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

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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 pyrines 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 pyrines 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*., 1994Galvez *et al*., 1996). (+)-Gallocatechin 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

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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 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, Meuller-Hinton Broth (MHB) and Meuller-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 ans 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 hydropilla, 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 - 37ºC. 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 µl 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 Chlorampenicol (C 30 µg/disc) (Oxoid, UK), Streptomycin (S 10 µg/disc) (BBL, USA), Nalidixic Acid (N 30 µg/disc) (Oxoid, UK) and Erythromycin (15 µg/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, ½, 1, 2, 5 and 10× 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 µl of known culture samples dilutions on agar medium. Extracts were deemed bactericidal at the smallest dose which reduced the original inoculum by ≥ 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 x *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 ± 0.2°C) to obtain *ca* 10^8 CFU/ml. The three treatment conditions were (i) Deionized H_2O (control), (ii) deionized H_2O with 5% methanol (v/v), and (iii) deionized H_2O 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 µm 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). Electophoresis 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 10ug 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 6.00×10^{10} CFU/ml, while methanol treated cells count increased to 4.67×10^{10} CFU/ml in 28 h (Fig. 1). The control *L. monocytogenes* in normal broth increased to 4.67×10^9 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 3.0×10^7CFU/ml initially to 1.67×10^5 CFU/ml within the first 12 h and attaining a final concentration of 3.33×10^6 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.

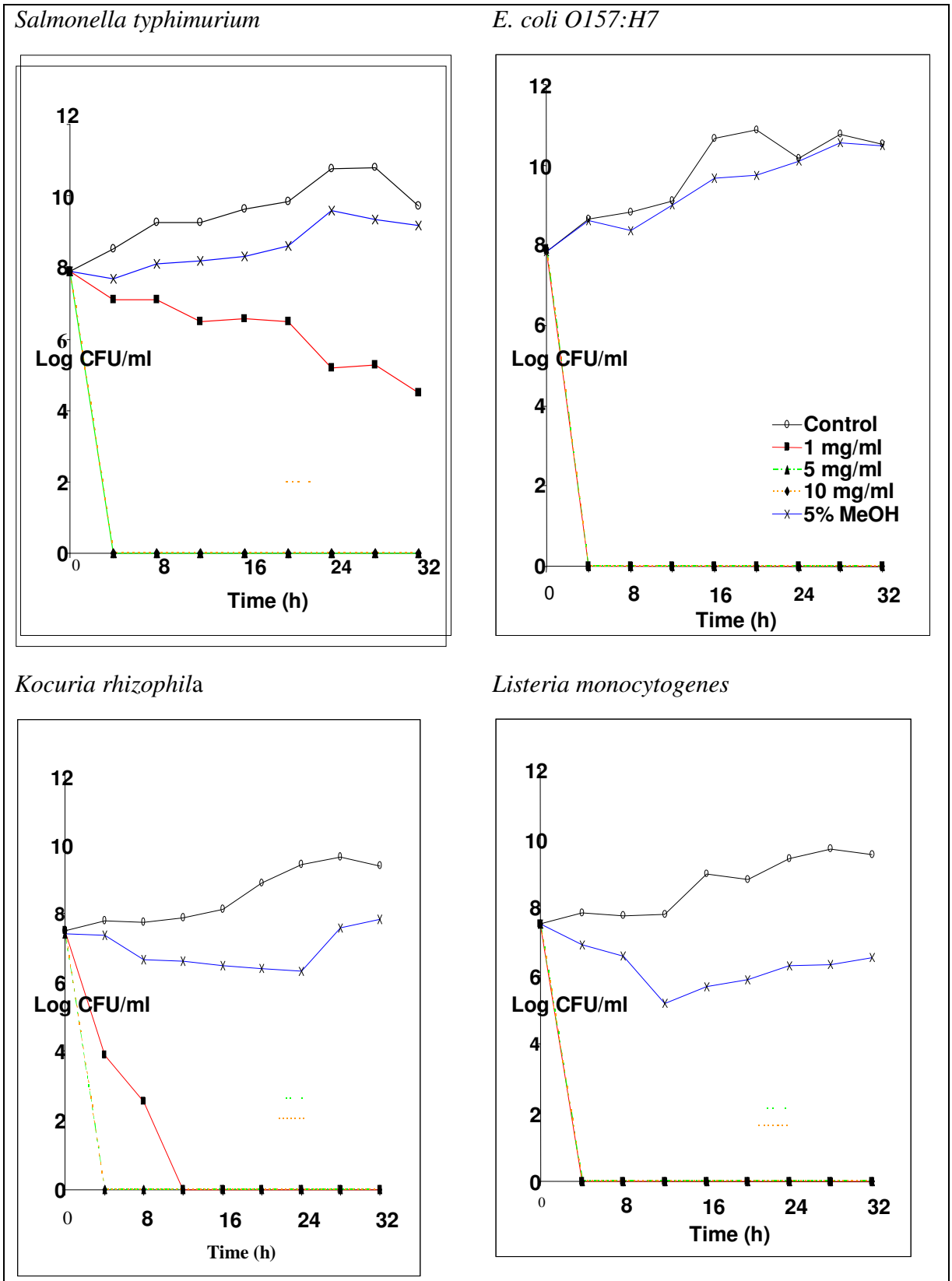

Figure 1. Killing kinetics of on various microorganisms treated with *P. guajava* leaf extract

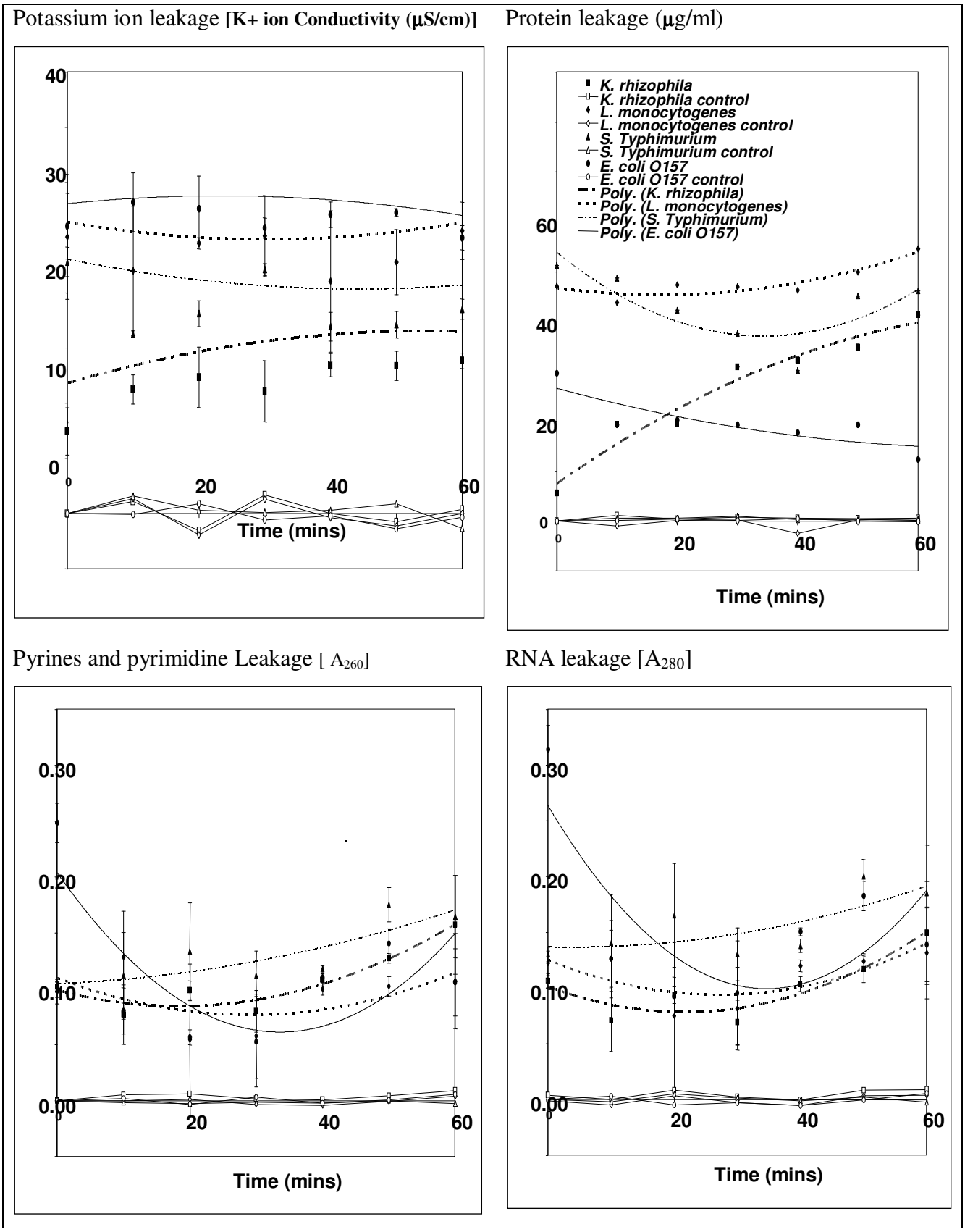

Figure 2. Leakage of various bacterial membrane upon exposure to *P. guajava* leaf extract

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

	Psidium guajava extracts at 10 µg in different solvents			
Microogranism	Hexane	Alcoholic Chloroform	Methanol	Positive Control
Staphylococcus aureus	0.0c	0.0c	18.7_a	$_S$14.3
Staphylococus epidermis	13.3_b	0.0_c	16.7_a	$_{NA}$22.0
Kocuria rhizophila	0.0c	0.0c	18.0_a	$_{NA}$10.0
Bacillus cereus	0.0c	0.0c	0.0c	$_C$23.3
Listeria monocytogenes	0.0c	14.7_a	15.3_a	$_S$27.0
Corynebacterium diptheriae	0.0c	0.0c	0.0c	$_E$27.7
Salmonella typhimurium	0.0c	0.0c	18.7_a	$_E$11.0
Salmonella Enteritidis	0.0c	0.0c	18.0_a	$_{NA}$15.7
Escherichia coli O157:H7	0.0c	0.0c	0.0c	$_C$27.0
Escherichia coli	0.0c	0.0c	0.0c	$_E$24.7
Aeromonas hydrophila	16.0_a	0.0_b	14.3_a	$_S$25.2
Aeromonas caviae	16.0_a	0.0_b	14.3_a	$_S$21.1
Citrobacter freundii	0.0c	0.0c	0.0c	$_C$21.3
Enterobacter aerogenes	0.0c	0.0c	9.3_b	$_{NA}$18.7
Vibrio parahaemolyticus	12.7_a	0.0_b	12.7_a	$_{NA}$10.0
Vibrio cholerae	0.0c	0.0c	0.0c	$_C$34.3

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)
	P. guajava	*Positive control*	P. guajava
Staphylococcus aureus	12.7_a	S 14.7	1
Kocuria rhizophila	13.3_a	NA 14.9	1
Listeria monocytogenes	11.7_a	S 26.2	5
Salmonella typhimurium	14.7_a	E 12.3	5
Escherichia coli O157:H7	0.0c	C 27.8	1
Aeromonas hydrophila	11.7_a	S 23.3	5
Vibrio parahaemolyticus	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 x 10^7 CFU/ml initially to 4.33 x 10^9 CFU/ml in 28 h before decreasing slightly to 2.33 x 10^9 CFU/ml within the next 4 h. *K. rhizophila* was significantly suppressed by the 5% methanol solution from an initial 2.67 x 10^7 CFU/ml, it gradually decreased to 2.33 x 10^6 CFU/ml over the first 24 h before overcoming the solvent effects and increasing to a concentration of 6.67 x 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±2.66 µS/cm to 24.99±1.10 µS/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±0.004 µg/ml to 41.33±0.002 µg/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 µg or 400 µg but was completely killed at 1mg/ml (1 x 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 anti-microbial 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 x and 1/2 x MIC of *A. indica* extract, a rapid initial decline in

Table 3. Equation for best fitted line and R^2 values for bacterial membrane leakage caused by *P. guajava* extract.

Microorganisms	R^2 Value *P.guajava*	Best fitted line guava leaf
Potassium ion leakage		
Kocuria rhizophila	0.8256	$-0.1811x^2 + 2.3265x + 6.1495$
Listeria monocytogenes	0.1657	$0.1940x^2 – 1.5607x + 26.101$
Salmonella typhimurium	0.1864	$0.1548x^2 + 1.681x + 22.477$
Escherichia coli O157:H7	0.3879	$-0.1484x^2 + 0.9873x + 25.753$
Protein leakage		
Kocuria rhizophila	0.9525	$-0.5233x^2 + 9.5714x – 1.5455$
Listeria monocytogenes	0.8429	$0.4688x^2 – 2.5457x + 48.803$
Salmonella typhimurium	0.6863	$1.4202x^2 – 12.594x + 65.038$
Escherichia coli O157:H7	0.6979	$0.2239x^2 – 3.7304x + 30.145$
Nucleic acid, pyrines and pyrimidine leakage		
Kocuria rhizophila	0.9066	$0.0042x^2 – 0.0241x + 0.1187$
Listeria monocytogenes	0.3036	$0.0039x^2 – 0.0306x + 0.1366$
Salmonella typhimurium	0.7083	$0.0014x^2 – 0.0004x + 0.1036$
Escherichia coli O157:H7	0.5809	$0.0127x^2 – 0.1103x + 0.3017$
RNA leakage		
Kocuria rhizophila	0.8523	$0.0049x^2 – 0.0310x + 0.1276$
Listeria monocytogenes	0.5333	$0.0043x^2 – 0.0317x + 0.1529$
Salmonella typhimurium	0.5443	$0.0017x^2 – 0.0047x + 0.14$
Escherichia coli 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 pyrines 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 pyrines 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 (Lutterodt *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 pyrines and pyrimidines in all the organisms investigated. The amount of pyrines 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 pyrines 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±2.15 µS/cm to 13.85±1.05 µS/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 pyrines 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 pyrines 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 permeablity 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 Rb_1 and Rb_2 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 Microbioliogy 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, luoroquinolones, 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 f1. 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 (+)-galoocatechin 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 aintimicrobial 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.