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## ANTIBACTERIAL ACTIVITY OF GUAVA, *Psidium guajava* LINNAEUS, LEAF EXTRACTS ON DIARRHEA-CAUSING ENTERIC BACTERIA ISOLATED FROM SEABOB SHRIMP, *Xiphopenaeus kroyeri* (HELLER)

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### SUMMARY

Guava leaf tea of *Psidium guajava* Linnaeus is commonly used as a medicine against gastroenteritis and child diarrhea by those who cannot afford or do not have access to antibiotics. This study screened the antimicrobial effect of essential oils and methanol, hexane, ethyl acetate extracts from guava leaves. The extracts were tested against diarrhea-causing bacteria: *Staphylococcus aureus*, *Salmonella* spp. and *Escherichia coli*. Strains that were screened included isolates from seabob shrimp, *Xiphopenaeus kroyeri* (Heller) and laboratory-type strains. Of the bacteria tested, *Staphylococcus aureus* strains were most inhibited by the extracts. The methanol extract showed greatest bacterial inhibition. No statistically significant differences were observed between the tested extract concentrations and their effect. The essential oil extract showed inhibitory activity against *S. aureus* and *Salmonella* spp. The strains isolated from the shrimp showed some resistance to commercially available antibiotics. These data support the use of guava leaf-made medicines in diarrhea cases where access to commercial antibiotics is restricted. In conclusion, guava leaf extracts and essential oil are very active against *S. aureus*, thus making up important potential sources of new antimicrobial compounds.

**KEYWORDS:** Guava leaves; *Psidium guajava* Linnaeus; Methanol extract; Antibacterial activity; Essential oils; Plant remedy.

### INTRODUCTION

Amongst the various forms of treatment for diarrhea, the use of traditional plant remedies is common and widespread. The World Health Organization (WHO) has catalogued more than 20,000 plant species with medicinal properties providing treatments for such complaints as pneumonia, ulcers, diarrhea, bronchitis, colds and diseases of the respiratory tract. One method, amongst the many ways in which plants are used in popular remedies, is to extract and consume essential plant oils. Essential oils are complex chemical mixtures, typically composed of more than a hundred compounds, by and large, are responsible for plant aromas. They are obtained from different parts of the plant: flowers, leaves, seeds, bark and tubers and many have medicinal properties<sup>2</sup>.

Outbreaks of diarrhea are common in communities living in precarious conditions with poor sewerage and hygiene. A number of food types have been linked to outbreaks and act as the carriers of infectious microbes. Under such conditions, diarrhea is a common occurrence after eating contaminated fish products. Illnesses arise because the microbial causal agents, often native to the fish itself, have not been adequately controlled, or arise as a consequence of

incorrect handling and or storage during industrial processing<sup>13</sup>. Illnesses causing gastroenteritis and diarrhea are primarily associated with enteric bacteria. Enteric bacteria are responsible for high mortality rates in numerous developing countries with as many as 50,000 people dying daily as consequence of infection<sup>14,16</sup>. Plant remedies are increasingly being recognized by scientists as a very important low-cost alternative to industrially-produced antibiotics which are not available to all who need them because their high price<sup>5,25</sup>. Publishing findings on the antimicrobial activity of plant remedies is important because it raises awareness of alternative medicines which in turn drives biotechnology development.

The guava tree, *Psidium guajava* Linnaeus, is distributed from México down to São Paulo State, Brazil, being indigenous to Central América and part of South America between Colombia and Peru<sup>7</sup>. Worldwide literature information is available about the antimicrobial activity by *P. guajava* on different members of such bacterial families as Enterobacteriaceae, Vibrionaceae, Micrococcaceae and Propionibacteriaceae<sup>6,18,20,25</sup>.

The objective of this study was to evaluate the antimicrobial effects of guava tree leaf extracts on diarrhea-causing bacteria. Screening was

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carried out on *Staphylococcus aureus*, *Salmonella* spp. and *Escherichia coli* strains isolated from seabob shrimp as well as on laboratory culture strains to evaluate whether differences between laboratory and environmental strains existed.

## MATERIALS AND METHODS

**1. Shrimp sampling:** Approximately 3.5 kg of seabob shrimp were bought from the Mucuripe Fishmarket (Fortaleza, Ceará, Brazil) and were transported on ice to the laboratory. The shrimp were ground using a porcelain mortar and pestle. From this material, 25 g were separated for the isolation of *Salmonella* spp. A further 25 g was homogenized with 225 mL of an 0.85% saline solution for the isolation of other bacterial species.

**2. Isolation of *Staphylococcus aureus*, *Salmonella* spp. and *Escherichia coli*:** *S. aureus* and *Salmonella* spp. were isolated using the protocols described in the Compendium of Methods for the Microbiological Examination of Foods<sup>8</sup> and *E. coli* using the multiple tube-fermentation technique described by MEHLMAN *et al.* (1984)<sup>17</sup>.

**3. Obtaining plant extracts:** Guava leaves were collected from urban orchards at Fortaleza and registered at the Herbarium of the Department of Biological Science, Federal University of Ceará (registry no. 30387), having been identified by the botanical officer E. Nunes, on March 3, 2001. Afterwards, they were dried at room temperature. 100 g of leaf material were mechanically ground and wrapped in filter paper and fractions thereof were extracted using a Soxhlet extractor and solvents in the following order of polarity: hexane, ethyl acetate and methanol. The extracted solutions were concentrated in a rotary evaporator (R-114, Büchi, Switzerland) resulting in 1.87 g of viscous material from the hexane extraction and 2.95 g from the ethyl acetate extraction. This material was suspended in 20 and 30 mL of hexane and ethyl acetate respectively. Both extracts were brown in color. Viscous extract weighing 7.75 g was recovered using methanol and suspended in 80 mL of the same solvent. Extracts were stored in a refrigerator at 5 °C until used.

**4. Obtaining the essential oil:** The essential oil was obtained from fresh leaves of *P. guajava* using a Clevenger type doser and the extraction methodology of GOTTLIEB & MAGALHÃES (1960)<sup>11</sup>. 840 g of fresh leaves were submerged in 2.5 L of distilled water in a 5 L glass bowl and submitted to the hydro-distillation technique for 24 hours. The water and oil mixture were separated by drying with anhydrous sodium sulphate (Na<sub>2</sub>SO<sub>4</sub>) and then filtered, resulting in a final volume of 1.4 mL of essential oil. The essential oil was stored in a refrigerator prior to further investigation.

**5. Antimicrobial activity:** The antimicrobial activity of the extracts and the essential oil was evaluated by the disc diffusion method described by BAUER *et al.* (1966)<sup>3</sup> with modifications. The bacterial strains isolated from the seabob shrimp were *S. aureus*, *Salmonella* spp. and *E. coli*. The experimental controls were type strains of *E. coli* (ATCC 25922), *S. aureus* (IOC 3089) and *Salmonella* Anatum (IOC 4279-99). Test colonies were taken from a 24-hour culture, grown on slanted TSA agar and suspended in sterile saline solution (0.85%). Optical density was compared with a 0.5 tube on the McFarland scale (10<sup>8</sup> UFC/mL)<sup>12</sup>. With the aid of a moist sterile swab the suspensions

were spread on plates of Mueller Hinton (MH) Difco agar, left to get dry for about 15 minutes and incubated at 37 °C.

Filter paper discs (0.25, Schleicher & Schuell), which had previously been sterilized in an oven at 100 °C for two hours, were soaked with the three extracts at four concentrations (5, 10, 15 and 20 µL). Discs were placed on MH agar and inoculated with the test bacteria and incubated at 37 °C for 18-24 hours.

After incubation, inhibition haloes were measured to determine which concentrations of the extracts inhibited bacterial growth. The resulting haloes were compared with control discs, soaked in hexane, ethyl acetate and methanol solvents without plant extract. Control discs were not used in the tests with essential oil because no solvents were used with this treatment.

A parallel analysis with commercial antimicrobial agents was conducted in order to compare their antimicrobial efficiency with the plant extracts and essential oil. The same method of spreading a bacterial suspension on MH agar in Petri dishes was used. The sensibiodisc kit (CECON) was used for the comparison<sup>3</sup>. The antibiotics used for comparison with *S. aureus* were: Ampicillin-AMP (10 mcg), Cephalothin-KF (30 mcg), Chloramphenicol-CL (30 mcg), Erythromycin-EM (15 mcg), Lincomycin-LIN (2 mcg), and Vancomycin-VAN (30 mcg). The Enterobacteriaceae family isolates, *E. coli*<sup>24</sup> and *Salmonella* spp.<sup>26</sup> were tested against: Cefotaxime-CT (30 mcg), Cefoxitin-FX (30 mcg), Chloramphenicol-CL (30 mcg), Gentamicin-GEN (10 mcg) and Tetracycline-TC (30 mcg). All assays were made in triplicates and each experiment was repeated four times. The haloes of commercial discs were measured according to guidelines set up by the Sensibiodisc-CECON Manual whereby each strain was defined as sensitive, intermediate or resistant to the tested commercial antimicrobial substances.

**6. Statistical Analysis:** Analysis of the antibacterial action of the extracts of guava leaves was carried out at different concentrations, by comparing the mean diameter of the inhibition haloes as a variable. When the halo produced surpassed the measurement of the diameter of the disc (6.00-mm) we considered that there had been growth inhibition of the tested strains. The one-way Analysis of Variance was used to test the effect of the 484.30 µg/disc (5 µL), 968.70 µg/disc (10 µL), 1453.10 µg/disc (15 µL) and 1937.50 µg/disc (20 µL) concentrations of the methanol extract of guava leaves. The estimated means were compared two by two using the Tukey test, with a 5% significance level.

## RESULTS

Table 1 shows the concentrations of the extracts of guava leaves which were tested against strains of *Staphylococcus aureus*, *Salmonella* and *Escherichia coli* isolated from seabob shrimp. The inhibition haloes seen surrounding discs soaked in the extracts varied from 7.00 mm to 11.25 mm with all the bacteria being sensitive to at least one of the extracts. The methanol extract inhibited bacterial growth more than hexane and ethyl acetate. The solvents, without plant extract, were used as negative controls and did not inhibit bacterial growth.

The effect of guava leaf extracts was greater on *S. aureus* than on

*E. coli* and *Salmonella* spp. Based on statistical analyses, the antimicrobial action of the methanol extract for concentrations 484.30 µg/disc and 1,453.10 µg/disc (Table 1) did not differ significantly for any of the strains tested. For concentration 968.70 µg/disc, the diameter of the inhibition halo for the *Salmonella* spp. differed from that for *E. coli*, whereas for concentration 1,937.50 µg/disc, the inhibition of *Salmonella* spp. was significantly different from the others.

In Table 1, we note that the guava leaf essential oil inhibited the growth of *S. aureus* more than the methanol, hexane and ethyl acetate extracts. This antibiotic action was also observed for the type strain of *Salmonella* Anatum, but not observed with *E. coli*. Almost all of the commercial antibiotics evaluated in these experiments were shown to

efficiently inhibit *S. aureus*. Strains isolated from shrimp proved to be resistant to a number of commercial antibiotics (Table 2).

The *S. aureus* type strain was shown to be sensitive to erythromycin, chloramphenicol, lincomycin and vancomycin. Resistance to ampicillin and intermediary resistance to cephalotin were observed. In the tests carried out with antibiotics, the *Salmonella* spp. strains isolated from shrimp proved to be resistant to tetracycline, but sensitive to chloramphenicol, cefotaxime, cefoxitin and gentamicin. The type strain behaved but similarly demonstrating intermediate resistance to tetracycline (Table 2).

The *E. coli* strain isolated from seabob shrimp was resistant to

**Table 1**

Antimicrobial activity of guava leaf (*Psidium guajava* L.) essential oils and methanol, hexane, ethyl acetate extracts on *S. aureus*, *E. coli* and *Salmonella* Anatum, type strains, and isolates from seabob shrimp (*Xiphopenaeus kroyeri*)

Microorganisms	Essential oil	Extracts (µg/disc)											
		Hexane				Ethyl acetate				Methanol			
		467.5	935.0	1402.5	1870.0	491.6	983.3	1474.5	1966.6	484.3	968.7	1453.1	1937.5
<b>Shrimp isolates</b>													
<i>S. aureus</i>	18	8.75	8.0	8.25	7.0	7.25	7.50	na	na	8.50	9.25	8.25	8.25
<i>E. coli</i>	na	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	8.0	8.0	9.0	8.25
<i>Salmonella</i> spp.	na	na	na	na	na	na	na	na	na	8.75	9.75	9.5	11.25
<b>Type strains</b>													
<i>S. aureus</i>	11	7.0	7.0	7.0	7.0	8.0	8.0	8.0	8.0	8.25	7.75	8.25	9.0
<i>E. coli</i>	na	7.0	7.0	7.0	7.0	na	na	na	na	9.0	8.0	8.5	8.0
<i>Salmonella</i> Anatum	18	na	na	na	na	na	na	na	na	8.25	na	na	na

na - no activity.

**Table 2**

The susceptibility of *S. aureus*, *E. coli* and *Salmonella* spp. laboratory type strains and isolates from Seabob shrimp (*Xiphopenaeus kroyeri*) to commercial antimicrobial agents

<i>Staphylococcus aureus</i>								
Antimicrobials	Shrimp isolate				Type strain			
	Susceptibility		Halo(mm)		Susceptibility		Halo(mm)	
Ampicillin (AMP)	R		15.0		R		14.0	
Cephalotin (KF)	S		25.0		I		15.0	
Chloramphenicol (CL)	S		25.0		S		30.0	
Erythromycin (EM)	S		23.0		S		30.0	
Lincomycin (LIN)	S		25.0		S		25.0	
Vancomycin (VAN)	S		18.0		S		17.0	
Antimicrobials	<i>Salmonella</i> spp. and <i>S. Anatum</i>				<i>E. coli</i>			
	Shrimp isolate		Type strain		Shrimp isolate		Type strain	
	Sus.	Halo(mm)	Sus.	Halo(mm)	Sus.	Halo(mm)	Sus.	Halo(mm)
Cefotaxime (CT)	S	30.0	S	30.0	I	30.0	R	30.0
Cefoxitin (FX)	S	21.0	S	21.0	R	11.0	R	11.0
Chloramphenicol (CL)	S	22.0	S	28.0	R	12.0	R	11.0
Gentamicin (GEN)	S	22.0	S	23.0	S	21.0	S	21.0
Tetracycline (TC)	R	11.0	I	18.0	S	20.0	I	17.0

R - resistant, S - susceptible, I - intermediate.

cefoxitin and chloramphenicol and was sensitive to tetracycline and gentamicin (Table 2). *E. coli* strain also presented intermediary resistance to cefotaxime. Gentamicin had the greatest inhibitory effect of the antibiotics against the laboratory strain of *E. coli*. Resistance to cefoxitin, chloramphenicol, cefotaxime was also observed for *E. coli* type strain along with intermediary resistance to tetracycline.

## DISCUSSION

Our results support the findings of RABE & VAN STADEN (1997)<sup>19</sup>, where greater overall antimicrobial activity was seen with a methanol extract than with the essential oil and other extracts. While it is a common practice in traditional medicine to use plant extracts prepared with water, as is the case with infusions, decoctions and plasters, it seems unlikely that the active compounds present in water extracts are the same as those responsible for the action in the methanol extracts. The chemistry involved in chewing, boiling, soaking, rubbing and extracting plant medicines is complex. The results in Table 1 suggest that there are multiple and different anti-microbial agents present in each type of extract acting in different ways on different bacterial strains. VIEIRA *et al.* (2001)<sup>25</sup> have also reported the antibacterial effect of guava leaves extracts and found that they inhibited the growth of the *S. aureus*. GNAN & DEMELLO (1999)<sup>10</sup> testing guava leaf extract found good antimicrobial activity against nine different strains of *Staphylococcus aureus*. In contrast to the results reported here, JAIARJ *et al.* (1999)<sup>15</sup>, testing for antibacterial action of aqueous, methanol and chloroform extracts of *P. guajava*, on strains of *S. aureus* isolated from clinical patients, obtained better results from aqueous extracts than from methanol extract. BERDY *et al.* (1982)<sup>4</sup> and CACERES *et al.* (1993)<sup>5</sup> described the antibiotic activity of the aqueous extract of dried leaves of *Psidium guajava* to two compounds, namely guajaverin and psidiolic acid. Data on Table 1 both support and contradict previous findings concerning which type of plant extraction method results in greatest microbial inhibition. Such variability is perhaps explained in the light of multiple different active agents and different bacterial species. The secondary metabolism of plants is complex and results in an incredible variety of metabolic products. SANCHES *et al.* (2005)<sup>21</sup> evaluated the antibacterial activities of *Psidium guajava* against Gram positive and negative bacteria testing ethanol: water extract of *P. guajava* leaves, stem bark and root, and aqueous extract against *Staphylococcus aureus* were found to be more active by using ethanol:water extract than with just aqueous extract. High variability is not unexpected and serves to highlight gaps in our understanding of plant medicines, and provides questions and direction for further research. When comparing these data with others, what really matters is the powerful inhibitory effect of guava leaves on many common diseases causing enteric pathogens.

While the overall inhibitory effect of the essential oils in this experiment was less than for methanol, its individual effect on *S. aureus* was greater. According to ROBBERS *et al.* (1997)<sup>20</sup>, a higher concentration of active chemical compounds in essential oils explain their stronger inhibitory action (JAIARJ *et al.* (1999)<sup>15</sup>; ANDRADE-NETO *et al.* (1994)<sup>1</sup>. Within the genus *Psidium*, essential oil constituents are principally comprised of monoterpenes, 1.8-cineol,  $\rho$ -cimen and acetate of  $\alpha$ -terpenil. The complex composition of essential oils offers a variety of pharmacological resources and great potential for the development of novel drugs.

Investigating the antimicrobial effect of guava leaves involved a comparison of their effect (Table 1) with commercially developed antibiotics (Table 2) by comparing the inhibition haloes in those tables. We note that the commercial antibiotics had a larger inhibitory effect than the guava leaf extractions. This is not surprising and reinforces the position that commercially perfected and tested antibiotics should be used in treatments whenever available. Table 2 also provides data which support the findings of SILVA & HOFER (1995)<sup>22</sup> about *E. coli* isolated from marine fish, where multiresistance to commercial antibiotic was reported. Multidrug resistance is defined as resistance to at least two different classes of antibiotics<sup>9</sup>. An increase in the occurrence of bacterial strains resistant to more than one commercial antibiotic isolated from natural environments is cause for concern. Increased resistance has typically been ascribed to the indiscriminate use of drugs with antibiotic properties on human beings, livestock protection and food production, resulting in the development of resistant strains<sup>23</sup>. Our data demonstrate that multiple resistance is frequently found in strains from environmental samples where this type of exposure seems unlikely. In developing countries, especially in rural, coastal and estuarine environments, where child diarrhea is common and life-threatening, special attention is required when preparing fish for consumption. In conclusion, guava leaf extracts and essential oil are very active against *S. aureus*, thus making up important potential sources of new antimicrobial compounds.

## RESUMO

### **Avaliação da atividade antibacteriana de extrato de folhas de goiabeira, *Psidium guajava* Linnaeus, sobre bactérias entéricas diarreiogênicas, isoladas de camarão sete-barbas, *Xiphopenaeus kroyeri* (Heller)**

O chá de folhas de goiaba *Psidium guajava* Linnaeus é comumente usado como remédio nas gastroenterites e diarreias infantis por aqueles que não têm acesso a antibióticos. Esta pesquisa estudou o efeito antibacteriano sobre bactérias causadoras de diarreias, do óleo essencial e do extrato de folhas de goiabeira usando como diluente: metanol, hexano e acetato de etila. Os extratos foram testados sobre *Staphylococcus aureus*, *Salmonella* spp. e *Escherichia coli*. As bactérias testadas foram isoladas de camarão sete-barbas *Xiphopenaeus kroyeri* (Heller) usando-se como controle cepas padrão, de cada espécie. Das bactérias testadas, o melhor efeito inibitório foi observado sobre *Staphylococcus aureus*. O extrato de metanol apresentou maior inibição bacteriana. O óleo essencial mostrou ação inibitória contra *S. aureus* e *Salmonella* spp. Foi demonstrada alguma resistência das cepas isoladas de camarão aos antibióticos comerciais testados. Estes dados confirmam que remédios à base de folhas de goiabeira podem ser utilizados em casos de diarreia provocada por essas bactérias, quando o uso de antibiótico for restrito. Conclui-se que extratos de folhas de goiaba e o seu óleo essencial são muito ativos contra *S. aureus*, tornando-os importantes fontes em potencial de novos compostos antimicrobianos.

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