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Potencial antibacteriano de extractos antociánicos de fresa sobre Staphylococcus aureus asociado a la mastitis bovina

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Abstract: In this study, the effect of anthocyanic extracts of strawberry (Fragaria x ananassa Duch.) variety Jacona on the in vitro growth of Staphylococcus aureus associated to bovine mastitis was evaluated. Sensitivity tests were carried out on the anthocyanic extracts by disk difussion method (10 µg/disk-100 µg/disk), using the antibiotic Dicloxacillin® as positive control (50 µg/mL, 50 µL/disk) and percentages of inhibition of bacterial growth were determined. Anthocyanic extracts managed to inhibit the bacterial growth of the strain ATCC 27543 and the isolated STA28 of S. aureus up to 54% and 40%, respectively. The results showed the antimicrobial potential of anthocyanic extracts of strawberry against S. aureus associated to bovine mastitis.

Keywords: *Fragaria x ananassa* , secondary metabolites, anthocyanins, bacterial resistance.

Resumen: En este estudio, se evaluó el efecto de los extractos antociánicos de fresa (Fragaria x ananassa Duch.) variedad Jacona sobre el crecimiento in vitro de Staphylococcus aureus asociados a la mastitis bovina. Las pruebas de sensibilidad de los extractos antociánicos se realizaron por el método de difusión en disco (10 µg/disco a 100 µg por disco), utilizando el antibiótico Dicloxacillin® como control positivo (50 mg/ml, 50 µl/disco) y se determinaron los porcentajes de inhibición del crecimiento bacteriano. Los extractos antociánicos lograron inhibir el crecimiento de la cepa de S. aureus ATCC 27543 y del aislado STA28 hasta 54% y 40%, respectivamente. Los resultados mostraron el potencial antimicrobiano de extractos antociánicos de fresa contra S. aureus asociada a la mastitis bovina.

Palabras clave: Fragaria x ananassa , metabolitos secundarios, antocianinas, resistencia bacteriana.

INTRODUCTION

Bovine mastitis is considered one of the most common diseases that affects dairy cattle worldwide and causes great economic losses to

producers as well as to the dairy industry, due to the decline of milk quality and yield as well as the early disposal of diseased animals (Deb *et al*., 2013). The disease consists of inflammation of the mammary gland and its secretory tissues, caused by different etiological agents whose control is costly. Nowadays, more than 100 microorganisms are known to cause mastitis and are classified in contagious and environmental pathogens. One of the main contagious pathogens associated to cases of mastitis is *Staphylococcus aureus*, a Gram positive bacterium that has numerous pathogenicity factors that favor the invasion of the mammary epithelium (Deb *et al*., 2013; Rabello *et al*., 2005), frequently causing chronic intramammary infections; which start when bacteria reach the nipple hole, enter through the canal and reach the mammary gland adhering to the epithelium (Sutra & Poutrel, 1994).

The control of *S. aureus* has been achieved mainly through antibiotic therapy; however, the indiscriminate use of antibiotics has promoted the selection of bacteria resistant to such compounds, which results in therapy failure; consequently, the search for new sources of antibiotics as an alternative to such conventional therapy is necessary (Adesola, 2012; Ochoa-Zarzosa *et al*., 2008; Sutra & Poutrel, 1994).

In this regard, secondary metabolites of plant origin, many of which assist in the protection of plants against herbivores, pests and pathogens, are considered an excellent source of antibiotic compounds and have been the main providers of the pharmaceutical industry since they contribute with more than 40% of the compounds that exist in the market nowadays, either as natural products or in their synthetic versions (Crozier, Jaganath & Clifford, 2007; Ochoa-Zarzosa *et al*., 2008). Secondary metabolites of plants show a great chemical diversity, so the British Nutrition Foundation classifies them into four big groups: terpenoids, alkaloids, compounds that contain sulfur and phenolic compounds, the latter with about 8000 characterized metabolites (Crozier *et al*., 2007).

One of the most important sources of secondary metabolites of plant origin is fruits. The vast majority of the benefits of consuming fresh fruits has been attributed to the high concentrations of secondary metabolites that they contain and have been associated to the prevention or control of several chronic degenerative diseases (Poiroux-Gonord *et al*., 2010). In this regard, strawberry (*Fragaria x ananassa* Duch.) contains several phenolic compounds, among which are the anthocyanins, that constitute the largest group and probably the most important of water-soluble, natural pigments and that gives the red coloring to strawberry (Poiroux-Gonord, 2002).

The intense color that anthocyanins emit promote the dispersion of seeds and pollination due to attraction of animals and insects. Additionally, the anthocyanins participate in the protection of plants against damage caused by UV rays (Mazza & Miniati, 1993). Several studies have reported that anthocyanins possess biological activities as powerful antioxidant, anti-inflammatory and anticarcinogenic agents (Aaby, Ekeberg & Skrede, 2007; He & Giusti, 2010; Zhang, Seeram, Lee, Feng & Heber, 2008), apart from preventing cardiovascular diseases

and controlling diabetes and obesity (Aaby, Mazor, Nes & Skrede, 2012; He & Giusti, 2010). Knowledge of such properties has arisen interest in studying their antifungal and antimicrobial activities on humans' pathogens (Cisowska, Wojnizz & Hendrich, 2011; Kim *et al*., 2012; Nohynek *et al*., 2006). However, as far as it is known, no antimicrobial activity of strawberry anthocyanins against pathogenic bacteria of farm animals has been studied, particularly those associated to bovine mastitis. Therefore, the objective of the present work was to explore the *in vitro* antibacterial activity of anthocyanic extracts of strawberry on *S. aureus* ATCC 27543 and STA28 associated to bovine mastitis.

MATERIAL AND METHODS

Biological material

The certified strain ATCC 27543 and the isolated STA28 of *S. aureus* were provided by Dr. Joel Edmundo López Meza of the *Centro Multidisciplinario de Estudios en Biotecnología* (CMEB-FMVZ) of the *Universidad Michoacana de San Nicolás de Hidalgo*. Bacteria were kept under continuous subculturing in mannitol salt agar (Bioxon®) at 37 °C. The isolate of *S. aureus* STA28 was characterized by observation under an optical microscope and biochemical tests such as catalase, coagulase, gelatinase and fermentation in mannitol salt agar (López-Meza *et al*., 2006). Then, the isolate was also characterized by amplification of a sequence of the nuc gene, which encodes a thermostable nuclease that is highly specific for *S. aureus* (Brakstad, Aasbakk & Maeland, 1992; Ochoa-Zarzosa *et al*., 2008).

Strawberries used to obtain anthocyanic extracts were harvested from plants of F. x ananassa variety Jacona grown in the greenhouse of CIIDIR IPN Unidad Michoacan, in Jiquilpan, Michoacan, Mexico, located at an altitude of 1560 m s.n.m, delimited by the coordinates 20° 03' 02'' and 19° 52' 54'' of North latitude and meridians 102° 39' 33'' and 102° 56' 16'' West longitude.

Extraction and quantifying of anthocyanic extract of strawberry

In order to obtain the anthocyanic extract of strawberry, the methodology of Abdel-Aal & Hucl (1999) was used; it consisted of softening 1 g of fresh fruit in 5 mL of acidified ethanol (ethanol and HCl 1N; 85:15 v/v, J.T. Baker®). The pH of the mixture was adjusted to 1 with HCl 1N. The extract was shaken at 250 rpm in a rotary shaker (Heidolph®) during 16 h, at room temperature (25 ± 2 °C). The extract was centrifuged (Hettich®) at 6000 rpm for 15 min; the supernatant was recovered and diluted at 25 mL with acidified ethanol. For the extract quantifying, its absorbance was read at 535 nm in a spectrophotometer of UV/VIS light (Optizen POP®), using the pigment cyanidine 3-glycoside as standard and acidified ethanol as target. The samples were stored at -20 °C until their

use. The concentration of total anthocyanins present in the extract was determined by applying the following equation: C = (A/E) × (vol/1,000) × MW × (1/weight of sample) × 10^6, where C = total concentration of anthocyanins (mg/kg); A = absorbance at 535 nm; E = molar absorbance of cyanidin 3-glycoside = 25 965 cm^{-1} M^{-1}; and Vol = total volume of anthocyanins extract and MW= molecular weight of cyanidin 3-glycoside = 449.

In vitro inhibition tests of anthocyanic extract on S. aureus

Bioassays were carried out using the disk diffusion method for sensitivity tests on antimicrobials, with some modifications (Klančnik, Piskernik, Jeršek & Možina, 2010). Also, 100 µl of bacterial suspension, adjusted to a concentration of 1 × 10^8 UFC/mL, were used. This suspension was distributed over the surface of a Petri dish that contained Muller Hinton agar (Bioxon®), with the help of a sterile glass handle. Later, sterile disks of 6 mm of diameter (paper Whatman No. 1®) were saturated with the volumes corresponding to each concentration of total anthocyanins extract (10 µg, 25 µg, 50 µg, 75 µg and 100 µg = 6 µL/disk, 15 µL/disk, 30 µL/disk, 46 µL/disk and 61 µL/disk, respectively), they were dried on laminar flow bell (CHC Biolus®) during 1 h and placed on the surface of each dish.

Absolute ethanol was used as negative control (6 µL/disk, 15 µL/disk, 30 µL/disk, 46 µL/disk and 61 µL/disk) and the antibiotic Dicloxacillin® was used as positive control at a concentration of 50 µg/mL (50 µL/disk). The Petri dishes were incubated for 24 h at 37 °C. Then, the diameter of inhibition halo of treatments was measured with a digital Vernier Caliper® without considering the disk. All the treatments were performed in triplicate. The diameter of halo of inhibition was expressed as the percentage of extract inhibition which was calculated by the following formula: % of inhibition = (diameter of extract halo / diameter of positive control halo) × 100 (Corzo, 2012).

Statistical analysis

With the data obtained from the experiments, an analysis of variance (Anova) and a Tukey test ($p \leq 0.05$) were carried out, by means of the program SAS® version 9.0. Data were transformed with the formula √(% inhibition)/100 (Ruiz-Sánchez, Mejía-Bautista, Cristóbal-Alejo, Valencia-Botín & Reyes-Ramírez, 2014).

RESULTS AND DISCUSSION

Several phenolic compounds of fruits and vegetables such as quercetin, kaempferol, gallic acid, cinnamic acid and coumarins, among others, have been studied due to their antioxidant and antimicrobial qualities against several pathogen microorganisms (Hafidh *et al*., 2011; Lopes-

DaSilva, De Pascual-Teresa, Rivas-Gonzalo & Santos-Buelga, 2002; Puupponen-Pimia *et al*., 2001). It is worth noting that antibacterial activity of phenolic extracts of different fruits, including strawberry anthocyanic extracts against human pathogens, has been approached to characterize and develop new food ingredients, as well as medical and pharmaceutical products (Cisowska *et al*., 2011; Puupponen-Pimiä *et al*., 2001). However, this is the first report of the antibacterial effect of strawberry anthocyanins on pathogenic bacteria associated to bovine mastitis, such as *S. aureus*.

Anthocyanic extracts of F. x ananassa var. Jacona showed significant antibacterial activity ($p \leq 0.05$) on the growth of S. aureus ATCC 27543, since all the assayed amounts inhibited its growth. Inhibition percentages observed oscillated between 2.4% and 53.6%, which increased in proportion to the applied amount (table 1). Similarly, the assays with the isolated STA28 revealed a significant antimicrobial activity ($p \leq 0.05$) in all the amounts of anthocyanic extract assayed. Inhibition percentages observed were from 2.56% to 40.05% (table 1).

Table 1
Table 1

Table 1 Percentage of inhibition of anthocyanic extracts of strawberry var. Jacona on *S. aureus*

Treatments (µg)	Inhibition (%)*	
	ATCC 27543	STA28
10	2.4 ± 0.05 F	2.56 ± 0.17 E
25	13.9 ± 0.05 E	20.45 ± 0.2 D
50	32.5 ± 0.05 D	23.1 ± 0.36 C
75	39.6 ± 0.15 C	25.66 ± 0.36 C
100	53.6 ± 0.15 B	40.05 ± 0.6 B
**Positive Control	100 ± 0.2 A	100 ± 0.8 A

*Mean and standard deviation of inhibition percentages are presented. Different letters within a column indicate significant differences based on Tukey test ($P < 0.05$, n = 3).
**Dicloxacillin® at 50 µg/mL (50 µL/disk).
Source: Author's own elaboration.

Although the results of the present work revealed the antimicrobial effect of strawberry anthocyanic extract against bacteria associated to bovine mastitis for the first time, the use of plant extracts to control this disease has already been documented. Mubarack, Doss, Dhanabalan & Venkataswamy (2011) evaluated the antibacterial activity of ethanolic extracts of four medicinal plants of India (*C. ciliaris, C. grandis, Brachiaria* sp. and *A. indicum*) against pathogenic isolates that cause bovine mastitis (*Streptococcus agalactiae, Kleibsella pneumoniae, Escherichia coli* and *S. aureus*). The authors reported that, with concentrations of 100 mg/mL and 200 mg/mL, inhibition from 34% to 82% were generated, in contrast to positive control (Ciprofloxacin, 200 mg/mL). Dhanabalan *et al.* (2008) found that leaf extracts of *Tridax procumbans* inhibited growth of different strains of *S. aureus* isolated from clinical cases of bovine mastitis (29% to 70%), attributing this

biological activity to the flavonoids, tannins and alkaloids present in the extracts.

It is important to note that the low concentration of the anthocyanin extract evaluated in the present study (less than or equal to 100 µg/disc) allowed an inhibition greater than 40% in both strains of *S. aureus*., while in other reports, greater amounts of anthocyanin extracts were used on humans' pathogenic strains, Gram (+) as well as Gram (-). For example, anthocyanin extracts of *Sysygium cumini* fruits (5 mg/disk and 10 mg/disk) against *Enterococcus faecalis, Bacillus cereus, B. subtilis, Meticillin Resistant Staphylococcus Aureus* (MRSA), besides *Salmonella typhimorium, E. coli* and *S. paratyphi B; assays* targeted to *S. aureus* showed inhibition in both concentrations, with 10 mm and 12.5 mm of inhibition diameter (25% and 31%, respectively), contrasting with 40 mm of inhibition of positive control (Ampicillin 10µg/disk) (Priya, Devi, Eganathan & Kingsley, 2013). Similarly, the results matched the ones obtained by Nohynek *et al*. (2006), who reported antimicrobial activity of phenolic extracts of strawberry in dilution assays in liquid means against pathogenic bacteria of humans such as *B. cereus* E-127, *Campylobacter jejuni* E-1008, *Clostridium perfringens* E-861, *Helicobacter pylori* NCTC 11637, *S. aureus* E-045 and *S. epidermidis* E-768, at a concentration of 1 mg/mL. Strawberry phenolic compounds have shown a significant antimicrobial effect against Gram-negative bacteria, such as *Salmonella enterica* and *E. coli* (CMI = 1mg/ml) (Puupponen-Pimia *et al.*, 2001). The authors attributed this effect to the synergy between the anthocyanins and other phenolic compounds present in the extracts, added to the acidic conditions of these. Hafidh *et al.* (2011) also attributed the inhibitory potential of different pathogenic microorganisms to the anthocyanins of purple cabbage (*Brasicca oleracea*), including *S. aureus* Meticillin Resistant (MRSA) whose trials showed average inhibition halos of 11.5 mm (500 mg/mL). The authors suggested that the anthocyanins antioxidant action, their complex interactions with DNA and direct link to proteins could explain, together or separately, the antimicrobial potential of the methanolic extract of purple cabbage. On the other hand, Gyawali & Ibrahim (2012), suggested that the toxicity of various compounds present in strawberries (e.g. phenolic, flavonoids) is derived from both direct and indirect actions that these exert on microorganisms. The direct actions refer to the interactions of those compounds with the cell membrane, causing the inactivation of enzymes that are essential for the survival of the cell, whereas the substrate availability and the genomic expression that result from the deteriorated metabolism of the microorganism are considered indirect actions (Lacombe & Wu, 2017). However, the mechanisms by which strawberry anthocyanic extracts exert their inhibitory effect on S. aureus should be investigated.

The above results show the antibacterial potential of strawberry anthocyanic extract against *S. aureus*, the main pathogenic bacteria associated to bovine mastitis; this could increase the possible pharmaceutical uses of these compounds in veterinarian medicine. However, deeper research should be done to know the effect of the

application of greater amounts of anthocyanins on the growth of *S. aureus*, to determine the antibacterial activity of anthocyanic extracts of different varieties of strawberry on more *S. aureus* strains of clinical interest, and to identify the compounds responsible for inhibiting their growth.

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Additional information

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