Rev. Inst. Med. trop. S. Paulo
43 (3):145-148, May-June, 2001.

MICROBICIDAL EFFECT OF MEDICINAL PLANT EXTRACTS (*Psidium guajava* LINN. AND *Carica papaya* LINN.) UPON BACTERIA ISOLATED FROM FISH MUSCLE AND KNOWN TO INDUCE DIARRHEA IN CHILDREN

Regine Helena Silva dos Fernandes VIEIRA(1), Dália dos Prazeres RODRIGUES(2), Flávia Araújo GONÇALVES(1), Francisca Gleire Rodrigues de MENEZES(1), Janisi Sales ARAGÃO(1) & Oscarina Viana SOUSA(1)

SUMMARY

Out of the twenty-four samples of shrimp and fish muscle used for this study, twelve were collected near a large marine sewer for waste disposal, 3 km off the coast of Fortaleza (Brazil) and used for the isolation of *E. coli*. Other twelve were collected at the Mucuripe fresh fish market (Fortaleza, Brazil) and used for the isolation of *Staphylococcus aureus*. Ethanol, water and acetone-diluted extracts of guava and papaya leaf sprouts were tested on the bacteria in order to verify their microbicidal potential. The *E. coli* strains used in the trials were rated LT positive. The papaya leaf extracts (*Carica papaya* Linn) showed no microbicidal activity while the guava sprout extracts (*Psidium guajava* Linn) displayed halos exceeding 13 mm for both species, an effect considered to be inhibitory by the method employed. Guava sprout extracts by 50% diluted ethanol most effectively inhibited *E. coli* (EPEC), while those in 50% acetone were less effective. It may be concluded that guava sprout extracts constitute a feasible treatment option for diarrhea caused by *E. coli* or by *S. aureus*-produced toxins, due to their quick curative action, easy availability in tropical countries and low cost to the consumer.

KEYWORDS: *E. coli*; *S. aureus*; *Psidium guava*; *Carica papaya*; Medicinal plants.

INTRODUCTION

Gastrointestinal diseases are the most frequent causes of morbidity and mortality in developing countries. The presence of enterobacteria in foodstuffs and water is a common cause of diarrhea and dysentery among the infant population. *Escherichia coli* is a classic example of enteric bacteria capable of producing diseases[3].

Enterotoxigenic *E. coli* strains (ETEC) produce toxins that affect the small intestine, such as thermolabile enterotoxins (LT) resembling the toxin produced by *Vibrio cholerae*, along with other and thermostable toxins (ST). A single strain can produce one or both of these forms of toxins as determined genetically by the plasmids[6].

Fishery products have been implicated in toxic infections, especially when sold on local, unsanitary fish markets. A recent survey carried out at a fresh-fish market in Fortaleza analyzed 24 samples of marine seafood products and 24 samples of freshwater fish products and found that 62.5% of the former were contaminated by fecal coliform bacilli, as opposed to only 58.3% of the latter[7].

Staphylococcus aureus is another pathogen of interest to public health surveillance because of the intoxication it causes in consumers of foods containing preformed toxins[4]. In a study involving fresh samples of shrimp collected at the Mucuripe fish market[20], detected in a batch of 10 samples from a single stand no less than 3 samples containing *Staphylococcus aureus* in amounts above those permitted by the Brazilian legislation[2].

Popular Brazilian medicine has long made use of native plants for the treatment of gastrointestinal diseases, as in the case of guava sprouts (young leaves) (*Psidium guajava* Linn.). The increase in resistance against antibiotics as displayed by the bacteria causing these diseases constitutes a strong incentive towards research into new drugs[3].

In the present study, the antimicrobial effect of guava and papaya leaf extracts was studied using *Staphylococcus aureus* and *Escherichia coli* strains isolated from fresh shrimps sold at the Mucuripe fish market (Fortaleza, Brazil).

MATERIALS AND METHODS

Collection of samples: The fish and shrimp muscle samples were collected at the Mucuripe fresh-fish market (Fortaleza, Brazil) and near the outlet of a large sewer for waste disposal, 3 km off the coast of Fortaleza from November 1998 to June 1999. Twelve samplings were

(1) Instituto de Ciências do Mar-UFC, Ceará, Brasil.
(2) Dep. de Bacteriologia, Fundação Oswaldo Cruz, FIOCRUZ. Rio de Janeiro, RJ, Brasil
Correspondence to: Dr. Regine Helena S. dos F. Vieira, Av. da Abolição 3207, Meireles, 60165-081 Fortaleza, Ceará, Brasil. (e.mail- regpoema@labomar.ufc.br)

performed with a total of 24 samples collected.

***Isolation of strains* (*E. coli* and *S. aureus*)**: The strains of *E. coli* were isolated from the samples collected near the outlet of the sewer. The samples were subsequently submitted to colimetry using the multiple tube technique. For each positive tube in the fecal coliform test (EC broth) a plate of Eosin Methylene Blue agar (Levine-EMB-Agar) was inoculated, from which typical colonies of *E. coli* could be isolated after 24 hours of incubation at 35 °C. The identification of the strains was performed with conventional biochemical tests (IMViC)[12]. The thermolabile toxin (LT) producing capacity of the isolated strains was measured by ELISA[21].

The *S. aureus* strains were identified by isolating typical colonies (small and black with a halo) and atypical colonies (small or large, black, gray and without a halo) on Baird-Parker agar (Difco). Confirmation was obtained through biochemical tests such as coagulase, catalase, thermonuclease, Voges-Proskauer and the use of carbohydrates[18] .

Preparation of extracts: *Psidium guajava* (Linn.) and *Carica papaya* (Linn.) extracts: A 30g sample of each type of young leaves was dried for 1 hour in an oven at 120 °C and then ground. Three solvents were used for the preparation of the extracts, namely distilled water, acetone and ethanol, at four different concentrations (20%, 50%, 60% and 80%). Each extract was boiled for 15 minutes, filtered and then boiled for another 15 minutes for greater uniformity. The prepared extracts were kept in a refrigerator up to thirty days prior to being used. The evaluation of the antibacterial activity in the water, acetone and ethanol-based guava sprout extracts was performed by two methods: Radial diffusion in two layers of perforated agar (RDAP), and disk diffusion (DD).

***Radial diffusion in two layers of perforated agar* (RDAP)**: The RDAP procedure was performed using plate count agar (PCA), with 1.7 g of bacteriological agar being added for each 100 mL of base medium. An initial layer of this medium (25 mL) was distributed on the plates 24 hours prior to inoculation. Subsequently, the plates were stored in a refrigerator. Two LT positive strains of *E. coli* and two strains of *S. aureus*, isolated from the fish and shrimp muscle samples, were used for this step of the procedure. The cultures were grown for 24 hours in a Brain Heart Infusion (BHI) broth medium after which 0.25 mL were inoculated onto the first layer of culture medium in the spread plate fashion using a sterile bent glass streaking rod. After 10 minutes at room temperature during which the inoculum was allowed to absorb completely, a second layer of PCA medium (35 mL) at up to 45 °C was added. A complete solidification of the medium occurred under refrigerated conditions after about 10 minutes. The perforations in the medium (a maximum of six) were made with a metallic flame-sterilized cylinder (6 mm diameter). After filling up each well with 75 mL of extract the plates were placed in a refrigerator at 4 °C for 3 hours. This procedure allowed for the complete diffusion of the test extracts in the two layers of agar. Subsequently, the plates were incubated under aerobic conditions at 37 °C for 24 hours. The blank tests were performed by filling the central well of each plate with 75 mL of the solvent used for the extract and at the corresponding concentrations[16].

For the reading and interpretation of the results only clear halos of 13 mm or more and devoid of bacterial growth were considered as halos of inhibition. The results were validated when no halo was observed around the well of the blank test (only solvent was used for control), thus proving that the microbicidal action observed could be attributed to the compounds contained in the guava or papaya sprout extracts and not to the solvent itself.

***The disk diffusion method* (DDM)**: Using a Drigalski handle, the *E. coli* and *S. aureus* strains isolated from the samples were inoculated onto the surface of plates containing 25 mL of PCA medium (prepared as described above). Small filter paper discs (Whatman 90 mm) soaked from three to five times in the test extracts and dried in an oven were then placed upon this surface. Subsequently, the plates were incubated in an oven at 37 °C for 24 hours. The disc of the blank test was only soaked in the respective solvent.

RESULTS AND DISCUSSION

Out of the 18 *E. coli* strains isolated from 3 fish samples and 15 shrimp samples, as many as 13 contained LT toxins. These results differ from those found by VIEIRA *et al*., (1998)[20] who isolated 295 *E. coli* strains from the coastal ocean waters of Fortaleza (Brazil) but did not detect any LT or ST positive cases among the confirmed enteropathogenic strains. The presence of toxigenic *E. coli* bacilli in the fish sampled but not in water from the same location may be mainly due to the longer permanence of the bacilli on an adequate substrate, namely within a living organism.

The results of the microbicidal effect of the guava sprout extracts for different solvent concentrations as assayed by radial diffusion in two layers are shown in Table 1. The results obtained for the papaya sprout extracts are not presented since no inhibition halos were observed at any time when the bacteria were exposed to these extracts.

Table 1
Microbicidal effect of guava (*Psidium guajava* Linn.) sprout extracts (ethanol, acetone and water) upon toxigenic *S. aureus* and *E. coli* strains isolated from fish and shrimp muscle, as determined by radial diffusion in two layers of perforated agar

Extract	Concentration	Diameter of halos (mm)			
		Strains			
		S. aureus$_1$	*S. aureus*$_2$	*E. coli*$_1$	*E. coli*$_2$
Ethanol	20%	*	*	18	20
	50%	20	20	18	20
	60%	28	30	32	30
	80%	20	25	21	20
Acetone	20%	*	*	15	15
	50%	15	15	20	20
	60%	26	30	30	29
	80%	27	27	26	20
Water	-	26	28	31	27
	-	20	20	20	20

*tests not performed at this concentration; strain 1 isolated from fish; strain 2 isolated from shrimp.

Table 2
Microbicidal effect of diluted guava (*Psidium guajava* Linn.) sprout extracts (ethanol, acetone and water) upon toxigenic *S. aureus* and *E. coli* strains isolated from fish and shrimp muscle, as determined by disc diffusion

		Diameter of halos (mm)					
		Strains					
Extract	Concentration	*S. aureus*$_1$	*S. aureus*$_2$	*S. aureus*$_3$	*S. aureus*$_4$	*E. coli*$_1$	*E. coli*$_2$
Ethanol	50%	*	*	*	*	18	18
	60%	14	14	15	15	13	13
Acetone	50%	*	*	*	*	16	18
	60%	20	14	20	20	13	13
Water	-	19	18	20	20	14	14

*tests not performed at this concentration; strains 1 and 3 isolated from fish; strains 2 and 4 isolated from shrimp

All concentrations of guava sprout extracts on PCA plates showed inhibitory effects on the growth of LT+ *E. coli* and *S. aureus* strains. The effect was perceived through the presence of halos ranging from 15 to 32 mm for the former and from 15 to 30 mm for the latter. The extracts prepared with 60% alcohol and 60% acetone produced the largest halos for both species of bacteria. IROBI *et al*., (1994)[9] reported that the zone of inhibition of water and ethanol extracts of *Bridelia ferruginea* at a concentration of 5mg/mL in agar diffusion assays against some microorganisms ranged from 4 to 20 mm while the antibiotic chloramphenicol produced zones that measured 15-36 mm.

Table 2 shows the results of the inhibitory effect of alcohol, acetone and water-extracted guava sprouts upon strains of *E. coli* and *S. aureus* when the disc diffusion method was used. The ethanol, acetone and water-based guava sprout extracts produced halos with all four strains of *S. aureus*. However, the extracts prepared with water and 60% acetone produced the largest halos. These results agree with those obtained by JAIARJ *et al*., 1999[10], who observed growth inhibition of *S. aureus* strains when these were diluted in water, methanol and chloroform guava leaf extracts. GNAN & DEMELLO (1999)[8] obtained similar results when testing the growth inhibition of *S. aureus* by guava leaf and fruit water extracts. Comparing the effect of the extracts made of guava leaves and fruits at a concentration of 6.5 mg/ml upon strains of *Staphylococcus* spp. to the effect produced by known antibiotics used routinely in the treatment of a number of infectious disorders, they obtained results comparable to chloramfenicol (CF30), cefoxitin (CX30) and mefaxotin (ST25). As in the present study, the authors found all their *S. aureus* strains to be growth-inhibited by guava leaf sprout extracts, a significant finding when it is considered that *S. aureus* is often resistant to a variety of well-known antibiotics[11].

In relation to the two strains of *E. coli* (ETEC) exposed to the extracts, the best results were obtained with the ethanol extract and a little lower with the acetone extract, both at a 50% concentration.

CÁCERES *et al*. (1993)[3] tested guava leaf extracts obtained with three solvents of different polarities (n-hexane, acetone and ethanol) and discovered that the ethanol extract was the most efficient against the pathogenic enterobacteria tested.

The microbicidal activity of *Psidium guajava* is attributable to guajaverine and to psydiolic acid[1]. Furthermore, the leaves contain large amounts of tannin, triterpernoids (crategolics, guaijavolic, oleanolics and ursolic acids) and essential oils containing ß-sitosterol, ß-bisabolene, ß-cariophyllene, aromadendrene, ß-salinene, guaijaverine, nerolidiol and sel-11-en-4α-ol[13]. GNAN & DEMELLO (1999)[8] also reported a complete inhibition of *S. epidermitis* and *Salmonella typhimurium* when it was used extract of the guava leaves.

DIREKBUSARAKOM *et al.* (1997)[5] tested guava (*Psidium guajava)* extract for antiviral activity against the fish pathogenic viruses, infectious, haematopoietic necrosis virus (IHNV) infectious pancreatic necrosis virus (IPNV) and *Oncorhynchus masou* virus (OMV) using plaque reduction in CHSE-214 cell lines. Antiviral tests against the shrimp pathogenic virus, yellow-head virus (YHV), was carried out using the injection method. Also they tested the efficacy of guava extract using MIC of the extract against 24 strains of pathogenic bacteria including *Vibrio harveyi* (9 strains), *V. splendidus (*7 strains), *V. parahaemolyticus* (2 strains) and 1 strain of each *V. mimicus*, *V. vulnificus*, *V. fluvialis*, *V. cholerae*, *V. alginolitycus* and *Aeromonas hydrophila*. The extract of guava demonstrated antiviral activity against IHNV, OMV and YHV but was not effective for IPNV. The MIC of the extract ranged from 625-5,000 µg/mL against all pathogenic bacterial strains tested. According to the authors it might be possible to use guava extract for prevention of bacterial diseases in fish.

All this is in agreement with THANANGKOL & CHAICHANGPTIPAYUT (1987)[17] who found that guava leaves were more efficient than oxitetracycline in the treatment of acute diarrhea in humans.

In conclusion, in the treatment of diarrhea caused by *E. coli* or *Staphylococcus aureus*-produced toxins extracts made of guava leaf sprouts constitute a feasible option due to their quick curative action, the ease with which they can be obtained in tropical countries and their low cost to the consumer.

RESUMO

Uso de extrato de plantas medicinais (*Psidium guajava* Linn. e *Carica papaya* Linn.) frente a bactérias isoladas de pescado, causadoras de diarréias infantis

Foram coletadas doze amostras de camarão e peixes nas imediações do interceptor oceânico, em Fortaleza e igual número na Feira de pescado do Mucuripe, Fortaleza, para isolamento de *E. coli* e *Staphylococcus aureus*, respectivamente. Extratos aquosos, alcoólicos e cetônicos de broto de goiabeira e de folha de mamão foram testados frente às bactérias para se verificar suas ações antibióticas. As cepas de *E. coli* utilizadas nos ensaios foram as classificadas como LT positivas. Os extratos de folhas de mamão *(Carica papaya* Linn) não revelaram quaisquer atividades antibióticas enquanto que os preparados com broto de goiabeira (*Psidium guajava* Linn*)* apresentaram halos sempre >13 mm para as duas espécies, considerados como de inibição pelo método empregado. Os extratos de broto de goiabeira que apresentaram melhores resultados frente às cepas de *E. coli* ETEC foram os alcoólicos a 50% seguido do cetônico também a 50%. Concluímos que nos tratamentos de diarréias causadas por *E. coli* ou por toxinas elaboradas por *S. aureus* o extrato de brotos de goiabeira é uma opção devido a sua pronta ação curativa, seu fácil cultivo nos países tropicais e ao seu baixo valor aquisitivo.

ACKNOWLEDGMENTS

The authors would like to thank Prof. Gustavo Hitzschky Fernandes Vieira for his help training the students in the preparation of extracts.

REFERENCES

1. BÉRDY, J.; ASZALOS, A.; BOSTIAN, M. & MCNITT, K.L. - **CRC Handbook of antibiotic compounds.** Boca Raton, CRC Press, 1981. v. 8, Part 1.

2. BRASIL. Ministério da Saúde. Secretaria Nacional de Vigilância Sanitária. Divisão Nacional de Vigilância Sanitária de Alimentos. Portaria nº 451. **Diário Oficial de Brasília,** 22 de setembro de 1997, p. 8-15.

3. CÁCERES, A.; FLETES, L.; AGUILAR, L. *et al.*. - Plants used in Guatemala for treatment of gastrointestinal disorders. 3. Confirmation of activity against enterobacteria of 16 plants. **J. Ethnopharm., 38:** 31-38, 1993.

4. DAVIS, B.D.; DULBECCO, R.; EISEN, H.N.; GINSBERG, H.S. & WOOD JR., B. - **Principles of Microbiology and Immunology**. New York, Harper International, 1968.

5. DIREKBUSARAKOM, S.; HERUNSALEE, A.; YOSHIMIZU, M. *et al*. - Efficacy of guava (*Psidium guajava*) extracts against some fish and shrimp pathogenic agents. *In:* FLEGEL, T. W. & MAC RAE, I. H., ed. **Diseases in Asian Aquaculture III:** Fish Health Section. Manila, Asian Fisheries Society, 1997. p. 363.

6. FRANCO, B.D.G.M. & LANDGRAF, M. - **Microbiologia dos alimentos**. São Paulo, Atheneu, 1996. p. 44-53.

7. GASPAR JR, J.C.; VIEIRA, R.H.S.F. & TAPIA, M.S.R. - Aspectos sanitários do pescado de origem de água doce e marinha, comercializado na feira da Gentilândia, Fortaleza-Ceará. **Hig. Aliment., 11**(51): 20-23, 1997.

8. GNAN, S.O. & DEMELLO, M.T. - Inhibition of *Staphylococcus aureus* by aqueous goiaba extracts. **J. Ethnopharm., 68**: 103-108, 1999.

9. IROBI, O.N.; MOO-YOUNG, M.; ANDERSON, W.A. & DARAMOLA, S.O. - Antimicrobial activity of bark extracts of Bridelia ferruginea (Euphorbiaceae). **J. Ethnopharm., 43**: 185-190, 1994.

10. JAIARJ, P.; KHOOHASWAN, P.; WONGKRAJANG, Y. *et al*. - Anticough and antimicrobial activities of *Psidium guajava* Linn. leaf extract. **J. Ethnopharm., 67:** 203-212, 1999.

11. LOPES, C.A.M. & MORENO, G. - Comparative in vitro activity of three cephalosporins against *Staphylococcus aureus* isolated from bovine mastitis. **Res. Vet. Sci., 51:** 339-340, 1991.

12. MEHLMAN, I.J.; ANDREWS, W.H. & WENTZ, B.A. - Coliform bacteria. In: **Bacteriological analytical manual**. 6. ed.. Arlington, Association of Official Analytical Chemists, 1984. p. 5.01-5.07.

13. MORTON, J.F. - **Atlas of medicinal plants of Middle America**. Springfield, Charles C. Thomas, 1981.

14. OLAJIDE, O.A.; AWE, S.O. & MAKINDE, J.M. - Pharmacological studies on the leaf of *Psidium guajava*. **Fitoterapia, 70:** 25-31, 1999.

15. OSMAN, A.M.; YOUNES, M.E. & SHETA, A.E. - Triterpenoids of the leaves of *Psidium guajava*. **Phytochemistry, 13:** 2015-2016, 1974.

16. PEREIRA, M.L.; BASTOS, E.M.; MONTEIRO, E.P.; AMANCIO, G.C.S. & SERRANO, A.M. - Antibiotic activity of Brazilian green propolis against bacteria from human clinical etiology. In: CONGRESS APIMONDIA 99, Vancouver, 1999. **Anais**. p. 225.

17. THANANGKOL, P. & CHAICHANGPTIPAYUT, C. - Double-blind study of *Psidium guajava* L. and tetracycline in acute diarrhoea. **Siriraj Hosp. Gaz., 39:** 253-267, 1987.

18. VANDERZANT, C. & SPLITTSTOESSER, D.F. - **Compendium for the microbiological examination of foods**. 3. ed. Washington, American Public Health Association, 1992.

19. VIEIRA, R.H.S.F.; RODRIGUES, D.P.; EVANGELISTA, N.S.S.; THEOPHILO, G.N.D. & REIS, E.M.F. - Colimetry of waters off Fortaleza (Ceará State, Brazil) and detection of enteropathogenic *Escherichia coli* strains. **Int. Microbiol., 1:** 221-224, 1998.

20. VIEIRA, R.H.S.F.; TAVARES, L.A.; GAMBAR, R.C. & PEREIRA, M.L. - *S. aureus* em camarão fresco e superfícies de bancadas da feira livre de pescado do Mucuripe, Fortaleza, CE. Registro de pontos críticos e medidas de controle. **Hig. Aliment., 12**(55): 47-50, 1998.

21.. YOLKEN, R.H.; GREENBERG, H.B.; MERSON, M.H.; SACK, R.B. & KAPIKIAN, A.Z. - Enzyme linked immunosorbent assay for detection of *Escherichia coli* heat-labile enterotoxin. **J. clin. Microbiol., 6:** 439-444, 1977.

Received: 19 September 2000
Accepted: 21 February 2001