

Bacterial membrane disruption in food pathogens by *Psidium guajava* leaf extracts

Henie, E.F. P., Zaiton, H. and *Suhaila, M.

Faculty of Food Science and Technology Universiti Putra Malaysia, 43400 Serdang Malaysia

Abstract: The mode of action and activities of guava leaf extracts against various food pathogens were studied. The killing kinetics, viability and cell leakage of *Kocuria rhizophila*, *Salmonella typhimurium*, *Listeria monocytogenes* and *Escherichia coli* O157:H7, measured after exposure to guava methanolic extracts (GME) revealed a significantly higher ($p \leq 0.05$) release of bacterial nucleic acids, K^+ ions and protein than that of untreated microbes, indicating disruption of the bacterial membrane. GME caused a significantly higher ($p \leq 0.05$) release of RNA in gram-negatives compared to gram-positives. GME caused a relatively small but significant release of pyrimidines and pyrimidines in all organisms investigated. GME probably disrupted the integrity of the Gram-negative microorganism lipopolysaccharide (LPS) layer. Unlike all the other microorganisms tested, *E. coli* O157:H7, demonstrated the lowest protein leakage, the highest K^+ leakage, the highest pyrimidines and pyrimidines leakage within the first 10 min of extract exposure, but the lowest after 30 minutes, which may indicate their good homeostasis ability or adaptability. Understanding the mode of action of this flavonoid rich guava leaf extract, would help develop it as an alternative biodegradable and safe, antimicrobial for food and medicine, and as a by-product of the guava industry.

Keywords: mechanism, microbial membrane disruption, *Psidium guajava*

Introduction

Psidium guajava L. (Mirtaceae) leaves reportedly have antibacterial, anticandidal, antidysenteric, antifungal, antimalarial, ameobicidal, antiseptic, analgesic, antioxidant, antispasmodic, antiulcerous, cardio depressant, cardio tonic, central nervous system depressant, cough suppressant, gastro tonic, hypotensive, sedative, vasoconstrictive, anti-anxiety, anticonvulsant, astringent, blood cleansing, digestive stimulant, menstrual stimulant, nervine and vermifuge effects (Abdelrahim *et al.*, 2002; Lozoya *et al.*, 2002; Goncalves *et al.*, 2006). Guava leaves contain tannins, pentacyclic triterpenoid guajanoic acid, uvaol, oleanolic acid, ursolic acid maslinic acid, volatile oils, triterpenoids, and

flavonoids (Meckes *et al.*, 1996; Begum *et al.*, 2004), guaijavarin, isoquercetin, hyperin, quercitrin, quercetin 3-O-gentobioside, and quercetin (Lozoya *et al.*, 1990). Quercetin acts as calcium antagonist, affecting intestinal smooth muscle fibers and is responsible for the antispasmodic and anti-motility effect of guava leaf (Lozoya *et al.*, 1994; Morales *et al.*, 1994; Galvez *et al.*, 1996). (+)-Galocatechin from guava leaves was antimutagenic against UV-induced mutation in *E. coli* (Matsuo *et al.*, 1994). 3,4-dihydroxybenzoic acid produced by peroxidase-dependent oxidation of quercetin (Takahama and Hirota, 2001) enhanced their antibacterial and antioxidant effectiveness. The antimicrobial mechanisms of plant extracts on microorganisms may involve one or more disturbances such as cytoplasm

*Corresponding author.

Email: suhaila@food.upm.edu.my

Fax: + 603-89423552

granulation, cytoplasmic membrane rupture, inactivation or synthesis inhibition of intracellular and extracellular enzymes (Cowan, 1999). The aim of this study is to evaluate a possible mode of action of guava leaf extracts against food borne pathogens by measuring the microbes' potassium ion, bacterial nucleic acids and protein leakage in solution post treatment in relation to the MIC and killing rate. Understanding the mode of action of this flavonoid rich guava leaf extract, will help develop it as an alternative biodegradable and safe, antimicrobial for food and medicine.

Methodology

Materials

Tryptic Soy Broth (TSB), Tryptic Soy Agar (TSA), Plate Count Agar (PCA), Buffered Peptone water, PALCAM Listeria selective agar base, PALCAM listeria selective supplement, XLT4 agar base, XLT4 agar supplement, Mueller-Hinton Broth (MHB) and Mueller-Hinton agar (MHA) were from Merck, Darmstadt, Germany. The solvents methanol, hexane, chloroform and ammonia were from Fisher Scientific, UK and J.T. Baker, USA. Sorbitol MacKonkey Agar with BCIG and supplement containing Cefixime Tellurite was from Oxoid, UK.

Plant materials and extraction

Guava (*P. guajava*) leaves were harvested from the University Research Orchards at Universiti Putra Malaysia (UPM), dried at room temperature and ground using an electric blender (Model MX-896TM, National, Japan) (Samy *et al.*, 1998). Plant material (159 g) was fractionated sequentially in hexane, ammoniacal chloroform (Culvenor and Fitzgerald, 1963) and methanol for 48 h at room temperature then rotary evaporated to dryness.

Microorganisms and culture conditions

The solvent extracts (1 - 50 mg/ml) were tested against 16 bacterial strains namely; Gram-positive (*Staphylococcus aureus*, *Staphylococcus epidermis*, *Kocuria rhizophila*, *Bacillus cereus*, *Listeria monocytogenes* and *Corynebacterium diphtheriae*) (Meckes *et al.*, 1996) and Gram-negative (*Salmonella typhimurium*, *Salmonella* Enteritidis, *Escherichia coli* O157:H7, *Escherichia coli*, *Aeromonas hydrophilla*, *Aeromonas caviae*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Vibrio parahaemolyticus*, and *Vibrio cholerae*) (Galvez *et al.*, 1996). Pure cultures were from the Microbiology Lab, Faculty of Food Science and Technology, UPM or from the Malaysian Medical Research Institute (IMR) (confirmed by gram staining, Biolog Microstation System and biochemical tests). *Kocuria rhizophila* (*Micrococcus luteus*) was isolated from oxalic acid treated chicken stored at 4°C (Anang *et al.*, 2006). Working bacterial stock cultures (strains) were maintained on Mueller Hinton agar (MHA) (Oxoid, UK) slants at 4°C, and subcultured fortnightly. The cultures were kept in nutrient broth (NB) containing 15% glycerol at -80°C (Smail and Jones, 1984). To activate, 0.05 ml of frozen stock culture were transferred into 10 ml of tryptic soy broth (TSB) (Oxoid, UK), incubated overnight at 35-37°C, then streaked onto tryptic soy agar (TSA) (Oxoid, UK) plates and incubated for 24 h at 35-37°C. One loopful of colony material from TSA plates into 10 ml of TSB and incubated on a gyratory/orbital shaker (100 rpm) for 18-24 h at 35 - 37°C. One ml of this batch culture were transferred into 100 ml of fresh TSB and incubated for 16h under similar conditions for the stationary phase of growth.

One ml of the 24 h old culture was then transferred to freshly prepared TSB, adjusted until turbidity was equal to a ca 0.5

McFarland Standard, yielding a cell concentration of about $10^6 - 10^7$ cfu/ml. Initial counts were obtained by serial dilution and spread plating 0.1 ml of this inoculum on MHA immediately prior to incubation (Okemo *et al.*, 2001).

Antimicrobial sensitivity test

Agar disc diffusion method (Bauer *et al.*, 1966) using sterile 6.0 mm diameter discs (Becton Dickinson Microbiology System, USA) impregnated with 20 μ l of 1, 20 and 50 mg/ml guava leaf extract and the solvent evaporated, on petri dishes containing 10 ml of MH agar medium, seeded with 24 h old microorganism cultures were incubated for 24–36h at 35–37°C and the inhibition zone diameters measured. Discs impregnated with blank solvents served as negative controls while Chloramphenicol (C 30 μ g/disc) (Oxoid, UK), Streptomycin (S 10 μ g/disc) (BBL, USA), Nalidixic Acid (N 30 μ g/disc) (Oxoid, UK) and Erythromycin (15 μ g/disc) (Oxoid, UK) were used as positive controls.

MIC and time-kill determination

The MIC (Minimum inhibition concentration) of the extract against all visible growth (Gradelski *et al.*, 2001) was confirmed after plating for up to 36h incubation at 35–37 °C on PCA and agar selective to the test organisms using broth dilution method (NCCLS, 1999).

For time-kill kinetics (Pankruch *et al.*, 1994), a starting bacterial density of approximately 10^6 to 10^7 CFU/ml containing extracts equal to 1/5, 1/2, 1, 2, 5 and 10 \times MIC, were incubated at 35–37°C on Mueller-Hinton broth (MHB) test media. For each organism, five assays were prepared (three experimental and two controls), with one control containing 5% methanol. Viable counts were taken every four hours up to 32 h, and plated immediately before and after the addition of

extract, using 100 μ l of known culture samples dilutions on agar medium. Extracts were deemed bactericidal at the smallest dose which reduced the original inoculum by $\geq 3 \log_{10}$ CFU/ml (99.9%), and bacteriostatic if the inoculum was reduced by $< 3 \log_{10}$ CFU/ml. *Listeria* was enumerated on PALCAM *Listeria* selective agar base (Van Netten *et al.*, 1989). Plates were incubated for 36h before counting. *Salmonella* counts were determined on XLT4 agar base containing sodium tetradecylsulphate, after 24 h incubation. Enumeration of *E. coli* O157:H7 was done on Sorbitol MacKonkey (SMAC) Agar with BCIG, containing Cefixime Tellurite. All other organisms were enumerated on PCA medium.

Stationary-phase cells, harvested by centrifugation at 12,000 \times g for 10 min at 4°C (Grosvenor *et al.*, 1995), were washed twice with sterile phosphate-buffered saline (PBS) (pH 7.0) (OXOID, UK), centrifuged, diluted in distilled deionized water and resuspended in 50 ml of treatment solution at room temperature (RT; $25 \pm 0.2^\circ\text{C}$) to obtain *ca* 10^8 CFU/ml. The three treatment conditions were (i) Deionized H₂O (control), (ii) deionized H₂O with 5% methanol (v/v), and (iii) deionized H₂O with methanol extract at a concentration of 5,000 ppm. The bacterial population was verified by plating 0.1 ml portions of the appropriately diluted culture on TSA and PCA plates, and incubated as follows; *K. rhizophila* and *L. monocytogenes* at 35°C for 36h and *S. typhimurium* and *E. coli* O157:H7 at 37°C for 24 h.

Cellular leakage measurements

Each solution containing treated inoculum (3.0 ml) was filtered through a 0.2 μ m pore size Nalgene nylon syringe filter (CanLab, Ontario, Canada) at 0, 10, 20, 30, 40, 50, and 60 min, and measured for 260nm and 280nm absorbing bacterial nucleic acids (Anthelie Advanced spectrophotometer;

Secoman, Domont Cedex, France). Electrophoresis of 1.0 ml aliquots of treatment solution on 0.75% agarose gel was carried out to detect the presence of nucleic acid (Sampathkumar *et al.*, 2003). The release of proteins was quantified at 595nm using Bradford assay (Bradford, 1976). Leakage of cytoplasmic K⁺ was determined using a Ciba–Corning Checkmate® Hand-Held Analysis System Conductivity/TDS meter (Corning Inc. NY, USA), fitted with a potassium ion selective electrode, calibrated with 2% KCl standard solution prior to sampling. One ml aliquots of each treated cell suspension were removed at 0, 10, 20, 30, 40, 50, and 60 min, serially diluted in 0.1% peptone water and counted for CFU.

All experiments were performed in triplicates and repeated at least once more to ensure reproducibility. Statistical significance of data obtained was determined using the SAS software (Version 8) (SAS Institute, Cary, NC.). Analysis of variance was carried out and Duncan's *t* test was used for differentiation of means.

Results

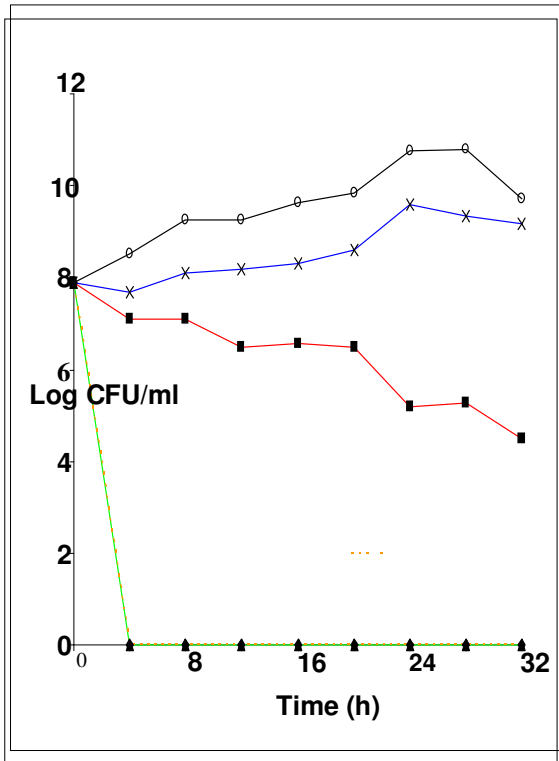
Table 1 shows the effects of various extracts against 16 bacteria. The leaf extract showed inhibition zones at 10 µg against four Gram-positive organisms (*S. epidermis*, *S. aureus*, *K. rhizophila* and *L. monocytogenes*) and five Gram-negative organisms (*S. typhimurium*, *S. Enteritidis*, *A. hydrophila*, *A. cavaie* and *V. parahaemolyticus*). Both hexane and methanol extracts were active against *S. epidermis*, *A. hydrophila*, *A. cavaie* and *V. parahaemolyticus* while the greatest inhibition zones were obtained from the polar methanol extracts. *P. guajava* extracts showed no inhibition zones against *B. cereus*, *C. diphtheriae*, *E. coli O157:H7*, *E. coli*, *C. freundii* and *V. cholera* at 10µg and other extract concentrations tested (Table 1).

A. hydrophila and *A. cavaie* were inhibited by the non polar hexane extracts at 4 µg, while *Listeria monocytogenes* was inhibited by the ammoniacal chloroform alkaloid extracts at 4 µg. *E. coli O157:H7*, *E. coli*, *E. aerogenes*, *Aeromonas hydrophila* and *Vibrio parahaemolyticus* were most resistant to the polar methanol extracts. The overall inhibition effectiveness of the solvent extract appeared to follow the sequence: Methanol (polar) > Ammoniacal chloroform (alkaloid) > Hexane (non-polar). The MIC of the methanol extract of *P. guajava* against the different microorganisms ranged between 1-5 mg/ml. *P. guajava* showed efficacy against three (*E. coli O157:H7*, *S. aureus* and *K. rhizophila*) out of seven (42.9%) test microorganisms at 1 mg/ml while *L. monocytogenes*, *S. typhimurium*, *A. hydrophila* and *V. parahaemolyticus* showed a higher MIC of 5mg/ml (Table 2). Chloramphenicol (30 µg/disc) was effective against all organisms except the oxalic acid-resistant *K. rhizophila*, which was inhibited only by Nalidixic Acid (30 µg/disc).

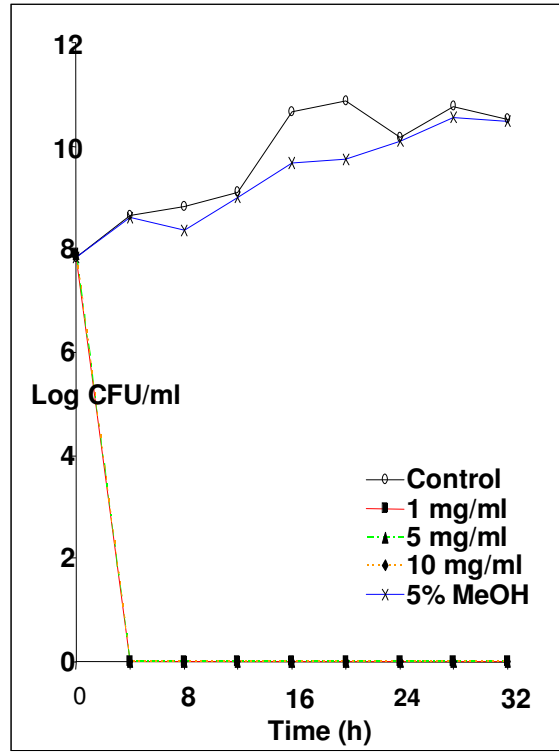
Killing kinetics

Normal cells increased to 6.00 x 10¹⁰ CFU/ml, while methanol treated cells count increased to 4.67 x 10¹⁰ CFU/ml in 28 h (Fig. 1). The control *L. monocytogenes* in normal broth increased to 4.67 x 10⁹ CFU/ml in 28 h before a drop in concentration was noticed, while the 5% methanol extract solution exhibited bacteriostatic effects with exposed culture decreasing from 3.0 x 10⁷CFU/ml initially to 1.67x10⁵ CFU/ml within the first 12 h and attaining a final concentration of 3.33 x 10⁶ CFU/ml at 32 h. After only 4 h, *E. coli O157:H7* and *L. monocytogenes* were completely killed by all concentrations of *P. guajava* contrary to the inhibition zone results, while *S. typhimurium* at a concentration ≤ 5 mg/ml.

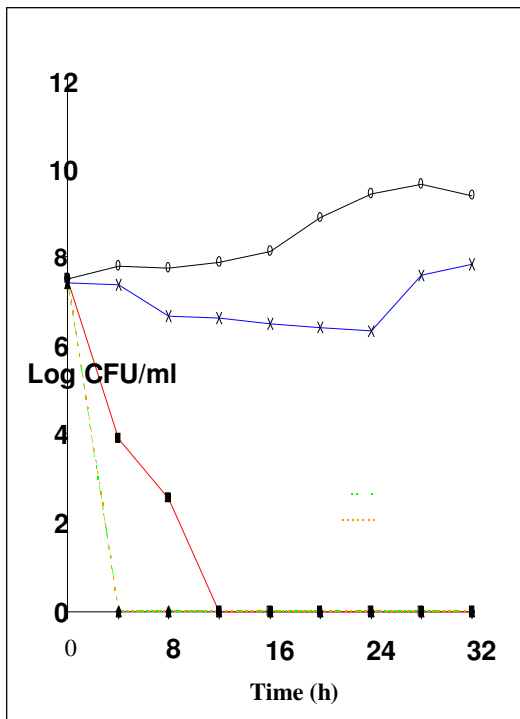
Salmonella typhimurium



E. coli O157:H7



Kocuria rhizophila



Listeria monocytogenes

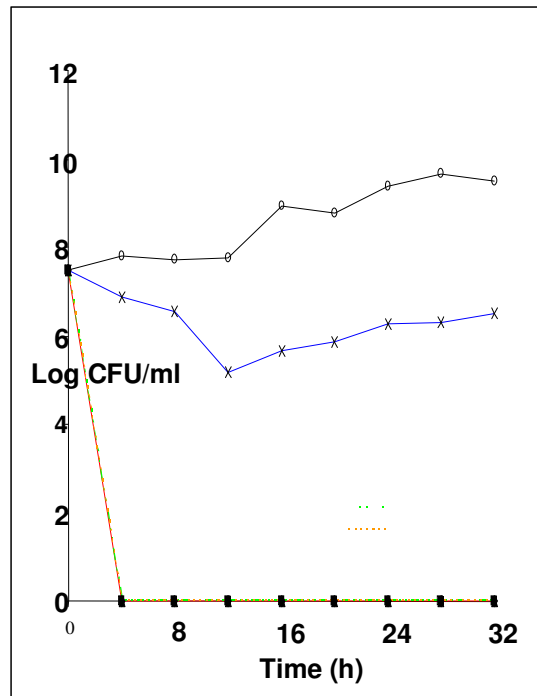


Figure 1. Killing kinetics of on various microorganisms treated with *P. guajava* leaf extract

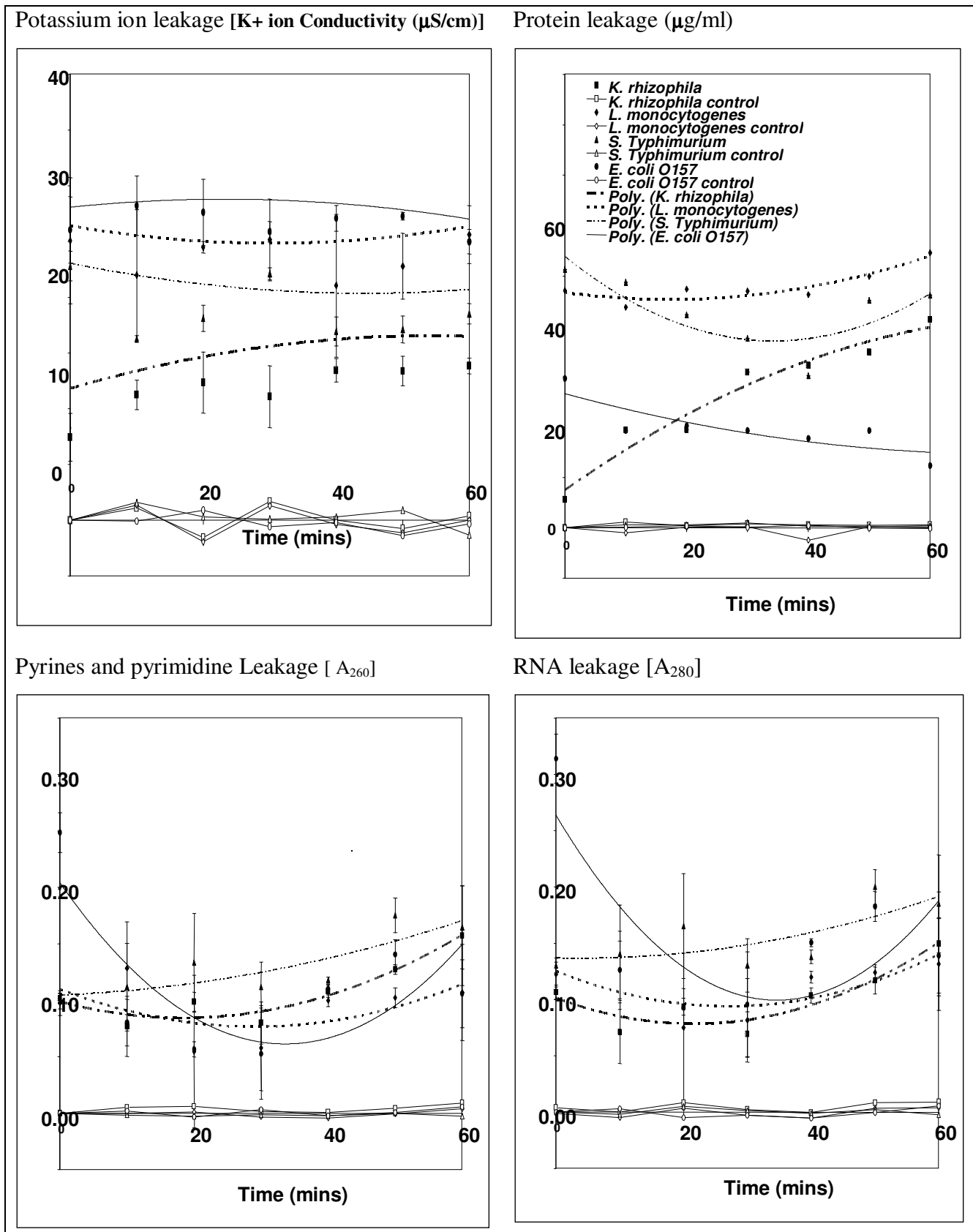


Figure 2. Leakage of various bacterial membrane upon exposure to *P. guajava* leaf extract

Table 1. Inhibition zone diameter [mm]) of different solvent extracts of *P. guajava*.

<i>Psidium guajava</i> extracts at 10 µg in different solvents				
Microorganism	Hexane	Alcoholic Chloroform	Methanol	Positive Control
<i>Staphylococcus aureus</i>	0.0 _c	0.0 _c	18.7 _a	S14.3
<i>Staphylococcus epidermis</i>	13.3 _b	0.0 _c	16.7 _a	NA22.0
<i>Kocuria rhizophila</i>	0.0 _c	0.0 _c	18.0 _a	NA10.0
<i>Bacillus cereus</i>	0.0 _c	0.0 _c	0.0 _c	C23.3
<i>Listeria monocytogenes</i>	0.0 _c	14.7 _a	15.3 _a	S27.0
<i>Corynebacterium diphtheriae</i>	0.0 _c	0.0 _c	0.0 _c	E27.7
<i>Salmonella typhimurium</i>	0.0 _c	0.0 _c	18.7 _a	E11.0
<i>Salmonella Enteritidis</i>	0.0 _c	0.0 _c	18.0 _a	NA15.7
<i>Escherichia coli</i> O157:H7	0.0 _c	0.0 _c	0.0 _c	C27.0
<i>Escherichia coli</i>	0.0 _c	0.0 _c	0.0 _c	E24.7
<i>Aeromonas hydrophila</i>	16.0 _a	0.0 _b	14.3 _a	S25.2
<i>Aeromonas caviae</i>	16.0 _a	0.0 _b	14.3 _a	S21.1
<i>Citrobacter freundii</i>	0.0 _c	0.0 _c	0.0 _c	C21.3
<i>Enterobacter aerogenes</i>	0.0 _c	0.0 _c	9.3 _b	NA18.7
<i>Vibrio parahaemolyticus</i>	12.7 _a	0.0 _b	12.7 _a	NA10.0
<i>Vibrio cholerae</i>	0.0 _c	0.0 _c	0.0 _c	C34.3

Note:

Positive Control (per disc): S= Streptomycin 10 µg; E=Erythromycin 15ug, C=Chloramphenicol 30ug; NA=Nalidixic acid 30ug;

_{a-c} Subscripts of different alphabet indicate significant difference (p<0.05) of the means of triplicate data.

Table 2. Inhibition zone diameter [mm]) and minimum inhibitory concentration (MIC) of methanolic extracts of *P. guajava*.

Strain of Bacteria	Inhibition zone diameter (mm) of 400 µg methanol extract		MIC (mg/ml)
	<i>P. guajava</i>	<i>Positive control</i>	<i>P. guajava</i>
<i>Staphylococcus aureus</i>	12.7 _a	S 14.7	1
<i>Kocuria rhizophila</i>	13.3 _a	NA 14.9	1
<i>Listeria monocytogenes</i>	11.7 _a	S 26.2	5
<i>Salmonella typhimurium</i>	14.7 _a	E 12.3	5
<i>Escherichia coli</i> O157:H7	0.0 _c	C 27.8	1
<i>Aeromonas hydrophila</i>	11.7 _a	S 23.3	5
<i>Vibrio parahaemolyticus</i>	10.3 _b	NA 10.1	5

Note:

Positive Control (per disc): S= Streptomycin 10 ug; E=Erythromycin 15ug, C=Chloramphenicol 30ug; NA=Nalidixic acid 30ug;

_{a-c} Subscripts of different alphabet indicatesignificant difference(p<0.05) of thetriplicate data.

The growth of *K. rhizophila* in normal broth increased gradually from 3.33×10^7 CFU/ml initially to 4.33×10^9 CFU/ml in 28 h before decreasing slightly to 2.33×10^9 CFU/ml within the next 4 h. *K. rhizophila* was significantly suppressed by the 5% methanol solution from an initial 2.67×10^7 CFU/ml, it gradually decreased to 2.33×10^6 CFU/ml over the first 24 h before overcoming the solvent effects and increasing to a concentration of 6.67×10^7 over 32 h. *K. rhizophila* was killed within 4 h at 5 mg/ml methanol extract concentrations, while at 1 mg/ml, complete death occurred at a slower rate in 12 h.

Cellular leakage

Different microbes showed different trends for K^+ leakage. Extracellular pools (K^+) remained fairly constant for all organisms with no significant difference ($p < 0.05$) observed in the leakage throughout the duration of exposure (Fig.2). Highest K^+ leakage ($28.22 \pm 2.66 \mu S/cm$ to $24.99 \pm 1.10 \mu S/cm$) was observed in *E. coli* O157:H7.

The amount of protein detected in post treatment solution increased gradually over time for all organisms except *E. coli* O157:H7. A stronger correlation between leakage and duration of exposure were revealed in Gram-positive than in Gram-negative organisms. The amount of protein in the solution of treated *K. rhizophila* cells increased steadily over 60 mins ($5.65 \pm 0.004 \mu g/ml$ to $41.33 \pm 0.002 \mu g/ml$) (Fig. 2). About 95% of protein leakage in *K. rhizophila* and 85% in *L. monocytogenes* are related to duration of exposure to the extract (Table 3). Highest protein leakage was observed in *L. monocytogenes* and *S. typhimurium*. Protein leakage in *L. monocytogenes* cells was relatively constant until about 40 mins before a gradual increase was observed. On the other hand, *S. typhimurium* demonstrated a constant decrease in the extracellular protein during

the first 40 mins of exposure followed by a rapid increase over the remainder of the experimental period. A stronger correlation between bacterial nucleic acids leakage and exposure time were demonstrated in the Gram-negatives than the Gram-positives (Table 3). Agarose gel electrophoresis confirmed the absence of free nucleic acids in the cell-free filtrates of cells treated with the extracts.

Discussion

The methanolic extract of *P. guajava* leaf (GME) was efficient against both Gram-positive and Gram-negative bacteria, indicating the broad-spectrum antibiotic activity. *E. coli* O157:H7 showed no inhibition zone at $10 \mu g$ or $400 \mu g$ but was completely killed at $1 mg/ml$ ($1 \times MIC$) within 4 hrs of exposure to the extract. The high MIC value of the crude extract may not make it practical to add it to foodstuffs, but it can be used as a first aid for food poisoning in regions where guava grows readily. The MIC value may be reduced substantially by purifying and identifying the active compounds. The size of the molecules of the active components of the different solvent extracts assayed may affect the inhibition zone values obtained. The methanol extract produced the highest antimicrobial activity pointing to the possible relatively polar nature of the active compounds, probably polyphenols or aldehydes (Power, 1995).

E. coli O157:H7 and *E. coli* were the most resistant of the organisms tested, but recent findings showed *P. guajava* extracts demonstrated significant antimicrobial activity against different strains of *E. coli*, including 6 strains of *E. coli* O157:H7 (Caceres *et al.*, 1990; Lin *et al.*, 2002; Voravuthikunchai *et al.*, 2004). When *E. coli* was tested at $1/4 \times$ and $1/2 \times$ MIC of *A. indica* extract, a rapid initial decline in

Table 3. Equation for best fitted line and R² values for bacterial membrane leakage caused by *P. guajava* extract.

Microorganisms	R ² Value <i>P. guajava</i>	Best fitted line guava leaf
Potassium ion leakage		
<i>Kocuria rhizophila</i>	0.8256	$-0.1811x^2 + 2.3265x + 6.1495$
<i>Listeria monocytogenes</i>	0.1657	$0.1940x^2 - 1.5607x + 26.101$
<i>Salmonella typhimurium</i>	0.1864	$0.1548x^2 + 1.681x + 22.477$
<i>Escherichia coli</i> O157:H7	0.3879	$-0.1484x^2 + 0.9873x + 25.753$
Protein leakage		
<i>Kocuria rhizophila</i>	0.9525	$-0.5233x^2 + 9.5714x - 1.5455$
<i>Listeria monocytogenes</i>	0.8429	$0.4688x^2 - 2.5457x + 48.803$
<i>Salmonella typhimurium</i>	0.6863	$1.4202x^2 - 12.594x + 65.038$
<i>Escherichia coli</i> O157:H7	0.6979	$0.2239x^2 - 3.7304x + 30.145$
Nucleic acid, pyrimidines and pyrimidine leakage		
<i>Kocuria rhizophila</i>	0.9066	$0.0042x^2 - 0.0241x + 0.1187$
<i>Listeria monocytogenes</i>	0.3036	$0.0039x^2 - 0.0306x + 0.1366$
<i>Salmonella typhimurium</i>	0.7083	$0.0014x^2 - 0.0004x + 0.1036$
<i>Escherichia coli</i> O157:H7	0.5809	$0.0127x^2 - 0.1103x + 0.3017$
RNA leakage		
<i>Kocuria rhizophila</i>	0.8523	$0.0049x^2 - 0.0310x + 0.1276$
<i>Listeria monocytogenes</i>	0.5333	$0.0043x^2 - 0.0317x + 0.1529$
<i>Salmonella typhimurium</i>	0.5443	$0.0017x^2 - 0.0047x + 0.14$
<i>Escherichia coli</i> O157:H7	0.5880	$0.0137x^2 - 0.1224x + 0.3725$

population for 2 h was observed, followed by an increase almost at the same rate as the control (Okemo *et al.*, 2001). A higher dosage (1 x MIC) also had the initial growth inhibition in the first 2 h but this was followed by an inconsistent growth effect and a final continued slow population decrease (Lutterodt *et al.*, 1999). In this study, at 1 mg/ml (1x MIC for *P. guajava*) a complete death was obtained in 4 h, suggesting that the effect of the extract component on the bacteria was permanent and did not allow for the development of resistance by the organism over time. The antimicrobial potential of the polar (ethanolic and aqueous) leaf extracts of *P. guajava* have been demonstrated against

three strains of *E. coli* O157:H7 (Voravuthikunchai *et al.*, 2004). MIC values ranging from 0.19 – 0.78 mg/ml for the aqueous extract and 6.25-12.5 mg/ml for the ethanolic extract, underscored the efficacy of the extracts against *E. coli* O157:H7.

A consistently higher amount of K⁺ ions was detected in solution of *E. coli* O157:H7 cells treated with GME than any other bacteria, indicating that even if the extract did not produce inhibition zones, it caused severe membrane or metabolic disruption at the same concentration. The protein leakage in post treatment solution for *E. coli* O157:H7, remained relatively constant. The protein leakage from *E. coli* O157:H7 was the lowest, only about 1/3 that

from other microbe and related well to their relative resistance to the methanol extract with no inhibition zone observed even at 400 µg. The leakage of pyrimidines and pyrimidines from *E. coli* O157:H7 was noticeably higher than that in other test organisms within the first 10 min after exposure to the extract. However after 30 min of exposure *E. coli* O157:H7 showed the lowest pyrimidines and pyrimidines leakage which may indicate a good homeostasis ability by the organism. Duration of exposure did not strongly influence the leakage of *E. coli* O157:H7 membrane (Tables 3).

The effectiveness of polar extracts of *P. guajava* leaves to inhibit microbial growth was demonstrated against different strains of *Salmonellae* including *S. typhimurium*, at a MIC of 5mg/ml which was lower than the reported MIC of 10 mg/ml (Litterodt *et al.*, 1999). In this study, the cellular leakage also increased with duration of exposure to the extracts but the levels of bacterial nucleic acids from *S. typhimurium* were about twice that observed in *L. monocytogenes*. This implies that *S. typhimurium* suffered greater nuclear/membrane damage than *L. monocytogenes*. Although each organism showed different RNA leakage with time, GME caused a relatively high release of RNA in the Gram-negative organisms, *S. typhimurium* and *E. coli* O157:H7. GME also caused a relatively high release of pyrimidines and pyrimidines in all the organisms investigated. The amount of pyrimidines and pyrimidines (260nm absorbing materials) released into the cell suspensions, showed similar patterns in *L. monocytogenes*, *S. typhimurium* and *K. rhizophila*. The duration of exposure strongly influenced the release of pyrimidines and pyrimidines in *K. rhizophila* and *S. typhimurium* which was also found to have a large inhibition zone diameter at 10 µg methanol extract. The effect of guava extract on *S. typhimurium* for the first 40

mins of the experiment was similar to microwave-radiated *E. coli* and *B. subtilis* cells which caused an opposite pattern for the proteins and nuclear materials released (Woo *et al.*, 2000). The size of pore formed would cause a relatively slower release of proteins than bacterial nucleic acids, as the extract take effect with increasing exposure time. It appeared that *L. monocytogenes* and *S. typhimurium* suffered greater membrane damage than *E. coli* O157:H7 when treated with GME. When several outer membrane proteins significantly decreased after 120 min infection of cells with c2 clear-plaque mutant, cell death were still attributed to membrane damage (Lin *et al.*, 2001).

The unexpectedly low level of protein leakage and relatively higher viability observed in *E. coli* O157:H7 exposed to the extract compared to *L. monocytogenes*, could be due to the difference in composition of the cell envelope or homeostatic ability or adaptability. Duration of exposure did not strongly influence membrane leakage in *L. monocytogenes* (Table 3). The high activity of *P. guajava* against the four bacteria tested, all of which, with the except *K. rhizophila*, may allow it to be used in the treatment of related foodborne diseases, or prevent death due to the dehydration caused by continuous diarrhoea especially in children (Litterodt *et al.*, 1999). *K. rhizophila* has not previously been identified as a pathogen, but was isolated from chicken treated with oxalic acid (Anang *et al.*, 2006), was resistant to many microbial agents and was therefore of interest. This organism was completely inhibited by GME at 1 mg/ml in 12 h. This suggests a possible disinfection role for the guava leaves extract against *Kocuria* spp. The antimicrobial compounds such as flavonoids contained in herbal preparations, are able to reach the small intestine without processing and are further metabolized in the large intestine by the

human intestinal micro-flora (Bae *et al.*, 2000). As rotaviruses and bacteria such as *S. typhimurium* and *E. coli* O157:H7 cause severe diarrhea resulting in damage to cells in the small intestine, bioactive substances should be delivered quickly to the target site, for protection against infection to some degree (Goncalves *et al.*, 2005). This study suggest that the plant extracts can deny the time needed by the respective organisms to produce toxins, and this would imply that, if administered to patients in non-cytotoxic portions, such concoctions could significantly lower the morbidity and mortality, especially when infection occurs in remote places without hospital facilities.

While K^+ leakage in *L. monocytogenes* and *S. typhimurium* could be attributed to other factors including concentration of extract, leakage in *K. rhizophila* cells was strongly correlated ($R^2 = 0.82$) to exposure time (Table 3). The lowest K^+ leakage ($7.45 \pm 2.15 \mu\text{S/cm}$ to $13.85 \pm 1.05 \mu\text{S/cm}$) was observed in *K. rhizophila*, yet it is one of the microbes that were most vulnerable to the extract. This may indicate that the extract also affect the microbes metabolism causing them to die. No leakage was observed in untreated cells. The release of potassium ions in *K. rhizophila* and *S. typhimurium* were more strongly correlated to exposure time than in *L. monocytogenes* and *E. coli* O157:H7.

The increase in bacterial nucleic acids detected in solution after 30 minutes exposure to the GME as demonstrated by all bacteria, could be attributed to nuclear damage or a complete stoppage of all cellular processes, which may have been taking place while the cells were already inactivated. From the MIC assays, it was expected that leakage of bacterial nucleic acids would have been greater in the *K. rhizophila* and *E. coli* O157:H7 cells treated with the GME since they were exposed to the extract at a concentration of 5x MIC.

While the expected result was obtained for *E. coli* O157:H7 and *K. rhizophila* demonstrated the lowest leakage among the organisms tested, suggesting that nuclear material damage may not be the killing mechanism of GME against this organism.

Methanol itself, demonstrated a slight bacteriostatic effect against *Listeria* and *Kocuria* and this, coupled with the effect of the slightly acidic environment could have aided the efficacy of the extract. Mechanisms of antimicrobial activity include inhibition of enzymes, membrane function, nuclear material damage, nutrient transport and overall metabolic disruptive activities (34).

The amount of RNA (280nm absorbing materials) released into solution was higher than that of pyrimidines and pyrimidines but followed a similar pattern for all organisms tested (Fig. 2, Table 3). The leakage of bacterial nucleic acids, were low for all organisms but were significantly different ($p \leq 0.05$) from untreated cells which showed no leakage. All organisms tested, with the exception of *E. coli* O157:H7, demonstrated increased pyrimidines and pyrimidines leakage at a decreasing rate after 30 min of exposure.

Total plate count of samples taken at 10-minute intervals after treatment showed no visible growth for all organisms after the first 10 min of exposure, indicating that a 100% reduction in viability was obtained within the first 10 min of exposure to extracts. The stability in the release of the K^+ in cells exposed to GME suggests that the duration of exposure did not influence K^+ ions efflux, and it does not correlate completely with the onset of cell death. The results of this study also suggest that the membrane damage, in both Gram-positive and Gram-negative bacteria, occurred immediately after contact with the extracts and that no further damage was inflicted and the leakage of K^+ ions was most likely as a

result of diffusion of the ions from the cytoplasm.

In the presence of the extracts, it is possible that disruption of normal functioning of the selective membrane permeability mechanism cause the cations to be transported across their concentration gradient into the cell. Cell death may not have been induced by K⁺ ions leakage only, but rather an extract-induced cation influx. The results also suggest an initial disruption in selective membrane permeability of the cell causing an increase in absorption of materials from the surrounding medium followed by an increased leakage of bacterial nucleic acids after about 40 min of exposure.

In this study *P. guajava* at 10µg was effective against 67% of the Gram-positive and 50% of the Gram-negative bacteria and most likely relate to the differences in their cell walls composition (Grosvenor *et al.*, 1995; Nostro *et al.*, 2000; Ojala *et al.*, 2000). The resistance of *Escherichia* spp. may relate to cell membrane permeability, genetic factors (Okemo *et al.*, 2001) or a good homeostasis or adaptability.

Phenolic antioxidants react with the cellular membrane, impairing both its functions and integrity (Raccach, 1984; Blaszyk and Holley, 1998). The loss of cell viability may not be attributed to a single particulate event, but to multitarget inactivation, resulting in several lesions that together cause death. The key target may also be affected when a secondary structure is previously damaged. The presence of flavonoids and terpenes and a certain degree of lipophilicity might determine toxicity by the interactions with the membrane constituents and their arrangement (Tomas-Barberan *et al.*, 1990). The high flavonoids and polyphenols content of GME (Koo and Mohamed, 2001) may have contributed to the antimicrobial activity (Meckes *et al.*, 1996; Koo and Mohamed, 2001).

Antimicrobials may interact with the cell surface, followed by penetration or may act at various external target sites (Sampathkumar *et al.*, 2003).

References

- Abdelrahim, S.I., Almagboul, A.Z., Omer, M.E.A. and Elegami, A. 2002. Antimicrobial activity of *Psidium guajava* L. *Fitoterapia* 73: 713–715.
- Anang, D.M., Rusul, G., Radu, S., Bakar, J. and Beuchat, L.R. 2006. Inhibitory effect of Oxalic acid on bacterial spoilage of raw chilled chicken. *Journal of Food Protection* 69 (8):1913-1919
- Bae, E. A., Park, S. Y., and Kim, D.-H., 2000. Constitutive β -glucosidases hydrolyzing ginsenoside Rb₁ and Rb₂ from human intestinal bacteria. *Biology and Pharmacology Bulletin* 23: 1481-1485.
- Bauer, A.W., Kirby, W.M.M., Sherris, J.C., and Turck, M., 1966. Antibiotic susceptibility testing by standardized single disc method. *American Journal of Clinical Pathology* 45: 493– 496.
- Begum, S., Hassan, S.I., Ali, S.N. and Siddiqui, B.S. 2004. Chemical constituents from the leaves of *Psidium guajava*. *Natural Product Research* 18 (2):135-40.
- Blaszyk, M., and Holley, R.A. 1998. Interaction of monolaurin, eugenol and sodium citrate on growth of common meat spoilage and pathogenic organism. *International Journal of Food Microbiology* 39: 175-183.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantification of

- microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochemistry* 72: 248-254.
- Caceres, A., Cano, O., Samayoa, B. and Aguilar, L. 1990. Plants used in Guatemala for the treatment of gastrointestinal disorders: Screening of 84 plants against enterobacteria. *Journal of Ethnopharmacology* 38: 55-73.
- Cowan, M.M. 1999. Plant products as antimicrobial agents. *Clinical Microbiology Review* 12: 564-582.
- Culvenor, C.C.J. and Fitzgerald, J.S. 1963. Field test for alkaloids in plant material. *J. Pharmacology Science* 52: 303-304.
- Galvez, J., Duarte, J. and Sanchez, F. 1996. Inhibitory effects of quercetin on guinea pig ileum contractions. *Phytotherapy Research* 10: 66-69.
- Goncalves, J.L.S., Lopes, R.C., Oliveira, D.B., Costa, S.S., Miranda, M.M.F.S., Romanosa, M.T.V., Santos, N.S.O., and Wigg, M.D. 2005. *In vitro* anti-rotavirus activity of some medicinal plants used in Brazil against diarrhea. *Journal of Ethnopharmacology* 99: 403-407.
- Gradelski, E., Valera, L., Kolek, B., Bonner, D., and Fung-Tomc, J. 2001. Comparative killing kinetics of the novel des-fluoro (6) quinolone BMS-284756, fluoroquinolones, vancomycin and β -lactams. *International Journal of Antimicrobial Agents* 18: 43-48.
- Grosvenor, P.W., Suprino, A. and Gray, D.O. 1995. Medicinal plants from Riau Province, Sumatra, Indonesia. Part 2: antibacterial and antifungal activity. *Journal of Ethnopharmacology* 45: 97-111.
- Hawrelak J. 2003. Medicinal herb monograph: Guava (*Psidium guajava*). *Journal of the Australian Traditional-Medicine Society* 9; 25-29.
- Jaiarj, P., Khoohaswan, P., Wongkrajang, Y., Peungvicha, P., Suriyawong, P., Sumal Saraya, M.L. and Ruangsomboon, O. 1999. Anticough and antimicrobial activities of *Psidium guajava* Linn. leaf extract. *Journal of Ethnopharmacology* 67: 203-212.
- Koo H.M. and Mohamed S. (2001) The Flavonoids (Myricetin, quercetin, kaempferol, luteolin, apigenin) content of edible tropical plants. *Journal of Agriculture and Food Chemistry* 49(6) :3106-3112
- Lin, J., Puckree, T. and Mvelase, T.P. 2002. Anti-diarrhoeal evaluation of some medicinal plants used by Zulu traditional healers. *Journal of Ethnopharmacology* 79: 53-56
- Lin, S-H., Chen, W-P., and Kuo, T-T. 2001. Mechanism of host cell death induced by infection of *Escherichia coli* with the c2 clear-plaque mutant of phage ϕ 1. *Botany Bulletin Academy Singapore*. 42: 45-52.
- Lozoya, X., Reyes-26 Morales, H., Chavez-Soto, M.A., Martinez-Garcia Mdel, C., Soto Gonzalez, Y. and Doubova, S.V. 2002. Intestinal antispasmodic effect of a phytodrug of *Psidium guajava* folia in the treatment of acute diarrheic disease. *Journal of Ethnopharmacology* 83: 19-24.
- Lozoya, X., About-Zaid, M.N., Nozzilillo, C., Arnason, J.T., 1990. Spasmolytic effect of the methanolic extract of *Psidium guajava*. *Planta Medica* 56: 686-689.

- Lozoya, X., Meckes, M., Abou-Zaid, M., Tortoriello, J., Nozzolillo, C. and Arnason, J.T., 1994. Quercetin glycosides in *Psidium guajava* L. leaves and determination of a spasmolytic principle. Archives of Medical Research 25: 11–15.
- Lutterodt, G.D., Ismail, A., Basheer, R.H. and Baharudin, H.M. 1999. Antimicrobial effects of *Psidium guajava* extract as one mechanism of its antidiarrhoeal action. Malaysian Journal of Medical Science 6 (2): 17-20.
- Matsuo, T, Hanamure, N, Shimoi, K., Nakamura, Y. and Tomita, I. 1994. Identification of (+)-galocatechin as a bio-antimutagenic compound in *Psidium guajava* leaves. Phytochemistry 36 (4): 1027-1029.
- Meckes, M., Calzada, F., Tortoriello, J., Gonzalez, J.L. and Martinez, M., 1996. Terpenoids isolated from *Psidium guajava* hexane extract with depressant activity on central nervous system. Phytotherapy Research 10: 600–603.
- Morales M.A, Tortoriello J. and Meckes M. 1994. Calcium-antagonist effect of quercetin and its relation with the spasmolytic properties of *Psidium guajava* L. Archives of Medical Research 25: 17–21.
- NCCLS. 1999. Methods for determining bactericidal activity of antimicrobial agents. Approved standard M26-A. Wayne, PA: National Committee for Clinical laboratory Standards.
- Nostro, A., Germano, M.P., D'Angelo, V., Marino, A. and Cannatelli, M.A., 2000. Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. Letters in Applied Microbiology 30: 379–384.
- Ojala, T., Remes, S., Haansuu, P., Vuorela, H., Hiltunen, R., Haahtela, K., and Vuorela, P. 2000. Antimicrobial activity of some coumarin containing herbal plants growing in Finland. Journal of Ethnopharmacology 73: 299–305.
- Okemo, P.O., Mwatha, W.E., Chhabrab, S.C., and Fabryc, W. 2001. The kill kinetics of *azadirachta indica* A. Juss. (Meliaceae) extracts on *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. African Journal of Science and Technology (AJST) 2 (2): 113-118.
- Pankuch, G. A., Jacobs M. R., and Appelbaum P. C. 1994 Study of comparative antipneumococcal activities of penicillin G, RP 59500, erythromycin, sparfloxacin, ciprofloxacin, and vancomycin by using time-kill methodology Antimicrobial Agents Chemotherapy 38(9): 2065-2072.
- Power, E.G.M. 1995. Aldehydes as biocides. Progress in Medical Chemistry 34: 149–201.
- Raccach, M. 1984. The antimicrobial activity of phenolic antioxidants in foods: a review. Journal of Food Safety 6: 141-170.
- Roller, S. 2003. *Natural Antimicrobials for the Minimal Processing of Foods*, pp. 176-191. Woodhead Publishing and CRC Press, Cambridge, UK and Boca Raton, FL, USA, 2003.
- Sampathkumar, B., Khachatourians, G.G. and Korber, D.R. 2003. High pH during trisodium phosphate treatment causes

- membrane damage and destruction of *Salmonella enterica* serovar Enteritidis. Applied and Environmental Microbiology 69 (1): 122-129.
- Samy, R.P., Ignacimuthu, S. and Sen, A. 1998. Screening of 34 Indian medicinal plants for antibacterial properties. Journal of Ethnopharmacology 62: 173–181.
- Smail, E. H., and J. M. Jones. 1984. Demonstration and solubilization of antigens expressed primarily on the surfaces of *Candida albicans* germ tubes. Infection and Immunity 45: 74-81.
- Takahama, U. and Hirota, S. 2001. Deglucosidation of quercetin glucosides to the aglycone and formation of antifungal agents by preoxidase-dependent oxidation of quercetin. Plant and Cell Physiology 41: 1021-1029.
- Tomas-Barberan, F., Iniesta-Sanmarti, E., Tomas-Lorente, F. and Rumbero, A. 1990. Antimicrobial phenolic compounds from three Spanish *Helichrysum* species. Phytochemistry 29: 1093-1095.
- Van Netten, P., Perales, J., Van De Moosdijk, A., Curtis, G.D.W. and Mossel, D.A.A. 1989. Liquid and solid selective differential media for the detection and enumeration of *Listeria monocytogenes*. International Journal of Food Microbiology 8: 299-316.
- Voravuthikunchai, S., Lortheeranuwat, A., Jeeju, W., Sririrak, T., Phongpaichit, S. and Supawita, T. 2004. Effective medicinal plants against Enterohaemorrhagic *Escherichia coli* O157:H7. Journal of Ethnopharmacology 94: 49–54.
- Woo, I-S., Rhee, I-K. and Park, H-D. 2000. Differential damage in bacterial cells by microwave radiation on the basis of cell wall structure. Applied and Environmental Microbiology 5: 2243-2247.