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RESEARCH ARTICLE

Anti-hypertensive activity *in vitro* and *in vivo* on royal jelly produced by different diets

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ABSTRACT

Royal jelly is a glandular secretion produced by *Apis mellifera* L. bees, it is considered as a functional food with the ability to control and prevent chronic diseases such as high blood pressure, because in their composition there are peptides with biological activity; however, different investigations indicate that the composition of amino acids in royal jelly can be affected by the diet consumed by bees, which could influence the presence of peptides. The main objective of this study was to evaluate the effect of the food consumed by honey bees on the antihypertensive potential of the royal jelly produced. Honey bee colonies were fed with three different diet treatments: *Mucuna pruriens* flour and honey; pollen and honey and free feeding. The amino acid content was determined in the protein ingredients and the royal jelly obtained by every feed treatment. The antihypertensive activity was evaluated *in vitro* by the angiotensin converting enzyme technique. To assess the antihypertensive activity *in vivo*, Wistar rats were subjected to a biological model of metabolic syndrome, the rats were dosed with royal jelly and the blood pressure were measured every week. Significant differences ($P<0.05$) were found in the concentration of amino acids Arg and His between three types of royal jelly; regarding amino acids associated with antihypertensive activity, no significant differences ($P>0.05$) were found. The angiotensin converting enzyme inhibition values were less than 25%; however, the blood pressure in the groups of rats that received the royal jelly treatments was similar to the control group ($P>0.05$). These results indicate that the diet with pollen or *M. pruriens* consumed by the bees does not affect the bioactive compounds responsible for the *in vivo* antihypertensive activity, and we found that the continuous consumption of royal jelly prevents the elevation in the blood pressure values.

Keywords: Antihypertensive activity; *Apis mellifera*; *Mucuna pruriens*; pollen; royal jelly

INTRODUCTION

Royal Jelly (RJ) is the nourishment for the honey bee (*Apis mellifera* L.) larvae and for the queen through all of their lives; it is a milky substance secreted by the hypopharyngeal glandes of worker bees 5 to 12 days old, also called nurse bees (Xue et al., 2017). It is considered a functional food due to its components and nutrients, which can be used for prevention and/or control of some conditions such as chronic no communicable diseases (NCDs) (Maghsoudlou et al., 2019). The NCDs are one of the biggest health challenges worldwide, an example of this is cardiovascular diseases, which are a set of disorders of the heart and blood vessels, and they are the leading cause of death trough the world (Sankaran, 2012). High blood pressure represents a major public health problem worldwide, about a quarter of the adult population has hypertension; moreover, hypertension increases the possibility of cardiovascular disease (WHO, 2016). If not controlled, hypertension can lead to myocardial infarction, aneurysms, kidney failure, blindness, and cognitive decline (WHO, 2020).

There are different studies that have shown the antihypertensive activity of royal jelly. Fan et al. (2016) in their work carried out with mouse vascular smooth muscle cells (VSMC), concluded that Major Royal Jelly Protein 1

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(MRJP1), the protein with the highest concentration in royal jelly, can affect the VSMC that form the arterial wall in blood vessels, a phenomenon that helps to regulate blood pressure causing antihypertensive effects. Also RJ contains bioactive components such as biologically active peptides that help decrease levels of blood pressure (BP) through inhibiting vasoactive enzymes such as Angiotensin Converting Enzyme (ACE), responsible for increasing BP by converting angiotensin I to angiotensin II, which is a strong vasoconstrictor (Bhat et al., 2017). The following peptides with antihypertensive activity have been identified within the RJ: Trp-Val-Leu, Tyr-Tyr-Ser-Pro (Matsui et al., 2006), Ile-Tyr, Val-Tyr, Ile-Val-Tyr, Tyr-Tyr, Ile-Phe, Lys-Ser (Maruyama et al., 2003; Tokunaga et al., 2004), Asp-Gly-Leu and Leu-Thr-Phe (Matsui et al., 2002).

The quality of the RJ depends on the quality of the food consumed by the workers' bees; the honey bees naturally get protein from pollen (Wright et al., 2018), but in production systems protein supplements other than pollen can be offered because those are an economical and viable option for nourishment (Sereia et al., 2010); for example, *Mucuna pruriens*, which is a protein-rich legume with good acceptance by honey bees, has been used (Pech et al., 2006).

Previous work carried out by different researchers reported that the amino acid balance of the food consumed by the honey bees can influence the amino acid composition of the RJ (Balkanska and Zhelyazkova, 2015; Jie et al., 2016), it could be assumed that by feeding honey bees with a protein source that has the amino acids responsible for antihypertensive activity, an RJ that helps a better control BP will be obtained. The objective of this study was to evaluate the effect of different protein sources in the diets of bees and how it affects the amino acid profile of the royal jellies produced, as well as the antihypertensive activity of these measured trough *in vitro* and *in vivo* tests.

MATERIALS AND METHODS

Raw materials

Grains of the *M. pruriens* purchased in the municipality of Opichen, Yucatan, Mexico were used; the grains were left to soak for 24 hours in drinking water with a 1:2 w/v grain: water ratio to reduce non-nutritional factors (Tresina and Mohan, 2013), later they were dehulled and dried in a forced air stove (Felisa, México) for 24 hours at an average temperature of 55 °C. The grains were crushed in a mill (Ciclotec™ 1093) to obtain fine flour (833 μm). Pollen was obtained from an apiary at the municipality of Mocochá in Yucatan, Mexico. It was dried in a forced air stove at 60°C, was manually cleaned and ground in a commercial blender (Nutribullet® USA) to obtain fine flour.

Experimental diets

Two diets were developed to feed the honey bees, which were isoproteically balanced to a 14% protein rate. As protein source in diet 1, *M. pruriens* flour was used, and pollen flour in diet 2, honey was used as an energy source in both diets.

Royal jelly production

RJ production was carried out at the facilities of Campo Experimental Mocochá in Yucatán, México, which belong to the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias. The work was done with nine colonies of *A. mellifera* bees established in Langstroth hives with a single brood chamber, each hive having inside nine frames for breeding and food, and a single royal jelly production frame. The hives were divided into three groups, each including three hives: Treatment 1: *M. pruriens* and honey diet, treatment 2: pollen and honey diet and treatment 3: control, where the honey bees has no formulated diet, they were leave to get their food naturally; normally bees collect nectar and pollen from flowers around the hive to make their food (Haydak, 1970). The colonies had a period for adapting to the diets (two weeks), during which they received 40 g of food twice a week. After that adaptation period, the RJ production process began, and the colonies continued to be fed with the same quantity and periodicity. The larvae transfer method was used to produce RJ (Hu et al., 2017), the extraction was performed 72 hours after transfer; the resulting RJ was stored in clean amber coloured bottles at a -4 ° C temperature, in order of preserving its characteristics. The RJ produced by honey bees that received the diet with *M. pruriens* was identified as Mucuna Royal Jelly (MRJ), the one produced by honey bees that received the diet with pollen was identified as Pollen Royal Jelly of (PRJ) and the one produced by honey bees that did not receive any food was identified as Control Royal Jelly (CRJ).

Amino acids profile

To determine the amino acids contained in *M. pruriens* flour, pollen and each type of JR, acid hydrolysis was carried out using HCl in order to break peptide bonds and release all amino acids with their subsequent derivatization of the free amino acids with diethyl ethoxymethylenemalonate in order to were quantifiable (Alaiz et al., 1992). Samples were analysed on a 1260 Infinity quaternary pump HPLC (Agilent Technologies) with a C18 column (Nova-Pak®) 3.9x300 mm. For the determination of tryptophan, an alkaline hydrolysis was carried out with NaOH, without derivatization process (Yust et al., 2004).

Antihypertensive activity *in vitro*

Inhibitory Activity of Angiotensin Converting Enzyme was determined (ACE-I) according to Hayakari et al. (1978). each RJ sample was measured at different

protein concentrations (2, 3, 4 and 5 mg/mL), previous content was measured using method 954.01 reported by the AOAC (2012). The samples were read with a UV spectrophotometer Vis EvolutionTM 300 (Thermo Fisher Scientific, USA) at 382 nm. ACE-I activity was expressed as %ACE-I and was calculated with the following formula:

$$\%ACE\text{-}I = 100 - \left[\frac{(AS - ABS) \times 100}{(AE - ABE)}\right]$$

Where AS is the optical density of ACE with sample and substrate, ABS is the optical density of ACE with sample, AE is the optical density of ACE with substrate and ABE is the optical density of substrate without ACE or sample.

Antihypertensive activity *in vivo*

This determination was made in the Electrophysiology and Pharmacological Bioevaluation laboratory of the Faculty of Medicine at the Universidad Autónoma del Estado de Morelos, México. The experimental protocol was approved by the Commission for the Care and Use of Laboratory Animals (CCUAL, for its acronym in Spanish) of the Faculty and met the Mexican Official Standard NOM-062-ZOO-1999 "Technical specifications for the production, care and use of laboratory animals" as well as all applicable federal and institutional regulations. The work with animals was carried out in accordance with the guidelines for the care and use of experimental animals of the Directive 86/609/CEE of the Council of the European Union. Thirty 2-month-old Wistar rats (*Rattus norvegicus*) with an average weight of 220 g were used. The rats were kept in a controlled environment and a biological model of metabolic syndrome was used in twenty five of them, the model consisted of offering free demand water with 20% sucrose (Guzmán-Gerónimo et al., 2017) and a base diet of 500 g of pellets (Laboratory Rodent diet 5001) per week over a two-month period. At the end of the induction time, they were randomly divided into five treatment groups with 5 animals each; treatment 1 received MRJ; treatment 2 received PRJ; treatment 3 was designated CRJ. Rodents from treatment 4 were given captopril, and this group served as a reference parameter to compare the antihypertensive response of RJ treatments. Treatment 5 rats underwent the metabolic syndrome model and they received no treatment. Treatment 6 rats only received 1 mL of distilled water as placebo; this group was made up of the five rodents that were not part of the biological model of metabolic syndrome, so they were considered as rats with no pathological history. The groups and dosages in treatments are indicated in Table 1.

Each rodent was weighed, and BP was measured using a Kent Scientific CODA Non-invasive Standard sphygmomanometer for rats' BP (Científica Senna), recording both the diastolic pressure (DP) and systolic pressure (SP). The treatments were carried out daily for a four-week period, during which the animals continued to receive the same diet and management as during induction period. All rodents were weighed, and BP was measured weekly, the dose of each treatment was calculated individually based on the weight information. The treatments were diluted in 1 mL of distilled water for their application, and they were administered directly to the stomach of each rat using a 2 mm diameter stainless steel cannula (Fig. 1).

Statistical analysis

The results obtained from the total amino acids from the protein ingredients and from each RJ as well as the antihypertensive activity *in vivo* were analyzed with the GraphPad Prism 6.01® computer program and they were subject of an analysis of variance under a simple random model to determine if there was a difference among the treatments and a comparison of means by the Tukey method with a confidence level of 95%.

RESULTS

It was found that the *M. pruriens* flour and pollen used to make the diets had all the essential amino acids (Table 2) that honey bees require for their growth and

Table 1: Treatments, doses and identification keys used for the evaluation of antihypertensive activity

Number of treatment	Treatment	Dose mg/Kg/day	Key
1	Mucuna RJ	15	MRJ 15
2	Pollen RJ	15	PRJ 15
3	Control RJ	15	CRJ 15
4	Captopril	50	Capt 50
5	Sucrose water	-	SW
6	Distilled water	-	Control

Fig 1. Intragastric administration of treatments in rats subjected to a biological model of metabolic syndrome.

Table 2: Amino acid content in *M. pruriens* flour and pollen used for the elaboration of diets

Amino acid	*M. pruriens* flour (g/100g)	Pollen (g/100g)
Arg *	6.63±0.03[b]	12.79±0.60[a]
His *	2.62±0.01[a]	2.78±0.41[a]
Ile *	6.08±0.06[a]	5.12±0.03[b]
Leu *	8.51±0.04[a]	7.87±0.24[a]
Lys *	15.40±0.01[a]	11.38±0.64[b]
Met *	0.76±0.01[a]	1.44±0.80[a]
Phe *	5.19±0.08[a]	4.64±0.65[a]
Thr *	4.88±0.20[a]	4.82±0.03[a]
Trp *	0.32±0.03[a]	0.41±0.11[a]
Val *	6.03±0.01[a]	5.62±0.38[a]
Cys	1.50±0.05[a]	1.07±0.02[b]
Tyr	4.64±0.12[a]	2.58±0.26[b]
Asp + Asn	10.74±0.65[a]	11.89±1.25[a]
Glu + Gln	9.77±0.10[a]	11.31±0.57[a]
Ser	5.43±0.51[a]	6.43±0.04[a]
Gly	5.51±0.06[a]	5.38±0.02[a]
Ala	4.74±0.02[a]	2.32±0.12[b]
Pro	1.23±0.03[a]	2.07±1.26[a]

[a-b]Different literals indicate significant differences between groups (P<0.05).
* Essential amino acids of honey bees.

development (De Groot, 1952). In most of these amino acids, the concentration found was statistically similar (P>0.05) between *M. pruriens* flour and pollen, however, the concentration of Ile and Lys was higher in *M. pruriens* (P <0.05) and Arg was higher in pollen, this three amino acids are essential for the honey bees (De Groot, 1952). Regarding non-essential amino acids, significant differences (P<0.05) between Cys, Tyr and Ala were founded, being the *M. pruriens* flour the one that presented a higer concentration of these. Furthermore, the amino acid profiles of the protein ingredients showed the presence of the amino acids that have been reported as components of the peptides with antihypertensive activity in the RJ, which are: Ile, Leu, Lys, Phe, Thr, Trp, Val, Tyr, Asp + Asn, Ser, Gly and Pro (Matsui et al., 2006; Maruyama et al., 2003).

The concentration of amino acids in each of the types of RJ are in the Table 3, it was found that regardless of the diet treatment received by the honey bees all amino acids were found in each of the jellies. Significance differences (P<0.05) were found in the amino acids Arg and His, JRM had the lowest concentration; these differences could be due to the content of these amino acids in mucuna flour, which has low values of these. When analyzing the presence of the amino acids associated with antihypertensive activity, it is observed that there are no statistical differences between the jellies (P> 0.05) in the concentration of each one of them or in their total concentration (MRJ 75.31%, PRJ 74.76 and CRJ 76.97%).

In the analysis of ACE inhibition, it was shown within the three types of RJ that the highest inhibition value was at

Table 3: Amino acid content of the royal jellies produced. MRJ: *Mucuna* Royal Jelly; PRJ: Pollen Royal Jelly; CRJ: Control Royal Jelly

Amino acid	MRJ (g/100g)	PRJ (g/100g)	CRJ (g/100g)	FAO (g/100g)
Arg *	5.55±0.06[b]	6.12±0.33[ab]	6.48±0.04[a]	
His *	2.73±0.03[b]	2.83±0.01[a]	2.91±0.01[a]	2
Ile *	5.98±0.01[a]	5.81±0.15[a]	5.90±0.19[a]	3.2
Leu *	8.77±0.08[a]	8.80±0.73[a]	9.37±0.22[a]	6.6
Lys *	15.15±0.07[a]	14.18±1.62[a]	15.93±0.89[a]	5.7
Met *	1.03±0.13[a]	1.02±0.58[a]	1.34±0.12[a]	2.7†
Phe *	4.97±0.02[a]	4.67±0.44[a]	4.99±0.35[a]	5.2‡
Thr *	5.28±0.02[a]	5.20±0.44[a]	5.71±0.02[a]	3.1
Trp *	0.18±0.01[a]	0.23±0.13[a]	0.13±0.01[a]	0.85
Val *	6.73±0.01[a]	6.82±0.26[a]	7.10±0.08[a]	4.3
Cys	0.65±0.02[a]	0.60±0.03[a]	0.42±0.20[a]	
Tyr	4.24±0.08[a]	3.79±0.57[a]	4.12±0.36[a]	
Asp + Asn	11.95±0.08[a]	13.47±4.82[a]	11.03±0.66[a]	
Glu + Gln	10.22±0.02[a]	10.15±0.54[a]	7.32±2.84[a]	
Ser	6.64±0.08[a]	6.49±0.20[a]	7.09±0.12[a]	
Gly	4.33±0.07[a]	4.30±0.19[a]	4.64±0.04[a]	
Ala	4.47±0.05[a]	4.48±0.10[a]	4.52±0.05[a]	
Pro	1.09±0.11[a]	1.00±0.17[a]	0.96±0.07[a]	

[a-b]Different literals indicate significant differences between groups (P<0.05).
* Essential amino acids of honey bees;
† Methionine + cysteine; ‡ Phenylalanine + tyrosine.

a concentration of 4 mg/mL protein (Fig. 2), PRJ was the one with the highest value (23.33%) and MRJ the one with the lowest (13.89%). Regarding antihypertensive activity *in vivo*, no statistical differences (P> 0.05) were found between the three types of RJ (treatments 1, 2 and 3) and the Control (treatment 6), both in DP and SP. However, the SW group (treatment 5), was statistically different from the rest of the groups (P <0.05) with the highest BP values in the entire study. In comparison, the Capt50 group (treatment 4) was the one with the lowest values (Fig. 3).

DISCUSSION

The amino acids essentials for honey bees are Arg, His, Ile, Leu, Lys, Met, Phe, Trp and Val (De Groot, 1952), in this work all of these amino acids were found in the *M pruriens* flour and pollen (Table 2). In twelve of these amino acids, the concentration found was statistically similar between *M. pruriens* flour and pollen, in the remaining six amino acids, only Arg is found in higer concentration in pollen; Ile, Lys, Cys, Tyr and Ala were found in a higer quantities in *M. pruriens* meal, indicating the legume is a good substitute for pollen. The protein ingredients used in this work for the elaboration of diets can be classified as a quality supplement, because the quality of the protein used can be measured through the presence of essential amino acids (Wright et al., 2018), and this are presented in adequate concentrations, which provided the necessary nutrients for the proper development of the hypopharyngeal glands (Di-Pasquale et al., 2013) and for the elaboration of the RJ.

The three types of RJ had all the essential and non-essential amino acids that honey bees require even the CRJ, which was produced by bees that got their food naturally. This could be due to the fact that when several pollen sources are available in the field, the forager bees, show preferences for some plants over others, foraging more intensely from those with high protein values (Donkersley et al., 2017), or to those with a higher concentration of essential amino acids (Cook et al., 2003). The presence of the amino acids associated with the antihypertensive activity there were no significative differences between RJ, so that is possible the presence of peptides with antihypertensive activity in the RJ produced; also, the amino acid profiles of the jellies show their quality as a protein source and it was observed that, with the exception of sulfur amino acids and tryptophan, the other amino acids cover the recommended intake needs for children from 6 months to adults; therefore, RJ can be considered as a functional food, not only due to the presence of peptides with antihypertensive activity, but also as a viable option to complement a balanced diet according to the necessary amino acid requirements reported by the FAO (FAO, 2011).

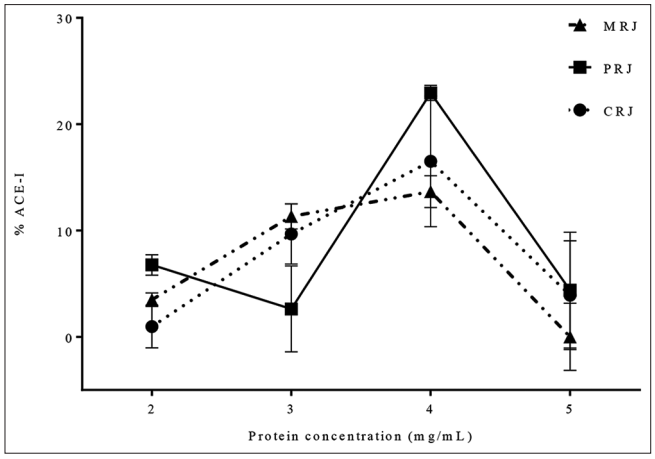

Fig 2. ACE-I percentage at different protein concentrations of each RJ. MRJ: Mucuna Royal Jelly; PRJ: Pollen Royal Jelly; CRJ: Control Royal Jelly.

Fig. 2 shows ACE-I at different protein concentrations. At 2, 3 and 4 mg/mL concentrations, a gradual increase in inhibition was detected; however, at 5 mg/mL a decrease in the inhibition was observed, this could be due to the fact that the reaction between ACE and the substrate present in royal jelly presents an uncompetitive type of enzymatic inhibition (Palmer and Bonner, 2007). Although the jellies presented all the amino acids that have been associated with the antihypertensive peptides and there are no significant differences between their concentrations, it would be expected that the ACE-I values would be similar; however, there were differences between the PRJ and MRJ values, this could be due to the availability of peptides with antihypertensive activity in each of the samples. It can be observed that despite the high values of amino acids involved in the antihypertensive activity (70% approximately), there is no relation between the activity of ACE-I and the amount of amino acids. One alternative to improve the ACE inhibition values can be the enzymatic hydrolysis or the action of digestive enzymes (Hernández-Ledesma et al., 2011; Guerra-Almonacid, et al. 2019) that could allow the release of bioactive peptides. In works where RJ has been used subjected to a trypsin hydrolysis process, the resulting peptide fractions showed a value of %ACE-I within an interval of 10-60% (Takaki-Doi et al., 2009).

As for the antihypertensive activity *in vivo*, the results show that without receiving any treatment, such as RJ or captopril, the BP of the rats would have increased, as occurred with the SW group. Some peptides have been reported to be generated and/or released by enzymatic reactions in the intestine, following ingestion of the food containing the precursor protein (Hernández-Ledesma et al., 2011; Murray and FitzGerald 2007), therefore, it is possible that the antihypertensive peptides had been released by the action of the digestive enzymes of the animal model and then reached the bloodstream; in this way, the joint action of peptides and the continuous administration of RJ caused

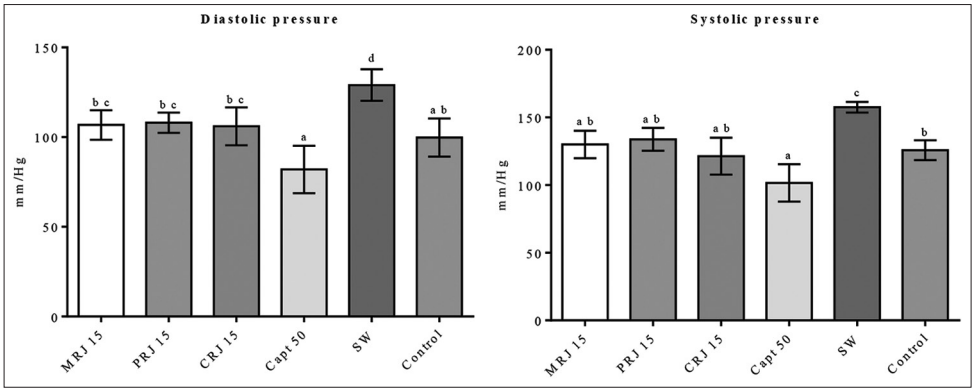

Fig 3. Diastolic and systolic pressure average (mm/Hg) obtained in each group during the administration of the treatments. Mean and SD.

the levels of BP in the groups that received RJ remained stable and similar to those of the Control group (Fig. 3). An advantage presented by the continuous intake of RJ is that it did not cause a decrease in BP as it did with captopril, whose recurrent consumption can cause a hypotensive effect. The next step would be to carry out the hydrolysis of RJ with enzyme like trypsin from to obtain peptides with antihypertensive activity (Takaki-Doi et al., 2009). On the other hand, it would be to carry out studies in people, in a controlled manner, in order to corroborate the release and action of peptides with antihypertensive activity when consuming RJ.

CONCLUSIONS

The components that make up the RJ allow it to have several applications, one of which is its use as a functional food with the ability to help prevent and control diseases such as hypertension. It is necessary to make an adequate selection of the protein with which the bee colonies will be fed, because it influences the presence of amino acids in the final product. The presence of all amino acids associated with antihypertensive activity in the RJ is an indicator that the antihypertensive response in this work is most likely due to the presence of bioactive peptides. The ACE-I% values of the three types of RJ were low in the *in vitro* determinations. However, *in vivo* tests showed that RJ has antihypertensive activity, despite the fact that hydrolysis treatment was not performed prior to ingestion. This was an indicative that the digestive enzymes of the animal model released antihypertensive peptides and these presented an effect in the organism. It is necessary to make an adequate selection of the protein with which the bee colonies will be fed, because it influences the presence of amino acids in the final product. With the knowledge of the amino acids present in the diet received by bees and in RJ, in the future production systems aimed at obtaining RJ with greater antihypertensive activity could become viable.

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Authors contributions

Karla Itzél Alcalá Escamilla is the principal autor and participed in the acquisition and analysis of data, and writing the paper. Yolanda Beatriz Moguel Ordoñez participated in the design of the research, analysis of data and was involved in revising the paper critically; Valentino Mukthar Sandoval Peraza participated in the adaptation of some analytical techniques and the acquisition of data; Juan José Acevedo Fernández participated in the design of the research and analysis of data. David Abraham Betancur participated in the design of the research, analysis of data and revising the paper critically.

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