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RESEARCH ARTICLE

Physiological and physicochemical behavior of guamara (*Bromelia pinguin*) and cocuixtle (*Bromelia karatas*) fruits, as well as the antibacterial effect of their pre-purified proteases

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ABSTRACT

The objective was to study the behavior of the respiration rate (RR) of guamara and cocuixtle fruits during storage, the effect or the maturity stage (green maturity and consumption maturity) of these fruits on physicochemical parameters and specific proteolytic activity, as well as the antibacterial effect of the prepurified proteases of these fruits. The guamara and cocuixtle fruits presented a RR of 15 mL CO_2/kg·h and 10 mL CO_2/kg·h, respectively; and because the ethylene production was not detected in any of the species, it was concluded that both fruits are of the non-climacteric type. The cocuixtle fruits presented the highest specific enzymatic activity in the state of green maturity and the guamara fruits in the state of consumption maturity, with 18.99 and 53.88 U/mg of protein, respectively. Likewise, pre-purified proteases from both fruits showed antibacterial activity against *E. coli* and *S. aureus*. It is concluded that ripened guamara fruits and green cocuixtle fruits can be an important source of proteases for the food industry and they can be used against pathogens.

Keywords: Cocuixtle and guamara fruits; Respiration rate; Specific proteolytic activity; Antibacterial activity

INTRODUCTION

The Bromeliaceae family includes the *Bromelia* genus that is constituted of plants and exhibit a great biotechnological potential due to the presence of bioactive compounds (phenolic compounds, flavonoids, stigmasterol, anthraquinones, and tannins) (Lugo-Vargas et al., 2016; de Oliveira-Júnior et al., 2014), and proteases such as pinguinain and karatasin (Ávalos-Flores et al., 2020; Escandon-Rivera et al., 2019; Payrol et al., 2008; Montes et al., 1990). *B. pinguin* (guamara) and *B. karatas* (cocuixtle) are plant species considered as wild (Aguilera-Aguirre et al., 2018; Segura et al., 2018; Meza-Espinoza et al., 2017a); however, the protein extracts of these bromelias have been particularly revalued in the last years by its potential industrial applications (Escandon-Rivera et al., 2019; Meza-Espinoza et al., 2018; Aguilera-Aguirre et al., 2018; Moreno-Hernández et al., 2017a, b, c; Looby et al., 2012). Further, the rheological (Osorio et al., 2017), nutritional and physicochemical properties (Pío-León et al., 2009) of these fruits have been characterized. Nevertheless, there were not found reports on the physiological behavior (respiration rate, ethylene production, and physiological weight loss) of these fruits when they were stored and neither the relationship with the protein content at different states of maturity of the fruits.

Osorio et al. (2017), reported that the *B. karatas* extract from ripe fruits had a higher concentration of phenolic compounds and antioxidant capacity (394 mg gallic acid equivalent/100 g, 35% inhibition of $O_2^{\cdot-}$, respectively) compared to the immature fruit extracts (280 mg EAG/100 g, 4% inhibition of $O_2^{\cdot-}$, respectively). It has also been reported that the ethanolic and aqueous extracts

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from *B. pinguin* fruits present an antimicrobial activity on pathogenic bacteria such as *Escherichia coli, Staphylococcus aureus, Enterococcus faecalis, Salmonella typhi,* and *Shigella flexneri* (Pío-León et al., 2009; Camacho-Hernández et al.., 2002). The authors attributed the antibacterial activity mainly to the presence of secondary metabolites such as tannins, flavonoids, and saponins in the extracts.

Ruiz-Ruiz et al. (2017) reported that protein extracts from *B. pinguin* fruits presented an antimicrobial activity for *E. coli* and *S. aureus* with a minimum inhibitory concentration of 0.35 mg/mL and 0.69 mg/mL, respectively. They suggested that the antibacterial activity may be due to the presence of proteases and bioactive peptides, that inhibited bacterial growth. On the other hand, the antibacterial activity from *B. karatas* extracts had not been evaluated. In the present study, the behavior of the respiration rate (RR) of guamara and cocuixtle fruits during storage was evaluated; the effect of the maturity stage (green and consumption) of the fruits on the physicochemical parameters and specific proteolytic activity was also studied, as well as the antibacterial effect of the pre-purified proteases of these fruits.

MATERIALS AND METHODS

Images of the overall experiment can be seen in Fig. 1.

Plant material

The guamara and cocuixtle fruits were harvested from rural areas of Tepic, Nayarit, Mexico. The fruits were selected with homogeneous characteristics in size and color. The experiment was divided into three stages. In the first stage, physiological parameters were measured. The green fruits were selected (physiological maturity) with total soluble solids of 13-14 ºBrix. The fruits were washed with water and sodium hypochlorite at 50 ppm, were dried at room temperature, and stored at 25 ° C and 85% relative humidity (RH). Respiration rate (RR) as well as physicochemical parameters were measured during the storage.

In the second stage, the physicochemical and nutritional parameters of cocuixtle and guamara fruits in two states of maturity were analyzed. Fruits were collected in two states of maturity: state of maturity I (physiological maturity: green color and firm fruits) with total soluble solids of 13-14 ºBrix (Fig. 2) and state of maturity II (fruits in consumption maturity: guamara fruits of yellow color and firm; cocuixtle fruits of white color with pink tones and firm) with 20-25 ºBrix (Fig. 2). In this regard, 50 fruits of each specie (state of maturity I and II) were pulped manually. A part of the fresh pulp of each raw material was used to evaluate physicochemical and nutritional parameters. The remaining pulps were lyophilized (LABCONCO model 77522020, Labconco Corporation, Kansas City, MI, USA) and then stored at -20 ºC to the third state.

In the third stage, the pre-purified proteases were obtained and the specific enzymatic activity and antibacterial activity were analyzed.

Methods

Physiological parameters

Respiration rate (RR) was measured daily, following the methodology proposed by Tovar et al. (2001), with slight modifications. Each fruit was placed in a chamber with septa and sealed for one hour. Thereafter, 1 mL was taken from the free space of the chamber head and injected into a gas chromatograph (HP model 6890, Santa Clara, USA) provided with two HP-PLOT capillary columns to detect CO_2 (divinylbenzene/styrene, 15 m x 0.53 mm and 40 µm film thickness) and ethylene (divinylbenzene/styrene, 15 m x 0.53 mm and 25 µm film thickness). Nitrogen was used as carrier gas at a flow rate of 7 mL/min. The flame ionization detector (FID) was used for ethylene detection and the thermal conductivity detector (TCD) for CO_2. The injector port and detectors remained at 250 °C; the oven temperature was kept at 50 ºC for 30 s, with a heating ramp of 30 ºC/min until reaching 80 ºC. The air and hydrogen flows were 400 mL/min and 30 mL/min, respectively. Respiration rate was expressed in mL CO_2/kg·h. Moreover,

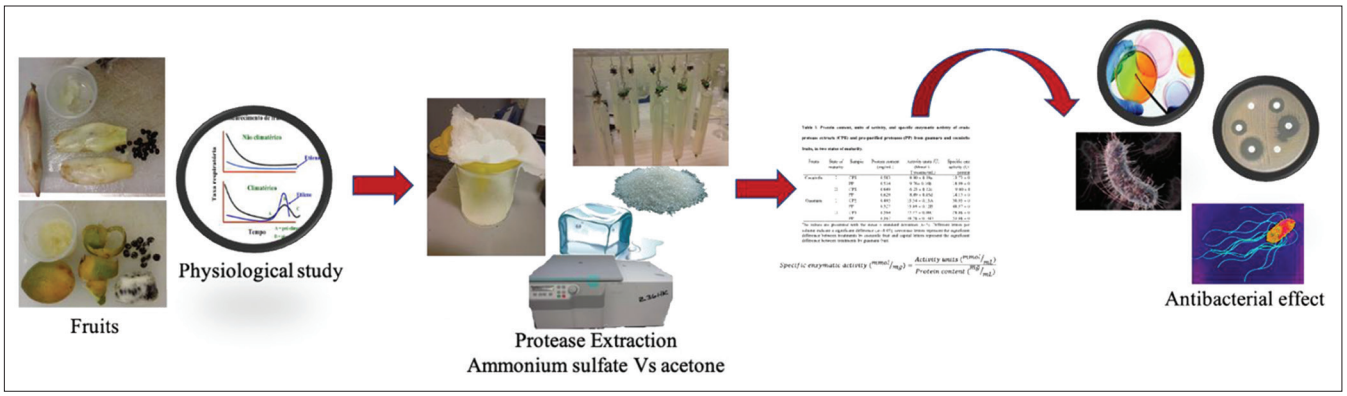

Fig 1. General diagram of the experiment.

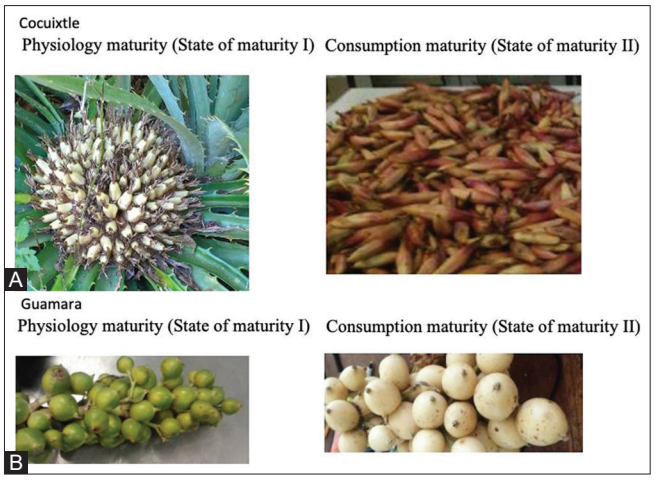

Fig 2. States of maturity analyzed in the second stage. A) Cocuixtle fruit in a state of physiological maturity and a state of consumption maturity. B) Guamara fruit in a state of physiological maturity and a state of consumption maturity.

the physiological weight loss of the fruits was evaluated during the days of storage and it was reported as a percentage (%).

Physicochemical and nutritional parameters

The analyzed physicochemical parameters were: pH (method 981.12, AOAC, 2005), total soluble solids (TSS) were reported in ºBrix (method 932.12, AOAC, 2005), and titratable acidity (TA) was reported in g milliequivalents of citric acid (MAC)/100 g of fresh weight (method 942.15, AOAC, 2005). The nutritional parameters were: the moisture content (method 934.06, AOAC, 2005), ash (method 940.26, AOAC, 2005), fat (method 950.54 of the AOAC, 2005), protein (934.06, AOAC, 2005), total soluble carbohydrates by the method of Dubois et al. (1956), and total dietary fiber by the enzymatic-gravimetric method (method 991.42) of the AOAC and modified by Mañas and Saura-Calixto (1995). The last results reported in g/100 g dry weight, except moisture.

Crude protease extracts

The lyophilized pulp (ground and sieved) of each fruit (10 g) was suspended in a phosphate buffer (0.1 M, 4 ° C, pH 6.0, 5 mM EDTA, and 5 mM L-cysteine) (García-Magaña et al., 2018; López et al., 2000). The solution was homogenized and subsequently filtered through a gauze and centrifuged at 7642 g for 30 min at 4 °C (Hermle, Z32HK, Labortechnik GmbH, Wehingen, Germany).

The supernatants were considered as the crude protease extracts of each fruit. A part of each crude protease extract (100 mL) was used to obtain the pre-purified proteases and the rest of them was lyophilized for the subsequent analysis.

Pre-purified proteases

To 100 mL of the crude protease extract from cocuixtle pulp, 20% (p/v) of ammonium sulfate was added, while for the crude protease extract from guamara pulp, 30% (p/v) of ammonium sulfate was added considering the reported recommendations for each fruit (Romero-Garay et al., 2020; García-Magaña et al., 2018; Moreno-Hernández et al., 2017b), the dialyzed precipitates were lyophilized and named pre-purified proteases (PP).

Specific enzymatic activity

The specific enzyme activity was calculated according to the method reported by Natalucci et al. (1996) with some modifications (Meza-Espinoza et al., 2018; García-Magaña et al., 2018). An L-tyrosine calibration curve was performed to calculate the activity units as the millimoles of L-tyrosine released in proteolysis.

One activity unit (U) was defined as the millimoles of L-tyrosine formed per minute per milliliter of the crude protease extracts or pre-purified proteases according to Meza-Espinoza et al. (2017b) (see equation 1).

Equation 1

Where:

$$U = \frac{\Delta\, Tyrosine\ concentration}{V_{enzyme} * t} * fd * V_{total\ reaction}$$

U = Activity units (mmol/mLmin)

Δ Tyrosine concentration = difference in the tyrosine concentration (mmol/mL) obtained from the enzyme-substrate reaction and the blank

V_{enzyme} = Volume of protease solution (mL)

t = hydrolysis time (min)

fd = dilution factor

$V_{reaction}$ = Total volume used in the reaction (mL).

The specific enzymatic activity was calculated as the activity units per mg of protein (U/mg of protein) per minute of the enzyme reaction (Meza-Espinoza et al., 2017b). Therefore, the protein content was measured by the Bradford method (1976) by using 100 µL of the protease solutions (1.73 mg/mL) and mixed with 600 µL of the Bradford reagent and then incubated at room temperature for 10 min. The absorbance was measured at 595 nm (Jenway 6705, Cole-Parmer Instrument Co., Felsted, UK) and the results were reported in mg/mL based on a calibration curve of bovine serum albumin.

The following equation was used to calculate the specific enzymatic activity.

Equation 2.

$$\text{Specific enzymatic activity } \left(\frac{mmol}{mg}\right) = \frac{\text{Activity units } \left(\frac{mmol}{mL}\right)}{\text{Protein content } \left(\frac{mg}{mL}\right)}$$

Antibacterial activity of pre-purified guamara and cocuixtle proteases

In sterile test tubes, 9.8 mL of 1% of peptone, 100 µL with 1X10^6 CFU of bacterial suspension (*E. coli* ATCC 8739 and *S. aureus* ATCC 33862), 25 µL of the solution of each pre-purified protease extract were placed at different concentrations (31.2, 62.5, 125, 250 and 500 mg/mL) and 20 µL of dimethyl sulfate (DMSO), resulting in a final reaction volume of 10 mL. Decimal dilutions were prepared (10^1 to 10^5) by using sterile 1% of peptone, solutions were poured into petri dishes with trypticase soy agar and the Petri dishes were incubated at 37 °C for 24 h. The control sample was treated as described above, but with the addition of 25 µL of peptone instead of the pre-purified proteases. To rule out the possible effect of DMSO on bacterial reduction, a tube with peptone diluent (9.9 mL) was prepared with a bacterial solution (100 µL) and it was inoculated following the methodology previously described (Wadhwani et al., 2009).

Ampicillin (5 mg/mL) was used as a positive control. The pre-purified protease solutions of each fruit (500 mg/mL + 20 µL of DMSO) were heat-treated (80 ° C/15 min) and they were used as a negative control (Ruiz-Ruiz et al., 2017). The CFU/mL count of all the treatments was performed to calculate the bacterial reduction (expressed as log CFU/mL) and the calculation of the reduction percentage (Briñes et al., 2006). All the materials, culture media, diluents, and reagents used were previously sterilized at 121 °C for 15 min.

Statistical analysis

The data analysis was examined by analysis of variance (One-way ANOVA, $\alpha = 0.05$) and Fisher´s LSD test ($p<0.05$). A Pareto chart was used to estimate the relationship of bromeliad fruits and maturity stage with the specific enzyme activity. All values were obtained from three independent experiments and each analysis was performed by triplicate. The results were expressed as the mean ± standard deviation. All data were analysed by the Statistica software (v.10 Statsoft®, Tulsa, USA).

RESULTS AND DISCUSSION

First stage. physiological and physicochemical behavior of guamara and cocuixtle fruits

The fruits were analyzed until 6 days due to the appearance and loss of moisture. The guamara (Fig. 3a) and cocuixtle

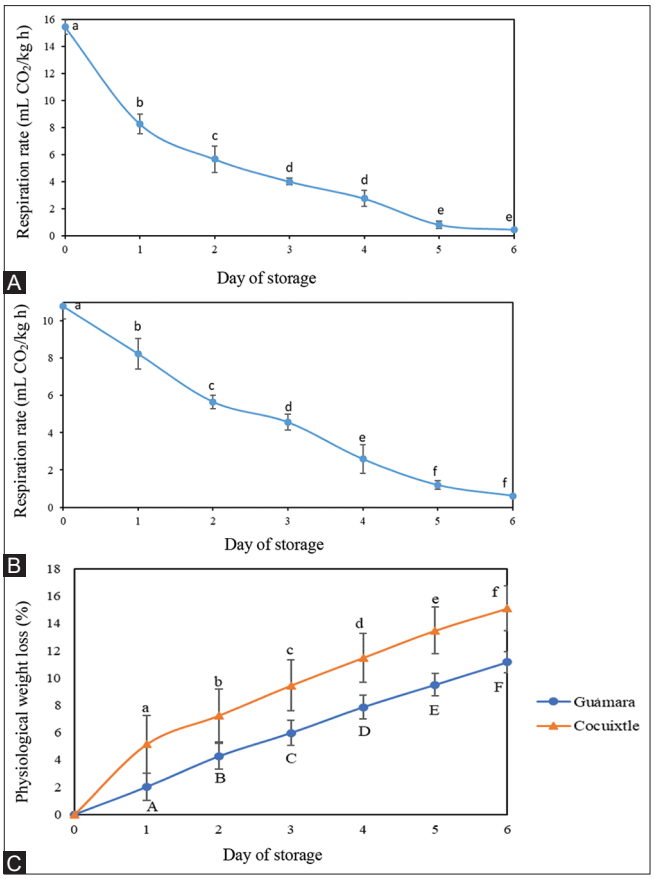

Fig 3. Respiration rate of guamara fruit (A), and cocuixtle fruit (B), and percentage physiological weight loss (C) of both fruits stored for 6 days at 25 ºC and 75% of relative humidity. Different letters indicate a significant difference (α=0.05).

(Fig. 3b) fruits presented an initial RR of 15 mL CO_2/kg·h and 11 mL of CO_2/kg·h on the day of harvest (day 0) respectively, but both fruits had a significant decrease ($p < 0.05$) in the RR during the 6 days of storage (<1 mL CO_2/kg·h).

According to Kader (1992), the fruits can be classified based on their respiration as low, moderate, and high RR. The fruits with low RR values present a production between 5 to 10 mL CO_2/kg·h, moderate RR between 11-20 mL CO_2/kg·h, and high RR with RR> 20 mL CO_2/kg·h (Liu et al., 2015; Kader, 1992). Therefore, the guamara fruit has a moderate RR, while the cocuixtle fruit a low RR. In addition, both fruits present a non-climacteric behavior, since the characteristic CO_2 peak was not observed and the autocatalytic ethylene was not detected (Mata-Montes de Oca et al., 2007). The results coincided with a non-climacteric fruit of the same bromeliads family, such as pineapple, which did not show an increase in the respiratory intensity (Morales et al., 2001). The guamara and cocuixtle fruits showed a physiological weight loss (Fig. 3c) of 15% and 10% during the 6 days of storage, respectively.

($p < 0.05$). This phenomenon could be related to transpiration among other factors such as the external conditions with a low RH and a high temperature that could impact this response variable of the fruits. Sargent et al. (2000), indicated that RH is an important factor to control the weight loss in fruits since low RH values favor a greater physiological weight loss. On the other hand, Hidalgo et al. (1996) reported a weight loss of 11% in 'Manila' mango fruits stored at 25 °C, concluding that the storage temperature accelerated the transpiration of the fruit, promoting a greater weight loss.

The volume and weight decrease of the fruits after cutting depends on the composition of the fruit cuticle; if the fruit has a thicker and woody cuticle (besides of waxes), physiological weight loss will be slower (Tafolla-Arellano et al., 2013). The cuticle of cocuixtle fruits is thicker than the cuticle of guamara fruits, which could explain that the physiological weight loss was greater in cocuixtle fruits than in guamara fruits (Fig. 2). Nonetheless, this same behavior has been observed in mango fruits (Siller-Zepeda et al., 2009), cucumber (Muy Rangel et al., 2004), and lemon (Zea-Hernández et al., 2016) with or without the application of natural waxes on the fruit surface. The pH, total soluble solids, and titratable acidity parameters had no significant changes ($p > 0.05$) at 6 days after harvest (Table 1). These results coincided with the behavior of the same parameters measured in non-climacteric fruits in postharvest. It has been demonstrated that if the non-climacteric fruits are harvested in a state of green maturity, the physicochemical changes are not found during the ripening process due to the low production ethylene, to decrease the RR during the storage and also there are not important metabolic changes because the fruits do not ripen (Mata-Montes de Oca et al., 2007; Alexander and Grierson, 2002; Kader, 1992; Fonseca et al., 2002;).

Second stage. physicochemical and nutritional parameters of cocuixtle and guamara fruits in two states of maturity

The results are shown in Table 2. The AT values did not show significant differences ($p > 0.05$) between states of maturity (3 g MAC/100 g) of cocuixtle fruits. However, significant differences were found ($p < 0.05$) in the TSS content (14.8 and 21.1 ºBrix, respectively) and pH (2.81 and 3.12) between the states of maturity I and II, respectively. These results agree with those reported by Osorio et al. (2017) in the parameters of pH, TSS, and AT of cocuixtle fruits in two states of maturity. The authors reported the TSS content in ripe fruits of 17.9 ºBrix compared with TSS of 5.3 ºBrix in immature fruits, The TSS increase was directly attributed to the ripening process.

Table 1: Variation of pH, total soluble solids (TSS), and titratable acidity (TA) in cocuixtle and guamara fruits during storage

Fruit	Parameter	Days		
		0	3	6
Cocuixtle	pH	2.87 ± 0.02a	2.84 ± 0.01a	2.86 ± 0.05a
	TSS	14.23 ± 0.05a	14.30 ± 2.17a	14.20 ± 0.01a
	TA*	3.07 ± 0.23a	3.18 ± 0.13a	3.00 ± 0.10a
Guamara	pH	3.54 ± 0.01a	3.53 ± 0.01a	3.53 ± 0.01a
	TSS	13.56 ± 0.05a	13.60 ± 0.10a	13.63 ± 0.11a
	TA*	2.58 ± 0.01a	2.60 ± 0.07a	2.54 ± 0.23a

The values are presented with their mean ± their standard deviation (n=3). Different letters per file indicate a significant difference ($\alpha = 0.05$).
*g/100g (milliequivalents of citric acid).

The guamara fruits in both maturity states, had no significant differences ($p > 0.05$) in the pH values (3.8), although the fruits presented differences ($p < 0.05$) in the TSS content (13.6 and 22.5 ºBrix, respectively) and AT (2.7 and 2.02 g MAC/100 g).

In general, the values found were similar to those reported by Pío-León et al. (2009) and Moyano et al. (2012) in guamara fruits (pH values approximately 3.7, AT 4.6 g MAC/100 g and 14 °Brix), and the variations may be due to various factors such as the growing area (Meza-Espinoza et al., 2017a; Looby and Eaton, 2014) or by variations in the state of maturity of the studied fruits (Osorio et al., 2017). Moreover, significant differences ($p < 0.05$) were observed in the nutritional composition (carbohydrates, fat, ash, protein, and total dietary fiber) of the fruits depending on the state of maturity (Table 2). The results are consistent with those reported by Pío-León et al. (2009) in guamara fruits (ash, lipids, and carbohydrates). The highest protein content (a component of great importance for this study) was obtained in fruits with the state of maturity I in both species, with values of 4.98 g/100 g from cocuixtle fruits and 6.42 g/100 g from guamara fruits. These values are similar to those reported by Pío-León et al. (2009) in guamara fruits (7 g/100g), although the authors did not specify the state of maturity of the fruits.

On the other hand, Moyano et al. (2012) reported a protein content of 13 g/100 g in green cocuixtle fruits, which is three times higher than the reported in this study. These differences could be due to the composition of the soil in which they were grown. It is known that nitrogen is necessary for protein synthesis in the plant and its presence in the soil is different depending on places (Kammann et al., 2015; Looby and Eaton, 2014).

Overall, the changes in the nutritional and physicochemical composition of the guamara and cocuixtle fruits in their different states of maturity may be due to various biological and metabolic phenomena that occur in the fruits during the ripening process (Osorio et al., 2017; Corte-Osorio et al., 2011; Chen et al., 2004;).

Third stage. specific enzymatic activity and antibacterial activity of crude protease extracts and pre-purified proteases

Table 3 shows that the protein content, activity units, and the specific enzymatic activity (SEA) of the crude protease extracts (CPE) and pre-purified proteases (PP) presented significant differences ($p < 0.05$) by type fruit, state of maturity, and the purification step.

The highest SEA was found in the pre-purified proteases from cocuixtle fruits in the state of maturity I with 18.99 U/mg of protein, while the pre-purified proteases from guamara fruits showed the highest SEA in the state of maturity II with 53.88 U/mg of protein. It has been demonstrated that the synthesis of proteases in developed plant organs is due to the need to hydrolyze proteins to obtain amino acids, and thus the plants can synthesize new proteins necessary for their growth (Kim et al., 2006).

The values are presented with the mean ± standard deviation (n=3). Different letters per column indicate a significant difference ($a=0.05$); lowercase letters represent the significant difference between treatments by cocuixtle fruit and capital letters represent the significant difference between treatments by guamara fruit.

The Pareto chart (Fig. 4) shows the effect of independent variables on specific enzymatic activity (SEA) of bromeliad fruits at a confidence level of 95%. In general, the type of fruit had the highest influence on the SEA, followed by the fruit's maturity stage and their interactions (fruit*maturity stage). The highest SAE was found in guamara fruits (30.95 U/mg de protein for CPE and 53.88 U/mg de protein for PP) compared to cocuixtle fruits (13.73 U/mg de protein for CPE and 18.93 U/mg de protein for PP) independently of the fruit maturity stage. Furthermore, a decrease in the SEA of the cocuixtle and guamara CPE was observed between mature stage I (from 13.73 to 9.60 U/mg de protein) and maturity stage II (from 30.95 to 28.86 U/mg de protein). It has been reported that the synthesis of proteases is also carried out as part of the defense mechanisms of plants against the attack of phytopathogens, insects, or animals (Kim et al., 2009). Meza-Espinoza et al. (2018) studied the SEA of proteases extracted from cocuixtle and guamara fruits. They reported a SEA of 4.45 and 7.91 U/mg of protein of the crude protease extract from cocuixtle and guamara fruits. Conversely, these authors carried out the

Table 2: pH, total soluble solids (TSS), titratable acidity (TA), and nutritional parameters of cocuixtle and guamara fruits in two states of maturity

Parameters	Fruits			
	Cocuixtle		Guamara	
	Maturity state I	Maturity state II	Maturity state I	Maturity state II
TA*	3.00 ± 0.284a	3.00 ± 0.261a	2.74 ± 0.214a	2.02 ± 0.318b
TSS (º Brix)	14.87 ± 0.833a	21.16 ± 2.863b	13.58 ± 1.481a	22.57 ±1.300b
pH	2.81 ± 0.054a	3.12 ± 0.342b	3.79 ± 0.648a	3.80 ± 0.047a
Nutritional parameters (g/100 g dry weight, except moisture)				
Moisture	96.38 ± 0.26a	95.73 ± 0.26a	96.40 ± 0.55a	96.37 ± 0.20a
Soluble carbohydrates	71.91 ± 1.07a	80.22 ± 1.91b	71.15 ± 1.80a	73.97 ± 0.68b
Fat	1.11 ± 0.85a	2.16 ± 0.52b	1.42 ± 0.28a	1.81 ± 0.74b
Ash	4.64 ± 0.32a	10.90 ± 0.24b	5.87 ± 0.34a	7.50 ± 0.12b
Protein	4.98 ± 0.22a	4.17 ± 0.23b	6.42 ± 0.15a	5.88 ± 0.30b
Total dietary fiber	11.10 ± 0.08a	8.81 ± 0.39b	15.14 ± 1.11a	10.84 ± 0.37b

The values are presented with their mean±their standard deviation (n=3). Different letters per file indicate significant difference ($\alpha=0.05$).
*g/100 g (milliequivalents of citric acid).

Table 3: Protein content, units of activity, and specific enzymatic activity of crude protease extracts (CPE) and pre-purified proteases (PP) from guamara and cocuixtle fruits, in two states of maturity

Fruits	State of maturity	Sample	Protein content (mg/mL)	Activity units (U) (Mmol L-Tyrosine/mL)	Specific enzymatic activity (U/mg de protein)	Purification (n-fold)
Cocuixtle	I	CPE	0.583	8.00 ± 0.19a	13.73 ± 0.33a	1.00
		PP	0.514	9.76± 0.16b	18.99 ± 0.31b	1.38
	II	CPE	0.649	6.23 ± 0.12c	9.60 ± 0.19c	1.00
		PP	0.629	8.89 ± 0.05d	14.13 ± 0.08a	1.47
Guamara	I	CPE	0.495	15.34 ± 0.15A	30.95 ± 0.30A	1.00
		PP	0.327	15.89 ± 0.12B	48.57 ± 0.37B	1.56
	II	CPE	0.594	17.17 ± 0.08C	28.86 ± 0.14C	1.00
		PP	0.367	19.78 ± 0.16D	53.88 ± 0.45D	1.86

The values are presented with the mean ± standard deviation (n=3). Different letters per column indicate a significant difference ($\alpha=0.05$); lowercase letters represent the significant difference between treatments by cocuixtle fruit and capital letters represent the significant difference between treatments by guamara fruit.

precipitation of proteases with organic solvents, which is a possible explanation of the differences found in the SEA.

Table 4 shows the antibacterial activity. The lethality values and the bacterial reduction percentage of *E. coli* and *S. aureus* were by the effect of pre-purified proteases at the different concentrations. It can be observed that there are significant differences ($p < 0.05$) between the concentrations of proteases from the same fruit, as well as between the species of fruit (guamara and cocuixtle) and type of microorganism. The positive control (ampicillin at 5 mg/mL) exhibited a population reduction of >99% in both pathogens, while the heat-treated pre-purified proteases (500 mg/mL) had population reductions from 0.65 to 2.76% in both pathogens. Likewise, the use of DMSO (0.2% v/v) in the assays did not influence the antimicrobial activity (<0.005% population reduction in both bacteria) (Wadhwani et al., 2009; Hili et al., 1997). Although the non-enzymatic proteins or metabolites that supported the pre-purification process could be dragged and they had antibacterial activity, it can be inferred that the major antibacterial effect is attributable to the pre-purified proteases, as it was reported by Ruiz-Ruiz et al. (2017); because as was mentioned above, when the antibacterial activity was carried out with the inactivated pre-purified proteases, it decreased considerably. Additionally, all the concentrations of pre-purified proteases shown inhibitory activity against both microorganisms ($p < 0.05$) (Table 4).

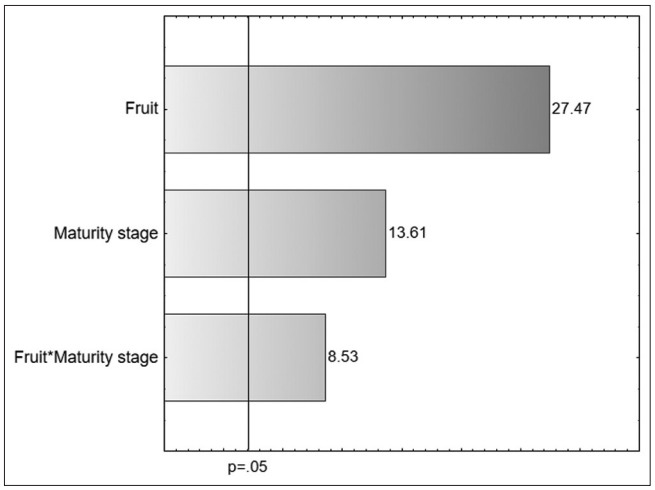

Fig 4. Pareto chart of effect on specific enzymatic activity of cocuixtle and guámara fruits at different maturity stage.

Pre-purified guamara proteases (500 mg/mL) shown the greater antibacterial activity against *E. coli* (1.64 log CFU/mL, 97.71% of reduction) than in *S. aureus* (1.45 log CFU/mL, 96.45% reduction); while pre-purified cocuixtle proteases (500 mg/mL) shown antibacterial activity against *E. coli* of 0.79 log CFU/mL (83.90% of reduction) and for *S aureus* of 2.17 log CFU/mL (99.32% of reduction). Therefore, the pre-purified cocuixtle proteases had the highest effect against *S aureus*. The antibacterial activity could be due to the role that the proteases play in the fruit as a defense mechanism against the attack of pathogenic microorganisms (Kim et al., 2006; Kim et al., 2009). However, more studies should be done to prove this statement. It has been reported that other plant proteases such as papain and bromelain (Eshamah et al., 2013) and a purified protease from guamara fruit (Ruiz-Ruiz et al., 2017), showed inhibitory activity against *E. coli* and *S. aureus* using sensitivity discs. Further, Ruiz-Ruiz et al. (2017) compared the growth profiles of *E. coli* and *S. aureus* in liquid medium added with guamara proteases, against the growth profiles of these same bacteria in the presence

Table 4: Effect of pre-purified proteases (PP) against *E. coli* ATCC 8739 and *S. aureus* ATCC 33862 in nutritive agar

Sample	Concentration (mg/mL)	*E. coli*		*S. aureus*	
		Lethality (Log CFU/mL)	Reduction (%)	Lethality (Log CFU/mL)	Reduction (%)
PP from cocuixtle fruit	31.2	0.105 ± 0.033a,A	21.26	0.211 ± 0.033k,l,B,C	38.61
	62.5	0.176 ± 0.025b,B	33.33	0.261 ± 0.006l,C	45.29
	125.0	0.264 ± 0.029c,C	45.40	1.173 ± 0.034m,F	93.29
	250.0	0.501 ± 0.022d,D	68.50	1.214 ± 0.099m,G	93.83
	500.0	0.793 ± 0.033e,E	83.90	2.171 ± 0.009n,H	99.32
PP from guamara fruit	31.2	0.926 ± 0.028f,I	88.15	0.192 ± 0.014k,N	35.76
	62.5	1.074 ± 0.082g,J	91.50	0.361 ± 0.026o,O	56.45
	125.0	1.307 ± 0.020h,K	95.07	1.090 ± 0.021p,J	91.88
	250.0	1.414 ± 0.055i,L	96.13	1.349 ± 0.007q,K	95.52
	500.0	1.641 ± 0.027j,M	97.71	1.450 ± 0.027r,L	96.45
PP from Cocuixtle fruit (80 °C/15 min) (C-)	500.0	0.010 ± 0.009	2.76	0.002 ± 0.001	0.65
PP from Guamara fruit (80 °C/15 min) (C-)	500.0	0.010 ± 0.008	1.74	0.004 ± 0.002	1.1
Ampicillin (C+)	5.0	4.153 ± 0.011	99.99	3.038 ± 0.028	99.90

The values are presented with the mean ± standard deviation (n=3). Per column, different lowercase letters indicate a significant difference between both proteases on a pathogen ($\alpha=0.05$). Per file, different capital letters indicate a significant difference between the effect of a protease on both pathogens ($\alpha=0.05$). C- = Negative control. C+ = Positive control.

of antibiotics (ampicillin, tetracycline, rifampin, and vancomycin).

The authors reported that the growth profile of *E. coli* in the presence of guamara proteases was similar to that obtained with rifampicin, while the growth profile of *S. aureus* was similar to the exhibited with tetracycline. Ávalos-Flores et al. (2020), reported that the pre-purified cocuixtle proteases exhibited antibacterial activity on *Listeria monicytogenesis* and *Salmonella Typhimurium*; however, there are no report of its effect on *E. coli* and *S. aureus*.

The foregoing suggests that proteases may present different mechanisms of action depending on the type of microorganism and therefore, on the antimicrobial activity. Eshamah et al. (2013) suggested that the composition of the cell wall of bacteria plays an important role in the effectiveness of the inhibitory activity of proteases. The action mechanism of proteases on the bacterial inhibition has not yet been elucidated, although it has been theorized about various mechanisms that could be involved in the bacterial inhibition by the action of plant proteases. Liliany et al. (2018) reported that the inhibitory effect of some proteases could be due to their interaction with the cell envelope of bacteria, causing instability in the electrostatic forces of the membrane, altering its permeability, forming pores, and affecting osmotic regulation, which is vital for the proper functioning of the cell (Lugo-Vargas et al., 2016). Other theories suggest that proteases could affect cellular respiration, decreasing ATP production (Thevissen et al., 1999; Cociancich et al., 1993). Likewise, proteases could be hydrolyzing or interfering in the synthesis of essential components of the cell wall (Odds et al., 2003; Camacho-Hernández et al., 2002; Nibbering et al., 2001; Montes et al., 1990). Nonetheless, specific studies are required to elucidate the action mechanism of pre-purified guamara and cocuixtle proteases in bacterial inhibition.

CONCLUSIONS

The guamara and cocuixtle fruits showed a non-climacteric respiratory behavior. The state of maturity I of cocuixtle fruits had the highest SEA, while the guamara fruits had the highest SEA in the state of maturity II. In this work was demonstrated that the pre-purified proteases from guamara and cocuixtle fruits showed an important inhibitory effect against the strains of *E. coli* ATCC 8739 and *S. aureus* ATCC33862. Therefore, these vegetal proteases can be an alternative for food industry. The perspective of this study is that more specific studies are necessary to establish the action mechanism of proteases on bacterial growth.

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Author contributions

Anaya-Esparza Luis Miguel; specifically performing the experiments and data/evidence collection. Martínez-Olivo Abraham Osiris; specifically performing the experiments and data/evidence collection. Abreu-Payrol Juan; Validation and supervision. Sánchez-Burgos Jorge Alberto; validation and supervision; Montalvo-González Efigenia; validation, formal analysis, writing-review, and edition. García-Magaña María de Lourdes; supervision, funding acquisition, methodology, formal analysis, writing-review and edition.

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