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Influence of variety and extraction solvent on antibacterial activity of roselle (*Hibiscus sabdariffa* L.) calyxes

Mercedes Morales-Cabrera[1], Javier Hernández-Morales[1], Gabriel Leyva-Rúelas[2], Yolanda Salinas-Moreno[3], L. Soto-Rojas[1] and Javier Castro-Rosas[4]*

[1]Fitosanidad-Fitopatología. Colegio de Postgraduados en Ciencias Agrícolas. Carretera México-Texcoco Km 36.5, Montecillo, 56230 Texcoco, Estado de Mexico, Mexico.
[2]Departamento de Ingeniería Agroindustrial. Universidad Autónoma Chapingo, Carretera México-Texcoco Km 38.5, 56230 Chapingo, Estado de Mexico, Mexico.
[3]Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, Laboratorios de Calidad, Carretera México-Texcoco Km 38.5, 56230 Chapingo, Estado de Mexico, Mexico.
[4]Centro de Investigaciones Químicas, Instituto de Ciencias Básicas e Ingeniería, Universidad Autónoma del Estado de Hidalgo, Carretera Pachuca-Tulancingo Km 4.5, 42183 Mineral de la Reforma, Hidalgo, Mexico.

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An analysis was done on the antimicrobial activity of calyx extracts from five *Hibiscus sabdariffa* varieties against *Salmonella typhimurium* and *Salmonella choleraesuis*. Calyx extracts produced with water, ethanol and methanol were applied to *Salmonella* cultures. All extracts exhibited antimicrobial activity against both *Salmonella* serotypes. The antimicrobial effect was highest in the variety named as "Alma blanca" (in which anthocyanin production has been almost completely suppressed). Extract pH had a significant ($p<0.05$) impact on extract antimicrobial effect. The variety had influence in the antimicrobial activity of *H. sabdariffa* calices.

Key words: Antimicrobial, Choleraesuis, plant extracts, *Salmonella typhimurium*, *Hibiscus sabdariffa*.

INTRODUCTION

Hibiscus sabdariffa L. (roselle) is a medicinal plant growing in Africa, South East Asia, and Central America. In México, it is known as "flor de jamaica" or "jamaica" and it is widely used for preparing beverages with medicinal and culinary purposes. The traditional medicine use the aqueous extract of this plant as diuretic, for treating gastrointestinal and liver diseases, fever, hypercholesterolemia, and hypertension (Monroy-Ortiz and Castillo-España, 2007). Extracts from *H. sabdariffa* L. flower calyces are reported to have an antimicrobial effect on different pathogenic and food spoilage micro-organisms (Fullerton et al., 2011; Hatil and Moneer, 2006; Kang et al., 2007; Liu et al., 2005; Moore et al., 2011). Aqueous and ethanol extracts from *H. sabdariffa* calyces exhibited a greater antimicrobial effect against a series of bacteria than those obtained with solvents of different polarities (Hatil and Moneer, 2006). On occasion, the individual susceptibility of strains, or that of a genus or species can also influence a plant compound's antimicrobial effect. Methanol extracts of *H. sabdariffa* calyces were reported to have a lower antimicrobial effect on a series of bacteria compared to

*Corresponding author. E-mail: jcastro@uaeh.edu.mx. Tel.: +52 771 717 2000 ext6501. Fax+52 771 717 2000 ext6502.

ethanol and aqueous extracts, but the methanol extract was more effective against *Streptococcus pneumonia* (Hatil and Moneer, 2006).

A wide variety of native and improved roselle *H. sabdariffa* varieties and genotypes are cultivated in Mexico and in different countries. Genetic improvement has produced varieties in México such as “Alma blanca” in which anthocyanin production has been almost completely suppressed, resulting in white flowers and green calyces. These improved varieties are extensively cultivated in Mexico. The primary roselle producing states within Mexico are Guerrero, Oaxaca, Michoacan and Nayarit (SAGARPA, 2010). It has been reported that the antimicrobial activity of a plant compound can be significantly affected by genotype, environment, harvest season, storage conditions, geographic location, altitude and extraction conditions, among other factors (Silva et al., 2007). No reported research exists on the antimicrobial activity of calyces from roselle varieties and genotypes grown in Mexico. Genetic variation between native and genetically-improved varieties leads to variability in the chemical composition of roselle calyces in Mexico. It is therefore possible that Mexican varieties produce calyces with antimicrobial activity differing from that reported for varieties grown elsewhere. The present study objective was to evaluate the antimicrobial effect of calyces from five *H. sabdariffa* varieties grown in Mexico and extraction solvent versus two *Salmonella* serotypes.

MATERIALS AND METHODS

H. sabdariffa calyces

Samples (2 kg) of dehydrated calyces were used from each five *H. sabdariffa* L. genotypes grown in Mexico: Tecoanapa (Guerrero state), Huajicori (Nayarit state), Chiautla (Puebla state), Criolla de Oaxaca (Oaxaca state) and Alma blanca (Oaxaca). All calyces were from the December 2010 harvest.

Extracts production

Methanol and ethanol extracts

Samples (25 g) of dried calyces from each genotype were aseptically weighed and placed in separate sterile glass flasks. Depending on the treatment, 225 mL 96% ethanol (Sigma-Aldrich, Mexico) or 225 mL methanol (Sigma-Aldrich, Mexico) were added to each flask. These were hermetically sealed and stored at room temperature for three days with manual agitation once daily. After this extraction period, the liquid phase was filtered through Whatman No. 4 filter paper and these filtered extracts concentrated in a rotary evaporator (BÜCHI, Vacuum Controller, V-800). Solvents (ethanol and methanol) were eliminated from the concentrates by placing them in a recirculating air incubator (Lab-Line, Ambi-Hi-Low Chamber, USA) for 24 h at 45 ± 1°C. Dilutions (10%) were prepared from these concentrates.

Aqueous extract

Samples (25 g) of dried calyces from each genotype were aseptically weighed and placed in separate sterile glass flasks. Distilled water (225 ml) was added to each flask, the flasks heated to boiling for 10 min and then allowed to cool to room temperature. An aqueous extraction was also done at room temperature. In this extraction, the same procedure as above was followed except the flasks were not heated and the calyces were left in water for three days at room temperature. A potentiometer (Thermo electron, Orion 3-Star, USA) was used to measure pH in all the extracts.

Bacterial strains

The *Salmonella* serotypes Typhimurium (ATCC 14028) and Choleraesuis (ATCC 10708) were kept in inclined trypticase soy agar (TSA) tubes at 3-5°C with monthly re-inoculation in trypticase soy broth (TSB).

Inoculum preparation

Tubes containing 3 ml trypticase soy broth (TSB, Bioxon, Becton Dickinson, Mexico) were inoculated with one of the tested *Salmonella* serotypes and incubated at 35°C for 18 h. The cultures were washed twice in sterile isotonic saline solution (ISS) by centrifuging at 3500 g for 20 min, and resuspending the pellets in sterile peptone water at about 10^9 CFU/ml. Decimal dilutions of these washed cultures were done with ISS to produce a final approximate concentration of 8 log CFU/ml.

Diffusion technique

From the first dilution of each *Salmonella* culture, 100 µL was inoculated on TSA plates and distributed over the agar using the surface extension technique. Aliquots (10 µL) of each extract were then placed on the inoculated plates (final doses per disc: 1 mg of extract), including a blank with saline solution at the pH value of each test. Four replicates were done per extract. Once the extracts were absorbed by the agar, the culture plates were incubated for 24 h at 35 ± 1°C. Discs of chloramphenicol (25 µg) were used as positive controls. The diameter of any resulting zones of inhibition (mm) was measured and average diameter values calculated for each extract.

Statistical analyses

A completely random experimental design was used with 21 treatments and four replicates each. All values were processed with an analysis of variance (ANOVA) and a Tukey comparison of means ($\alpha = 0.05$). Analyses were run with the SAS system ver. 9.1 package.

RESULTS AND DISCUSSION

Calyx extract pH was between 1.5 and 2.4, with differences (α=0.05) between genotypes (Table 1). The Alma blanca ethanol and methanol extracts had the overall lowest pH values. Plant extract antimicrobial activity can be seriously affected by pH. Some researchers claimed that the antimicrobial effect of *H. sabdariffa* calyx extracts is principally due to low extract pH, although this statement was not supported with experimental data (Abu-Tarboush, 1994). All the tested *H.*

Table 1. Values for pH in calyx extracts from five roselle *Hibiscus sabdariffa* varieties by solvent type.

Variety	Extracts (1:10 w/v concentration)			
	Et	Mt	Acc	Acta
Alma blanca	1.53^{f}*	1.67^{d,f,e}	2.36^{a}	2.38^{a}
Criolla de Oaxaca	1.88^{d,c}	1.79^{d,e}	2.02^{b,c}	2.21^{a,b}
Huajicori	1.75^{d,f,e}	1.88^{d,c}	2.23^{a,b}	2.24^{a,b}
Chiautla	1.63^{f,e}	1.81^{d,c,e}	2.31^{a}	2.35^{a}
Tecoanapa	1.67^{d,f,e}	1.76^{d,e}	2.24^{a,b}	2.30^{a}

*Means with different letters in the same column or same row are statistically different (Tukey, α ≤ 0.05); Et: ethanol extract; Mt: methanol extract; Acc; heated aqueous extract; Acta: room temperature aqueous extract.

Table 2. Antimicrobial effect against two *Salmonella* serotypes of calyx extracts from five roselle *Hibiscus sabdariffa* varieties by solvent type.

Salmonella serotype	Variety	Inhibition zone diameter (mm)			
		Et	Mt	Ac	Acta
Typhimurium	Alma blanca	24.5^{a}*	17.7^{b}	17.5^{b}	5.0^{h}
	Criolla de Oaxaca	16.0^{c,b}	13.0^{c,e,d}	13.2^{c,e,d}	12.0^{f,e}
	Huajicori	15.5^{c,b,d}	13.5^{c,e,d}	12.5^{f,e,d}	9.5^{f,g}
	Chiautla	13.7^{c,e,d}	13.5^{c,e,d}	9.5^{f,g}	6.7^{h}
	Tecoanapa	13.2^{c,e,d}	12.7^{c,f,e,d}	7.7^{h,g}	6.5^{h,g}
	Chloramphenicol	24	24	24	24
	Negative control	NE	NE	NE	NE
Choleraesuis	Alma blanca	20.2^{b}	23.7^{a}	17.5^{c,b}	5.0^{j}
	Criolla de Oaxaca	18.0^{c,b}	15.2^{c,d}	11.7^{f,g,h,e}	10.0^{h,i}
	Huajicori	18.0^{c,b}	13.7^{f,d,e}	10.5^{f,g,h,i}	10.2^{g,h,i}
	Chiautla	14.7^{c,d,e}	13.5^{f,g,d,e}	9.2^{h,i}	6.7^{j}
	Tecoanapa	14.0^{d,e}	14.0^{d,e}	8.2^{j,i}	8.2^{j,i}
	Chloramphenicol	25	25	25	25
	Negative control	NE	NE	NE	NE

*Means with different letters in the same column or same row are statistically different (Tukey, α ≤ 0.05); Et, Ethanol extract; Mt, methanol extract; Acc, heated aqueous extract; Acta, room temperature aqueous extract; NE, not inhibitory effect.

sabdariffa calyx extracts exhibited an antimicrobial effect against *S. typhimurium* and *S. choleraesuis* (Table 2), with differences (α=0.05) between solvents and genotypes. The Alma blanca variety had the greatest antimicrobial effect on both tested *Salmonella* serotypes. This genetically-improved variety has almost no anthocyanin content and therefore has green calyces that contrast with the red calyces (high anthocyanin content) of the other tested varieties.

The antimicrobial activity of *H. sabdariffa* calyces has been attributed to different compounds, such as protocatechuic acid and anthocyanins (Hatil and Moneer, 2006; Kang et al., 2007; Liu et al., 2005; Olaleye, 2007; Wong et al., 2010). Nonetheless, no published research has yet identified the specific compounds responsible for the antimicrobial effect of *H. sabdariffa* calyces. Both solvents and extraction procedure are important to consider when evaluating the antimicrobial effect of compounds from plants. Solvent type (for example, methanol, ethanol, hexane, acetone, water, chloroform among others is known to affect plant extract antimicrobial effect due to polarity differences between solvents (Muthuvelan and Balaji, 2008). This was also observed in the present study, where differences (α=0.05) in antimicrobial effect were determined by the solvent used in calix extraction (Table 2). The ethanol extracts had the greatest antimicrobial effect against *Salmonella*, independent of roselle genotype. Inhibition zone for the ethanol extracts were approximately 35% larger than the methanol extract inhibition zone and 65% larger than those of the aqueous extracts (Table 2). These results coincide with those reported elsewhere (Hatil and Moneer, 2006; Wong et al., 2010). The *S. typhimurium* and *S. choleraesuis* serotypes used here

exhibited different sensitivity to the roselle extracts. Inhibition one diameters were generally larger for *S. choleraesuis*, with the most marked differences produced by the ethanol extracts (Table 2). The antibacterial effect of *H. sabdariffa* calyx extracts was influenced by plant genotype and extraction solvent and condition. Extracts from the Alma blanca variety produced the largest *Salmonella* inhibition diameters, while the aqueous extracts from the Chiautla genotype resulted in the smallest inhibition diameters. The ethanol extracts had the highest antimicrobial effect. *S.* Choleraesuis was more sensitive to the roselle extracts' antimicrobial effect than was *S.* Typhimurium. In addition, it is the first report about antibacterial effect of a variety of *H. sabdariffa* in which anthocyanin production has been almost completely suppressed.

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ABBREVIATIONS

TSA, Trypticase soy agar; **TSB,** trypticase soy broth; **ISS,** isotonic saline solution.

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