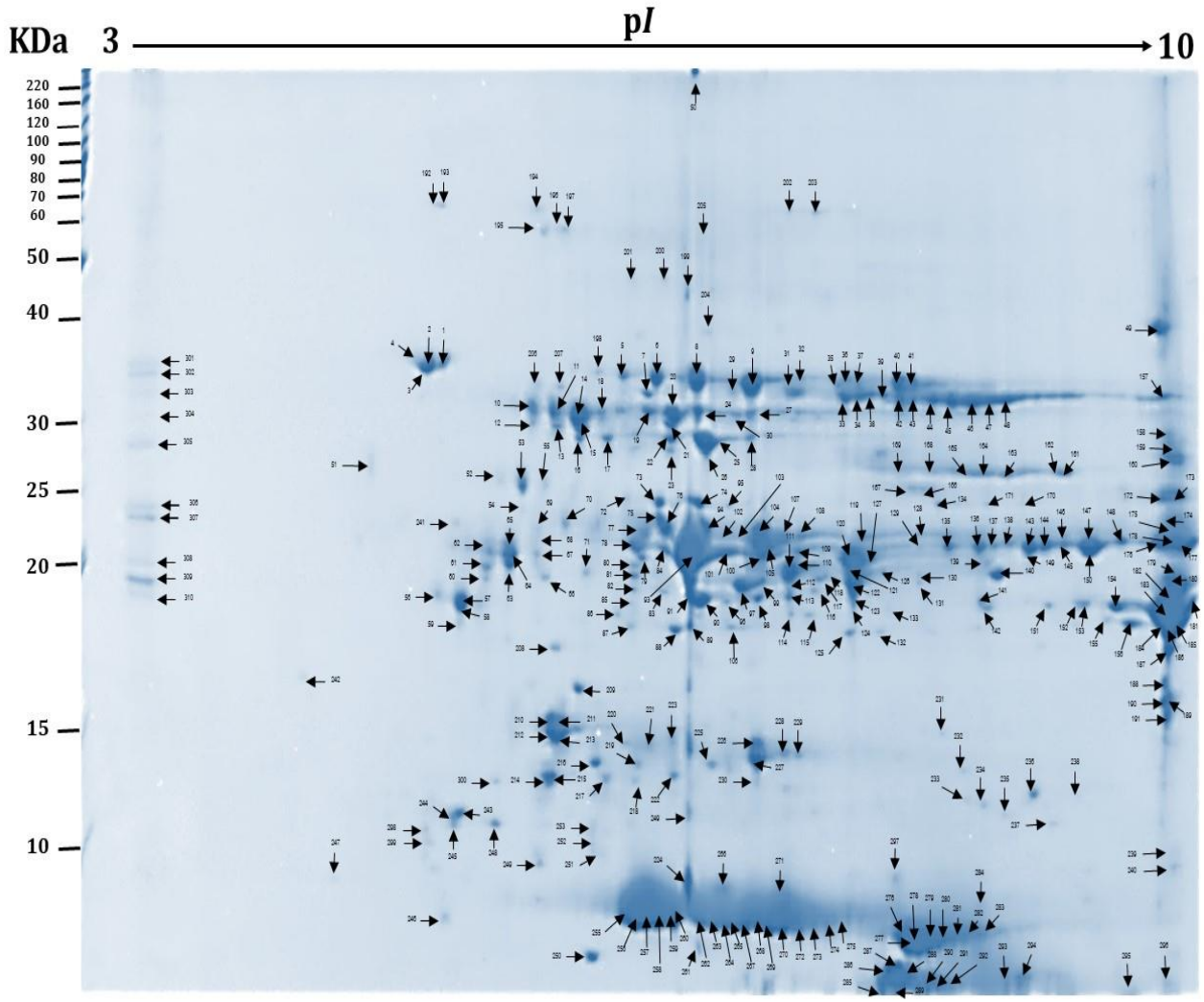

Figure 12. Supplementary S3. Representative 2-DE gel of *Jatropha curcas* globulins fraction. Numbers indicates the spot number assigned for LC-MS/MS analysis reported in Table 5.

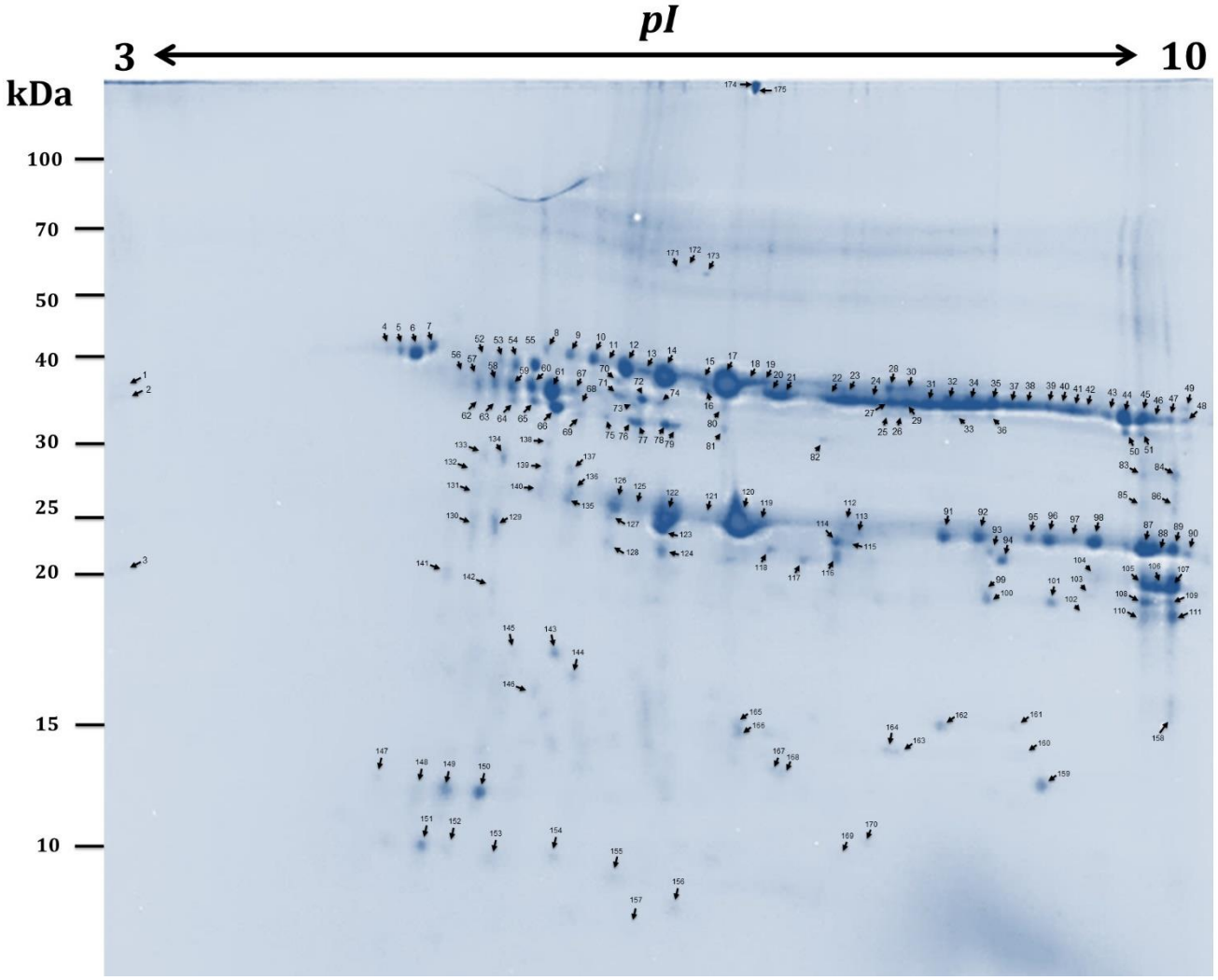

Figure 13. Supplementary S4. Representative 2-DE gel of *Jatropha curcas* glutelins fraction. Numbers indicates the spot number assigned for LC-MS/MS analysis reported in Table 6.