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Chemical characterization of alcoholic extract of guava leaf (*Psidium guajava*) and its effect as a mobility inhibitor for *Escherichia coli* O157:H7

Caracterización química de extracto alcohólico de hoja de guayaba (*Psidium guajava*) y su efecto como inhibidor de movilidad para *Escherichia coli* O157:H7

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ABSTRACT

The objective was to characterize and determine the mobility inhibitory effect in *Escherichia coli* O157: H7 of extracts of guava leaves (*Psidium guajava*). New alternatives of natural origin "plant extracts" have been sought to eliminate colonization of pathogenic bacteria in animals and prevent contamination of meat. Guava leaf extract (*Psidium guajava*) has broad-spectrum antibacterial activity, due to the active ingredient quercetin. *E. coli* O157: H7 enterohemorrhagic, is a pathogen of great importance in public health, which can cause hemolytic uremic syndrome, and ruminants are recognized as the main host of *E. coli* O157: H7. The extract was prepared with guava leaves in 70% ethanol, obtaining a crude extract (Extract A) and a concentrated extract using the soxhlet equipment (Extract B). Its chemical composition was determined by gas chromatography. Nursing ruminants with diarrheal syndrome were sampled, the samples were transported in Stuart medium. The bacteria were isolated in Mac Conkey medium and subsequently seeded in CHROMagar™ 0157 medium for the identification of *E. coli* O157:H7. Mobility tests of *E. coli* O157: H7 were carried out in SIM medium, with guava leaf extract and as a reference, concentrations of carvacrol of 0.3, 1 and 5 mM and quercetin 205, 102 and 51 mM were used. 78 *E. coli* O157: H7 were identified, which showed inhibition in mobility at different concentrations of carvacrol, in quercetin 205 mM and 102.5 mM and in extracts A and B. It is concluded that the alcoholic extract of guava leaves and its compound in a greater proportion (quercetin) they are effective in inhibiting the mobility of *E. coli* O157 H7.

Keywords: Extracts, carvacrol, quercetin, inhibition.

RESUMEN

El objetivo fue caracterizar y determinar el efecto inhibitorio de movilidad en *Escherichia coli* O157:H7 de extractos de hojas de guayaba (*Psidium guajava*). Se han buscado nuevas alternativas de origen natural "extractos de plantas" para eliminar la colonización de bacterias patógenas en animales y prevenir la contaminación de carne. El extracto de hoja de guayaba (*Psidium guajava*) tiene actividad antibacteriana de amplio espectro, debido al principio activo quercetina. *E. coli* O157:H7 enterohemorragica, es un patógeno de importancia en salud pública, que puede causar síndrome urémico hemolítico, además los rumiantes son reconocidos como el principal hospedero de *E. coli* O157:H7. El extracto fue preparado con hojas de guayaba en etanol al 70%, obteniendo un extracto crudo (Extracto A) y uno concentrado mediante el uso del equipo soxhlet (Extracto B). Se determinó la composición química por cromatografía de gases. Se muestrearon rumiantes lactantes con síndrome diarreico, las muestras fueron transportadas en medio Stuart. Las bacterias se aislaron en medio Mac Conkey y posteriormente fueron sembradas en medio CHROMagar™ 0157 para la identificación de *E. coli* O157:H7. Se realizaron pruebas de movilidad de *E. coli* O157:H7 en medio SIM, con extracto de hoja de guayaba y como referencia se utilizaron concentraciones de carvacrol de 0.3, 1 y 5 mM y quercetina 205, 102 y 51 mM. Se identificaron 78 *E. coli* O157:H7, las cuales mostraron inhibición en la movilidad a diferentes concentraciones de carvacrol, en quercetina 205 mM y 102.5 mM y en los extractos A y B. Se concluye que el extracto alcohólico de hojas de guayaba y su compuesto en mayor proporción (quercetina) son efectivos en la inhibición de movilidad de *E. coli* O157 H7.

Palabras clave: Extractos, carvacrol, quercetina, inhibición.

INTRODUCTION

Escherichia coli O157, is an important pathogen in public health, which can produce Shiga toxin (STEC) (Kaper *et al*., 2004). STEC products are transmitted through food, especially serotype O157: H7. The diseases caused to humans by the serotype that produces STEC range from mild diarrhea to hemorrhagic colitis and hemolytic uremic syndrome (HUS), which generally affects children, elderly and immunocompromised patients (Rodríguez-Angeles, 2002). The pathogenicity of STEC resides in different virulence factors, including Shiga toxins (Stx1 and Stx2), intimin, enterohemolysin, and the self-binding STEC adhesin (AEA) (Gyles, 2007).

Domestic ruminants such as cows, sheep and goats have been reported to be asymptomatic carriers, which can carry STEC and *E. coli* O157: H7 in their feces, which is why they are considered natural reservoirs of these pathogens (Blanco *et al*., 2004; Milton *et al*., 2018; Iweriebor *et al*., 2015; Bolukaoto *et al*., 2019). To eliminate the colonization of pathogenic bacteria in animals and prevent contamination of meat, there are a variety of chemical antimicrobial agents that are available for therapeutics in livestock. However, animal nutrition researchers have reported that with the increased use of antimicrobial agents in animals and humans, the prevalence of resistant strains has increased (Cattoir and Leclercq, 2017; Kim *et al*., 2019). The CTX-M β-lactamases genes have been reported in *E. coli*, from livestock for food purposes throughout the world; raising a potential threat to public health (Wittum *et al*., 2010; Botelho *et al*., 2015; Vitas *et al*., 2018).

From the increase in antibiotic resistant strains, new alternatives of natural origin have been sought, such as plant extracts; for example, the extract of guava leaf (*Psidium*

guajava), which has broad-spectrum antibacterial activity (Martínez *et al.,* 1997; Bermúdez-Vásquez *et al.,* 2019). Rattanachaikunsopon and Phumkhachorn (2010), report that the aqueous extract of guava leaf showed antibacterial activity in 35% of the cases, the alcoholic in 65% and the ketone in 100%, against pathogenic bacteria; including *Bacillus stearothermophilus*, *Brochothrix thermosphacta*, *E. coli* O157: H7, *Listeria monocytogenes*, *Pseudomonas fluorescens*, *Salmonella enterica*, *Staphylococcus aureus*, and *Vibrio cholerae*.

The aqueous extract of guava leaves reduces the production of labile toxins of *E. coli* and cholera (Birdi *et al*., 2010). Echemendía and Morón (2004) in their clinical trial concluded that the 20% tincture of *Psidium guajava* leaf has an antidiarrheal effect and Lozoya *et al*. (2002) evaluated the dust of the dry leaves, verifying this effect.

Various chemical compounds have been isolated from guava leaf extract, such as: pentacyclic triterpenoid, guajanoic acid; as well as ß-sitosterol, uvaol, oleanolic acid and ursolic acid and quercetin (Biswas *et al.,* 2013), to verify the antibacterial effect of guava leaves. Therefore, the objective of this work was the characterization and determination of the inhibitory effect of mobility in *E. coli* O157: H7, of extracts of guava leaves (*Psidium guajava*).

MATERIAL AND METHODS

Obtaining the alcoholic extract of Psidium guajava leaves

For each extract, 70% ethanol J.T. Baker, in a ratio of 25 grams of the ground sample for every 200 mL of solvent. The mixture was placed and sealed in one liter amber flasks, it was vigorously homogenized for 10 min; the extract was allowed to stand for a month at room temperature. The supernatant was passed through filter paper (Whatman No. 2), to remove the remains of the plant dust (Pesewu *et al*., 2008). A part of the prepared extract was used to concentrate it by using the soxhlet equipment for one hour, recovering half the initial volume, calling it Extract B. Extract A was named the most dilute extract, or as it was obtained after filtration.

Chemical composition of guava leaf extracts by gas chromatography

The chemical composition was determined using a gas chromatograph (GC; Agilent Technologies 6890N series manufactured in the USA), with a DB_WAXetr polar column, at 250 °C and 12.13 psi with a He flow of 36.5 mL min^{-1} after injection. The conditions for the column were: initial temperature 50 ° C, from zero to two min, increasing from 10 to 10 °C until reaching 250 °C, keeping the temperature constant for 5 min, and then descending to 50 °C for two min with a He flow of 1.6 mL min^{-1} at a pressure of 12.13 psi and an average velocity of 25 cm s^{-1}, using an ionizing flame detector (IFD) at a temperature of 210 °C, with a flow of H_2 of 40 mL min^{-1} and an air flow of 450 mL min^{-1}. Carvacrol and thymol standards (Sigma-Aldrich) (Bañuelos-Valenzuela *et al*., 2018) were used for the running of samples in the chromatograph.

Determination of the minimum hemolytic dose of the extracts

10 mL of blood was drawn into a heparinized tube; the tube with the uncoagulated blood was centrifuged at 2500 rpm x 10 min at 10 °C, the serum fraction was removed with a pipette; subsequently, three washes were carried out with a wash regulator [PBS 50% (v/v) and glucose 2.25% (w/v)] (López *et al*., 2017); then it was centrifuged in each wash at 2500 rpm x 10 min at 10 °C. The erythrocyte packet was recovered and resuspended to a concentration of 0.1% (v/v) with a suspension regulator, consisting of PBS at 50% (v/v), glucose at 2.25% (w/v) and gelatin at 0.05% (w/v). The assay was performed in triplicate in U-bottom 96-well plates; 100 µL of the 1% erythrocyte suspension were mixed with 100 µL of the suspension of each extract and with dilutions of 1:10, 1: 100 and 1: 1000.

The negative control was inoculated only 100 µL. This same procedure was carried out for each active principle in its different concentrations (carvacrol, thymol and quercetin), from the 1% erythrocyte suspension. For the positive control, 100 µL of the 1% erythrocyte suspension was mixed with 100 µL of 1% Triton X; Changes in the suspension of erythrocytes were observed every hour, up to 24 h. Based on the positive and negative controls, it was determined if there was hemolytic activity of the extract on the erythrocytes.

For the running of samples in the chromatograph, the standards of carvacrol and thymol, of the reagent grade SIGMA brand, were used. The guava leaf extract (40 mg/mL) was concentrated by boiling (Ext A).

Identification of bacteria in CHROMagar™ chromogenic medium

Rectal swabs were obtained from lactating ruminants, with the presence of diarrheal syndrome, under 21 days of age and with the assurance of having ingested colostrum. The samples were collected rectally with a sterile swab, they were labeled and transported in Stuar® medium made in Mexico City; each sample was seeded in the petri dish with MacConkey agar. A colony was taken to continue with the identification, which was carried out by streaking plating in a chromogenic medium CHROMagar™ O: 157, for *E. coli* O: 157: H7 (Moyne *et al*., 2011). They then selected 78 bacterial strains from feces of lactating ruminants with diarrheal syndrome under three months, identified as E. coli O157: H7 in CHROMagar™ which presented mauve pink coloration, due to chromogenic substrates in the medium; thus allowing the presumptive identification of the primary isolation plate and the differentiation of other organisms (Hirvonen *et al*., 2012; Lara *et al*., 2019).

Preparation of SIM medium in tube with extract

SIM medium was prepared for each type of standard and extract. For the preparation of the SIM medium, 30 g of agar were weighed for each liter of distilled water; the agar was sterilized in an autoclave at 121 °C for 15 min. The agar was allowed to cool to a temperature of approximately 35 °C, to add the extract and the corresponding standard; later, 4 mL of the previous mixture were added in sterile 10 mL tubes.

For each bacterium, a series of tubes was made in triplicate, as described below: Control (without extract), Carvacrol (C 0.3 mM, C 1 mM and C 5 mM), Ext A (diluted guava extract), Ext B (concentrated guava extract), Q 205 (205 mM quercetin), Q 102 (102 mM quercetin), Q 51 (51.25 mM quercetin) and OH (alcohol control).

Bacterial mobility tests

Each one of the bacteria identified by CHROMagar™ O: 157, was seeded on base agar by striae, with the aim of obtaining a single isolated colony to later carry out the sowing in tube. The sowing in the tube was carried out by stinging, which consists of taking an isolated colony of bacteria and making a sting in the SIM medium, crossing the agar to the bottom of the tube; the medium was prepared with the extracts, standards and control. Once the seeding by stinging was finished, all samples remained at a temperature of 37 °C, in a Thermo® incubator for a period of 24 h.

The sowing conditions were made with due sterility to avoid contamination. These were carried out in a laminar flow hood (Lab tech®). Bacterial mobility was measured, using a qualitative method; a) positive motility (+): presence of diffuse or total turbidity in the medium. b) Negative motility (-): absence or slight presence of growth, only at the site of the bite.

Statistical analysis

The statistical analysis performed was the contingency tables of dimension 2 × 2, between the variables extract A and extract B vs 0.3 mM carvacrol, 1 mM carvacrol, 5 mM carvacrol, 205 mM quercetin, 102 mM quercetin and 51 mM quercetin. The criteria used were the χ^2 independence tests (chi-square test), considering a level of significance of $p < 0.05$ and a 95% confidence interval (Good, 2000). The data were captured in Excel and analyzed in the Statistical Package for the Social Sciences (SPSS) version 17.

RESULTS AND DISCUSSION

Table 1 shows the presence of the active principles carvacrol and thymol in the alcoholic extracts of guava leaves, as well as their concentration by gas chromatography.

Table 1. Gas chromatography results expressed in mg/mL concentration units

Guava leaf extract	Carvacrol	Thymol
Extract A	3.0869	1.5130
Extract B	0	0.3525

Both active principles (carvacrol and thymol) were present in extract A; while extract B, only thymol was identified. The active principle with the highest concentration in the extracts was carvacrol with 3.0869 mg/mL in extract A. Medicinal plants are commonly rich in terpenes (carvacrol, citral, linalol and geraniol) and phenolic compounds, effective compounds as food additives (Nile *et al*., 2017).

The primary mode of antibacterial action of thymol is not fully understood but is believed to involve outer and inner membrane disruption and interaction with membrane proteins and intracellular targets. Studies by Wang and Yam (2018) have shown that thymol interacts with cell membranes, and these interactions affect the permeability of the bacterial membrane. For the hemolytic effect and based on the positive and negative controls, it was determined if there was hemolytic activity of the extracts of guava leaves and the active principles on the erythrocytes. In Table 2 it was observed that for carvacrol at a concentration of 0.3 mM, hemolysis did not occur, in extracts A and B there is only hemolysis until the 1:10 dilution. Finally, for quercetin, the minimum concentration of hemolysis was in a 1:10 dilution of its three concentrations. As mentioned by López *et al*. (2017) the term hemolysis refers to the process of destruction of erythrocytes, which generates the release of intraerythrocytic components; therefore, the hemolysis test is used to know the effect on the erythrocyte cell when confronting it with the extracts at different concentrations, which is what was intended with this experiment. The inhibition of hemolysis is due to the bioactive components, such as flavonoids and phenolic compounds present in the guava extracts.

Erythrocytes can change their normal shape to echinocytes or stomatocytes, which depends on cytoplasmic factors, including pH (Gedde *et al.,* 1997). The high concentrations of flavonoids found in fruits such as mango (García Bacallao *et al.*, 2001), guava leaves (Rodríguez *et al*., 2013) and flavonoids obtained from maqui (*Aristotelia chilensis*) with antioxidant properties, are inducers of echinocytes (Gironés-Vilaplana *et al.,* 2012; Durán *et al*., 2013).

Table 2. Hemolytic activity of guava leaf extracts

Sample dilution	Stock solution	1:10	1:100	1:1000
C 0.3 mM	+	-	-	-
C 1 mM	+	+	-	-
C 5 mM	+	+	+	-
EXT A	+	+	-	-
EXT B	+	+	-	-
Q 205 mM	+	+	-	-
Q 102 mM	+	+	-	-
Q 51 mM	+	+	-	-

(+) presence of hemolysis; (-) absence of hemolysis

With the use of CHROMagar™, *E. coli* O: 157 H7 was identified, differing by the color of the colony in the medium, it presents a mauve pink coloration (Lara *et al*., 2019). This medium was initially used in the food industry for the rapid release of pathogen-free foods, but is currently approved for the analysis of clinical samples and has been used in various studies (Bettelheim, 1998; Tang *et al*., 2014; Gutierrez *et al*., 2016; Parsons *et al*., 2016). 78 strains of *E. coli* O157: H7 were identified by selective CHROMagar ™, and these bacteria were inoculated in SIM medium.

The results of the *in vitro* mobility of the guava leaf extracts and the standards (active ingredients of guava leaf) on the bacteria are observed in table 3. This effect was qualitatively evaluated by the presence or absence of turbidity in the tube; The results showed that the carvacrol standards have broad antibacterial activity, against 65 microorganisms; inhibiting bacterial growth in 56 bacteria at a concentration of 5 mM (Table 3); in relation to the extract A of guava that showed an effect on the inhibition of the mobility of 62 bacteria; while extract B inhibits 46 bacteria. Quercetin at a concentration of 51 mM, presented the highest mobility inhibition in 60 bacteria.

3. Total results of bacterial mobility

	C 5 mM	C 1 mM	C 0.3 mM	EXT A	EXT B	Q 205	Q 102	Q 51	OH
+	56	60	65	62	46	59	51	60	0
-	22	18	13	16	32	19	27	18	78

(+): Presence of diffuse or total turbidity in the medium. (-): Absence or slight presence of growth

Gallegos- Flores *et al.* (2019) reported that carvacrol at a concentration of 0.3 mM decreases the mobility of the strain determined with the Wertern Blot technique, where they observed a decrease in flagellin synthesis; this protein is found in 8% of the total cellular protein. These results differ from those reported in table 2, mainly due to the fact that the 0.3 mM concentration did not inhibit the mobility in 100% of the *E. coli* O157: H7 bacteria.

From the point of view of Gallegos- Flores *et al.* (2019), E. coli cells grow in the presence of carvacrol at a concentration of 5 mM without flagella synthesis, causing the microorganism to grow without mobility; that is, when the bacterial cell is subjected to stress caused by toxic substances and its survival is at risk; which is capable of suppressing the production of the flagellin protein and conserving energy for other cellular functions, which can therefore be a survival tactic; however, at a concentration greater than 5 mM, the bacteria immediately cease mobility and cell death occurs; observing that the concentration of 5 mM is the one with the highest number of bacteria that inhibited growth.

The carvacrol-quercetin interaction that is present in extract A of guava leaves, shows a greater inhibition in the mobility of bacteria, as long as the concentrations of carvacrol and quercetin are those indicated above (Table 4). Both cases are significant, but a greater magnitude of comparison of extract A with carvacrol and quercetin is distinguished, due to the chemical compounds present. Smaller Chi square magnitude represents greater similarity to the extract; therefore, extract B represents more similar to quercetin standard, but does not have the inhibiting effect.

Table 4. Efficiency of the extracts

Concentration			Extract A				Extract B			
				χ^2	z	*p*		χ^2	z	*p*
Carvacrol (mM)	0.3	+	127	81.41	7.84	0.0001	111	71.15	5.28	0.0001
		-	29	18.59			45	28.85		
	1	+	122	78.21	7.04	0.0001	106	67.95	4.48	0.0001
		-	34	21.79			50	32.05		
	5	+	118	75.64	6.4	0.0001	102	65.38	3.84	0.0001
		-	38	24.36			54	34.62		
Quercetin (mM)	205	+	121	77.56	6.88	0.0001	105	67.31	4.32	0.0001
		-	35	22.44			51	32.69		
	102.5	+	113	72.44	5.6	0.0001	97	62.18	3.04	0.0023
		-	43	27.56			59	37.82		
	51.25	+	122	78.21	7.04	0.0001	106	67.95	4.48	0.0001
		-	34	21.79			50	32.05		

χ^2: calculated chi square value

Carvacrol damages the outer membrane of gram-negative bacteria and increases the permeability of the cytoplasmic membrane, causing ATP losses, ion leakage, and cell lysis (Meira *et al*., 2017). The fact that flagellated gram negative bacteria in the presence of carvacrol do not develop flagella could have implications for the use of this compound as an antibacterial additive for food products and/or for the generation of new antibiotics; since if the bacterial cell does not present flagella, this decreases or inhibits its pathogenicity mechanism, as it is less able to adhere to the host's epithelial cells.

Finally, the effect of quercetin on *E. coli* O157: H7 at a concentration of 102 mM, turns out to be optimal in the mobility of bacteria, having the same effect on the quercetin-extract B

interaction; This can be attributed to the fact that the main active compound in guava leaves is the flavonoid quercetin, to which an antibacterial effect is attributed (Echemendía and Morón, 2004).

CONCLUSION

It is concluded that guava leaf extract A and its compound in a higher proportion (quercetin) are effective in inhibiting the mobility of *E. coli* O157: H7; therefore it makes it an alternative of natural origin for the treatment of diarrheal syndrome in ruminants.

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