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FACS Analysis of Bacterial Responses to Extracts of *Vatica diospyroides* Fruit Show Dose and Time Dependent Induction Patterns

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Abstract

Antibacterial activity of *Vatica diospyroides* fruit extracts were screened against both Gram-positive and Gram-negative bacteria using disc diffusion method. Minimum Inhibitory Concentrations (MICs) were performed using a macro broth dilution assay. Bacteria were treated with the extracts at various dose levels (0.5, 1, and 2 MIC) and incubation times (3 and 6 h), and their responses were monitored by FACS analysis. The response patterns were analyzed in the counts of 4 subpopulations: viable cells (PI-, SSC-), membrane-damaged cells (PI+, SSC-), injured cells (PI-, SSC+), and dead cells (PI+, SSC+). The fraction of *Bacillus subtilis* responders to fruit cotyledon and pericarp extracts increased in a dose dependent manner, whereas *Staphylococcus aureus* treated with pericarp extract increased in a time dependent manner. Cells initially lost granularity (PI-, SSC+) and membrane integrity (PI+, SSC+). These results demonstrate that the FACS method can reveal the stages of bacterial responses to chemotherapeutic agents.

Keywords: Antibacterial activity, bacteria, FACS analysis, response, *Vatica diospyroides*

Introduction

Common microbiological techniques to study bacterial activity include Viable Plate Count (VPC), biochemical analysis, and turbidimetric methods, but these techniques cannot provide specific characteristics of the individual microbial cells [1], which is a severe limitation. However, the Fluorescence Activated Cell Sorter (FACS) is increasingly used to study bacterial activities, because it can reveal bacterial membrane alternations, intracellular enzyme activities, and mechanisms of antibiotic components against bacteria [2,3]. Moreover, FACS analysis has been used to study the behavior of bacteria responding to antibiotics [1]. Thus, this method has become a powerful tool for research on antibacterial effects. However, FACS is based on the binding of fluorescent dyes to cellular molecule targets, either within or outside the cell, and the results reflect the specific fluorescent stainings used. In the context of the response activities of bacteria, for example, Carboxyfluorescein diacetate (cFDA) has been used to detect enzyme esterase activity in live bacteria, whereas the Syto9/PI binding assay has been widely used to study membrane permeability/potential in both dead and live bacteria [2-4]. Unfortunately, these fluorescent dyes may themselves be toxic, and interfere with normal biological processes [5]. Therefore, it would be preferable to have other appropriate, perhaps easier to use, techniques that reveal the physiological responses of individual bacteria.

We investigated the suitability of PI-staining for the FACS method in studying the efficacy of plant extracts against bacteria, in terms of the bacterial responses. The goal was to demonstrate this technique in observing the individual bacterial response patterns, and in assessing the mechanisms of action of plant extracts.

Materials and methods

Microorganisms tested and their maintenance

The bacterial strains used to assess bacterial response patterns included both Gram-positive (*Bacillus subtilis* ATCC6633 and *Staphylococcus aureus* ATCC25923) and Gram-negative (*Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853) bacteria. The investigated bacterial strains were maintained at the Scientific Laboratory and Equipment Center (SLEC), Prince of Songkla University, Surat Thani campus, Thailand. The organisms were maintained on Nutrient Agar (NA, Oxoid, United Kingdom) slant at 4 °C. The bacteria were re-streaked on NA plates and incubated at 37 °C for 24 h. After that, the bacteria were harvested using a loop, inoculated into Nutrient Broth (NB, Oxoid, United Kingdom), and incubated at 37 °C for 16 - 18 h. Each type of bacteria was sub-cultured twice in the same medium prior to incubation until the mid-log phase.

Vatica diospyroides crude extraction

Fruit of *V. diospyroides* type LS were collected from the Nong Thung Thong non-hunting area, Kiansa district, Surat Thani province. Fruit parts were separated into pericarp and cotyledon and cut, then kept in shade until completely air-dried. The plant materials (270 g) were extracted for 5 days with acetone ((CH3)2CO), and then were filtered using 2 layers of cotton fabric [6]. The extracts were evaporated to obtain dry residue, which was stored in dark conditions at 4 °C prior to testing against bacteria.

Microbiological estimates of antibacterial effects

The antibacterial assay was of the disc diffusion type. The tested bacteria (1×10^7 CFU/ml) were inoculated on Trypticase Soy Agar (TSA) plates by smoothly swabbing the cell suspensions over the agar surface. Filter-paper discs (6 mm diameter) were gently placed onto the external layer of the agar using sterile forceps. Subsequently, 25 µl of a crude plant extract was filled into each disc, resulting in final concentrations in the range 10 - 1,000 µg/ml. The plates were incubated overnight at 37 °C for 18 - 24 h. Inhibition of bacterial growth was determined by measuring the diameter of the inhibition zone, recorded in millimeters. For each bacterial strain, 10 % Dimethyl sulfoxide (DMSO) and 30 µg/ml Oxytetracycline solutions were used as the negative and the positive control, respectively.

The Minimum Inhibitory Concentration (MIC) is the least concentration with observed inhibitory activity. The MICs for the *V. diospyroides* extracts were determined against the bacteria using a macro broth dilution assay. Stock solutions of the extracts were prepared to 2,000 µg/ml. Two-fold serial dilution generated 1,000 µl aliquots of each extract (1.95 - 1,000 µg/ml) stored in 10 sterile tubes. The diluent was Trypticase Soy Broth (TSB). Then, standardized 10^7 CFU/ml suspensions of each bacteria were incorporated into each tube, which were incubated at 37 °C in an incubator for 24 h. The lowest extract concentration that inhibited visible growth of bacteria was considered as the MIC value. The Minimum Bactericidal Concentration (MBC) was determined by spreading the TSB culture from each tube onto a sterile NA medium and incubating at 37 °C for 24 h. The lowest extract concentration that inhibited bacterial growth (no colony grown) was considered to be the MBC.

Determination of granular integrity and membrane permeability of bacteria

To determine bacterial responses using the FACS method, the cellular membrane integrity of treated cells was assessed using PI staining, and the retained granularity was assessed from scatter plots. The varied experimental parameters were time of incubation and extract dose level. First, extract stock for the final concentrations 0.5 MIC, 1 MIC, and 2 MIC was added to microcentrifuge tubes containing 1×10^7 CFU/ml of a given type of bacteria. The microcentrifuge tubes were incubated at 37 °C for 0, 3, and 6 h.

After treatment, the cells were separated by centrifugation at 8,000 rpm for 10 min, washed 2 times with Phosphate Buffer Saline (PBS buffer), and re-suspended in 950 µl of PBS buffer [7]. Then, 50 µl of PI (50 µg/ml) was added before FACS analysis was performed.

FACS analysis

FACS analysis was performed on a BD FACSCalibur flow cytometer (Becton Dickinson Biosciences (BDB), San Jose, CA) fitted with a 488 nm air-cooled argon laser. A total of 10,000 events per sample were acquired and the results plotted with CellQuest software (BDB). Red fluorescence of PI stained cells was detected in the FL2 channel (620±15 nm). Side scatter (SSC) and fluorescence signals (FL2) of each cell passing through the illuminated zone were collected as logarithmic signals. The populations of viable cells, membrane-damaged cells, injured cells, and dead cells in each sample were analyzed, and density plot diagrams were generated with WinMDI version 2.9 software (Scripps Institute, La Jolla, CA).

Results and discussion

Antibacterial activity

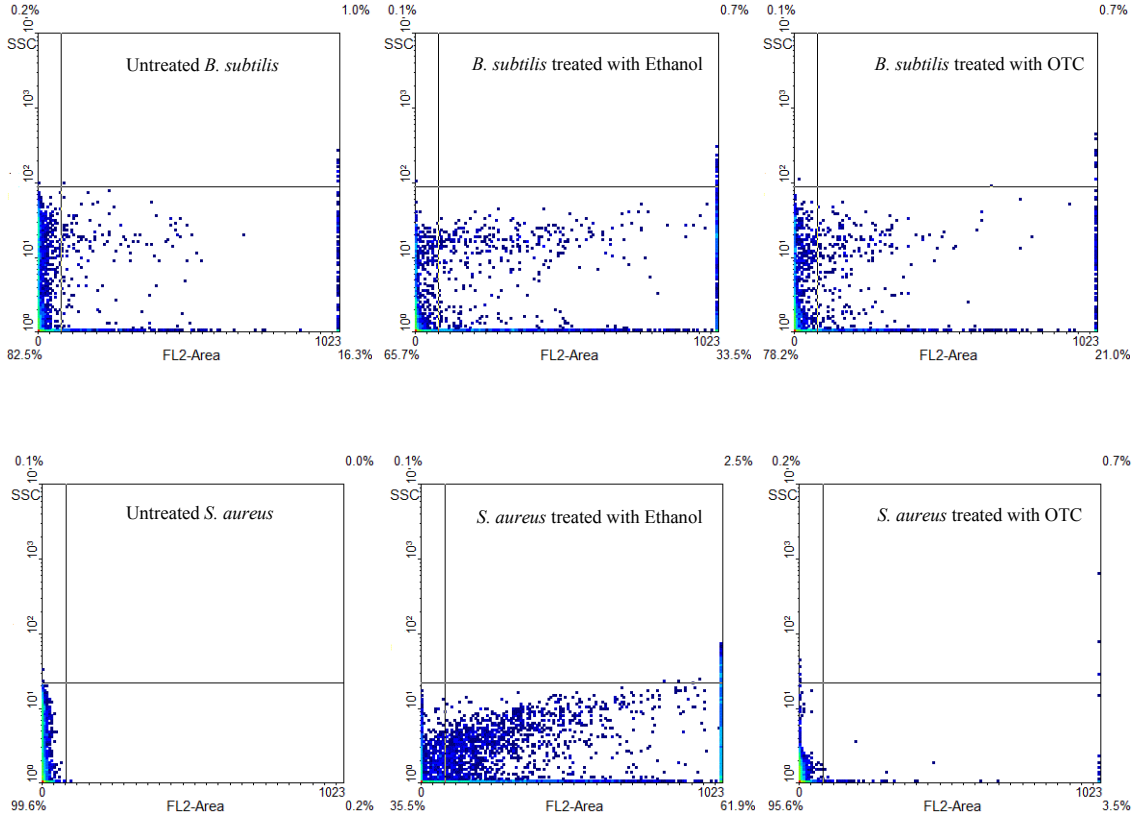
The extracts were measured for antibacterial efficacy against Gram-positive and Gram-negative bacteria. The inhibition zone diameters are presented in **Table 1**, for 4 types of bacteria, 2 types of extracts at the concentrations 50, 100, 250, 500, and 1,000 µg/disc, and with antibiotic Oxytetracycline at 30 µg/disc as the reference. The extracts exhibited varying degrees of inhibition against the Gram-positive bacteria tested. The inhibition zones of *B. subtilis* at 250, 500, and 1,000 µg/disc of cotyledon extract had 8.33, 9.33, and 11.66 mm diameters, respectively. The zones of inhibition exhibited by pericarp extract at 500 and 1,000 µg/disc against both *B. subtilis* and *S. aureus* had 12.00 and 12.66 mm diameter clear zones, respectively. The MIC values of both extracts against *B. subtilis* and *S. aureus* were 1,000 µg/ml. We could not detect the MBC values of either extract against these bacteria below 1,000 µg/ml, indicating MBC values higher than 1,000 µg/ml.

Table 1 Antibacterial activity (Mean±S.D.) of cotyledon and pericarp extracts against *B. subtilis, S. aureus, P. aeroginosa*, and *E. coli*, evaluated by the disc diffusion method.

Extract concentration (µg/disc)		*B. subtilis*	*S. aureus*	*P. aeroginosa*	*E. coli*
cotyledon	50	0	0	0	0
	100	0	0	0	0
	250	8.33 ± 0.57^{c}	0	0	0
	500	9.33 ± 2.30^{c}	0	0	0
	1,000	11.66 ± 1.52^{b}	0	0	0
	10 % DMSO	0	0	0	0
	30 % Oxytetracycline	21.33 ± 2.08^{a}	36.00±5.00	24.33±0.57	27.00±2.00
pericarp	50	7.66 ± 0.57^{e}	0	0	0
	100	9.67 ± 0.57^{cd}	10.00 ± 1.00^{d}	0	0
	250	10.66 ± 0.57^{bc}	10.60 ± 0.57^{cd}	0	0
	500	10.66 ± 2.30^{bc}	12.00 ± 1.00^{bc}	0	0
	1,000	12.00 ± 0.00^{b}	12.60 ± 1.15^{b}	0	0
	10 % DMSO	0	0	0	0
	30 % Oxytetracycline	21.33 ± 2.08^{a}	36.60 ± 2.08^{a}	24.00±1.00	26.67±4.16

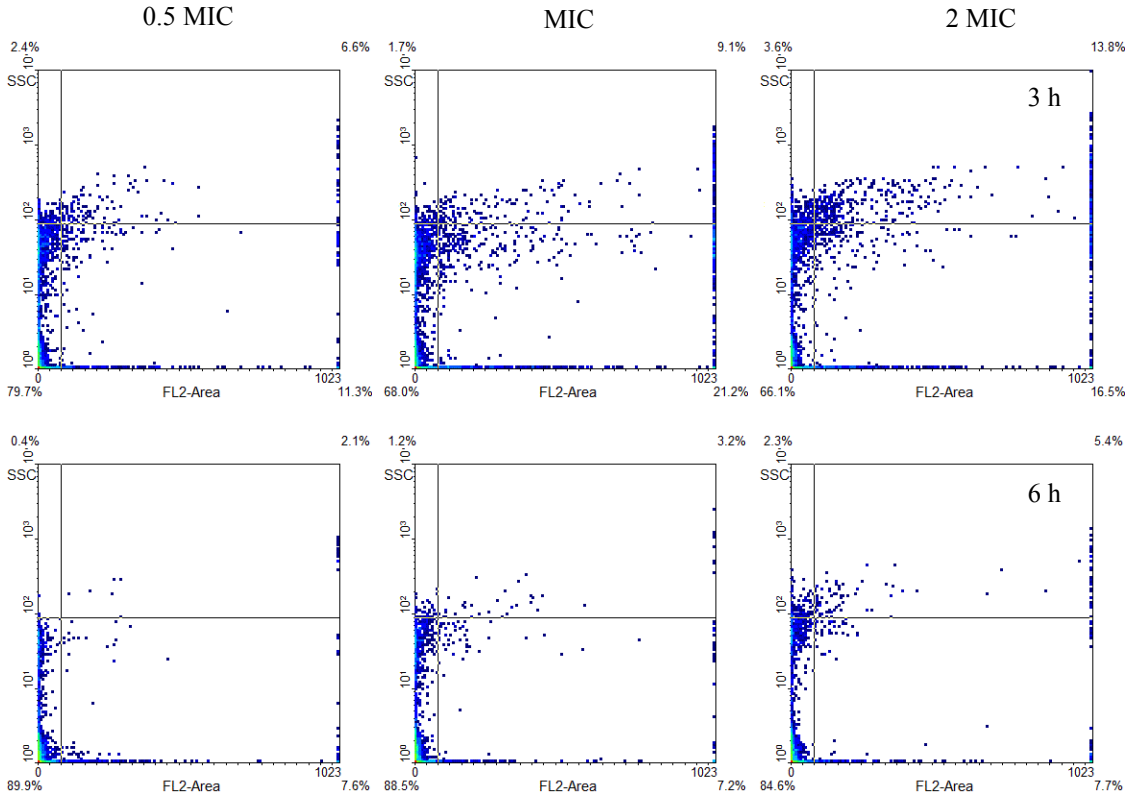
Values are mean inhibition zone diameter (mm) ± S.D from 3 replicates. Statistically significant differences between dose levels are shown by different superscripts, based on DMRT ($p = 0.05$).

These differences reflect the different cell wall and cell membrane permeabilities or penetrabilities of Gram-positive and Gram-negative bacteria. Gram-negative bacteria are more resistant, as their membranes pose an impermeable barrier to the plant extracts, whereas Gram-positive bacteria have cell membranes that are ineffective as barriers [8-10]. Also, in prior work, the extracts from a Dipterocarpaceae plant exhibited stronger antibacterial activity against Gram-positive than Gram-negative bacteria [11], because the plant extracts could infiltrate without disruption of the membrane and impact the inner cell structures, disrupting their processes [3].



(a) Control *B. subtilis* and *S. aureus*

Figure 1 PI-staining density plots of *B. subtilis* and *E. coli*. Control treatments of both bacteria (a), *B. subtilis* treated with cotyledon extract (b) and pericarp extract (c), and *S. aureus* treated with pericarp extract (d) at 0.5 MIC, 1 MIC, and 2 MIC concentrations for 3 and 6 h. Cells were categorized into 4 subpopulations: lower left (viable cells), lower right (membrane-damaged cells), upper left (injured cells), and upper right (dead cells).



(b) *B. subtilis* treated with cotyledon extract

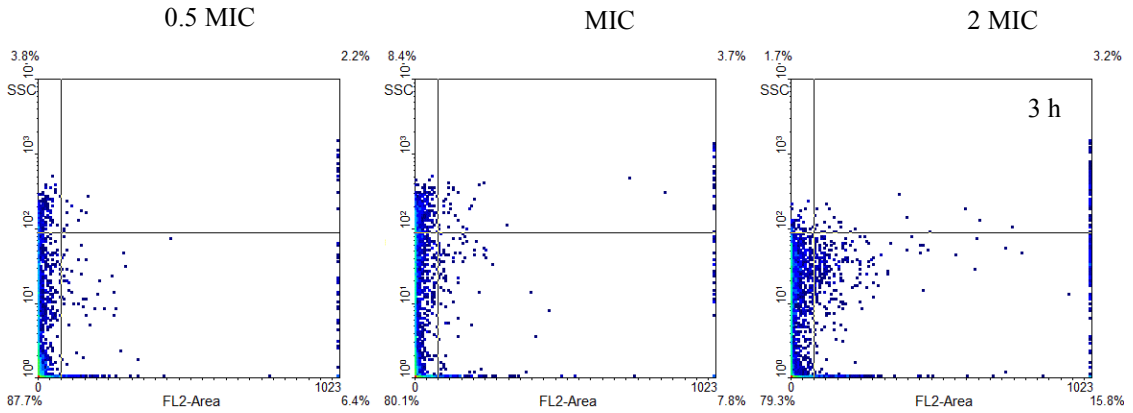
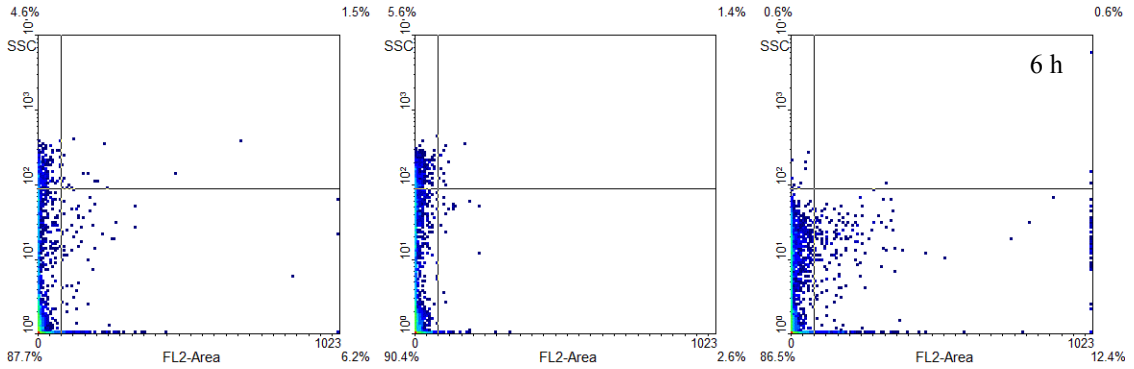
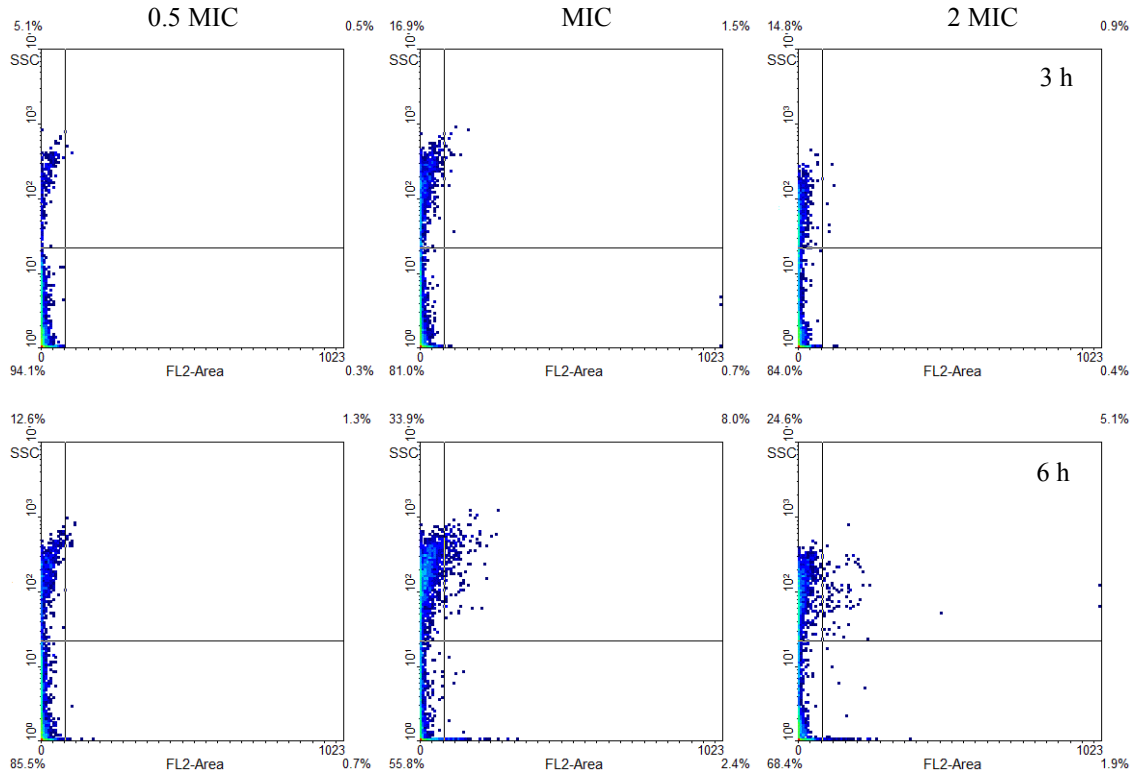


Figure 1 (cont.) PI-staining density plots of *B. subtilis* and *E. coli*. Control treatments of both bacteria (a), *B. subtilis* treated with cotyledon extract (b) and pericarp extract (c), and *S. aureus* treated with pericarp extract (d) at 0.5 MIC, 1 MIC, and 2 MIC concentrations for 3 and 6 h. Cells were categorized into 4 subpopulations: lower left (viable cells), lower right (membrane-damaged cells), upper left (injured cells), and upper right (dead cells).



(c) *B. subtilis* treated with pericarp extract



(d) *S. aureus* treated with pericarp extract

Figure 1 (cont.) PI-staining density plots of *B. subtilis* and *E. coli*. Control treatments of both bacteria (a), *B. subtilis* treated with cotyledon extract (b) and pericarp extract (c), and *S. aureus* treated with pericarp extract (d) at 0.5 MIC, 1 MIC, and 2 MIC concentrations for 3 and 6 h. Cells were categorized into 4 subpopulations: lower left (viable cells), lower right (membrane-damaged cells), upper left (injured cells), and upper right (dead cells).

Granular integrity and membrane permeability of bacteria

Extracts of *V. diospyroides* fruit were tested at various concentrations, observing the mortality rates of *B. subtilis* and *S. aureus* and other response patterns, to assess the antibacterial actions of the extracts and their dependence on dose level and exposure time (concentration and incubation time). The responses of individual bacteria were observed via FACS analysis. *B. subtilis* and *S. aureus* were treated with the extract concentrations 0.5 MIC, 1 MIC, and 2 MIC for 3 and 6 h, and the cells penetrated and those that lost granularity were counted. The density plots in **Figure 1** represent the bacteria counts, showing PI (X-axis) and SSC (Y-axis). The bacteria clustered into 4 subpopulations: PI-negative and SSC-negative (PI-, SSC-) representing viable bacteria, PI-positive and SSC-negative (PI+, SSC-) representing membrane-damaged bacteria, PI-negative and SSC-positive (PI-, SSC+) representing injury or loss of granularity, and PI-positive and SSC-positive (PI+, SSC+) representing dead cells (including those that lost granularity and had membrane damage). Membrane-damaged cells, injured cells and dead cells were counted as responders to the extracts.

In the negative control treatments of *B. subtilis* and *S. aureus* with 10 % DMSO, more than 80 % of the cell populations were viable. With 3 h of incubation, the fractions of responder *B. subtilis* cells were 20.3-32.0-33.9 % in the order of increasing doses (**Table 2**). Interestingly, at 6 h of incubation, the fraction of responder *B. subtilis* cells increased (10.1-11.6-15.4 %, by dose levels). At any fixed treatment time, the fraction of membrane-damaged or injured cells showed inconsistently with dose level. Thus, the cotyledon extract induced cell deaths to *B. subtilis* in a dose-dependent manner. On the other hand, by dose level, only the fraction of membrane-damaged *B. subtilis* cells was induced by pericarp extract at 3 h of incubation (6.4-7.8-15.8 %). Interestingly**,** with increasing time of incubation, the overall fraction of responder *S. aureus* cells at 0.5, 1, and 2 MIC increased dramatically, and consistently with time level (5.9 - 14.6, 19.1 - 44.3, and 16.1 - 31.6 %, respectively). The pericarp extract had a time-dependent effect, seen in the fraction of responder cells. All responder cells of *S. aureus* were inconsistently induced by this extract (**Figure 1** and **Table 2**). The response pattern of bacteria to the plant extracts proceeded in stages: the cells initially lost granularity and subsequently lost membrane permeability, leading eventually to death. The antibacterial effects of cotyledon and pericarp extracts were observed by the conventional plate count technique, as the number of viable cells decreased with extract concentration. However, prolonged treatment times (from 3 to 6 h) did not improve the antibacterial effects of the extracts (data not shown).

Using fluorescent dyes in flow cytometry provides bacterial response patterns by informing about cell physiology and enabling counts of viable cells, injured cells, or damaged cells and dead cells [7,9]. In this study, we demonstrated bacterial responses in the counts of 4 subpopulations, namely viable cells, membrane-damaged cells, injured cells, and dead cells. The effects of an antibacterial agent on the various subpopulations within a bacterial culture are important [1]. The PI is normally used to evaluate bacterial membrane permeability, because loss of membrane, or damage to it, allows the PI to enter the cell, where it binds to nucleic acids [4]. On the other hand, the high SSC counts after exposure to the extracts might indicate interference with intracellular functions. This could be related to Programmed Cell Death (PCD) of bacteria after exposure to antibiotics and self-digestion of the cell wall [12]. Unfortunately, little is known about the role of *V. diospyroides* fruit extract in the PCD of bacteria. If the plant extract acts as a DNA-damaging agent, it would cause increased SSC intensity in the FACS profile. After DNA damage, cell division will be inhibited, followed by the activation of various lysis-induced proteins such as autolysin [13]. The subsequent cell wall and membrane digestion will then lead to the death of the bacterium. The pattern of this chain of events, observed by the FACS method, would show increasing PI-intensity after SSC increase. It is noted that, while DNA damage may lead to death, alternatively, the damaged DNA might be repaired if the DNA-damaging agent becomes ineffective. The injured bacteria could then recover, and proliferate by cell division. It is important to observe the various subpopulations of a bacterial culture, and consider the recovery of injured cells. In further experiments to continue the work reported here, the physiological roles of proteins or genes related to PCD could be studied in treated bacteria, and the mechanism of cell killing by the plant extracts could be further clarified.

Table 2 The acetone extracts of *V. diospyroides* fruit at 0.5 MIC, 1 MIC, and 2 MIC were cytotoxic to bacterial cells, with different modes of action. The cell count fractions in each response to treatment were determined by the FACS method.

Treatments		Response pattern of *B. subtilis* to both extracts				
		Non-responder (viable)	Membrane-damaged cells	Injured cells (lost granularity)	Dead cells	Total of responder cells
0 h	10 %DMSO	82.5	16.5	0.2	1.0	17.7
	Ethanol	65.7	33.5	0.1	0.7	34.3
	OTC	78.2	21.0	0.1	0.7	21.8
3 h	10 %DMSO	76.4	21.3	0.1	2.2	23.6
	Ethanol	65.0	32.2	0.0	2.8	35.0
	OTC	61.4	35.3	0.2	3.2	38.7
cotyledon	0.5 MIC	79.7	11.3	2.4	6.6	20.3
	MIC	68.0	21.2	1.7	9.1	32.0
	2 MIC	66.1	16.5	3.6	13.8	33.9
pericarp	0.5 MIC	87.7	6.4	3.8	2.2	12.4
	MIC	80.1	7.8	8.4	3.7	19.9
	2 MIC	79.3	15.8	1.7	3.2	20.7
6 h	10 %DMSO	77.4	20.6	0.2	1.9	22.7
	Ethanol	33.3	61.1	0.1	5.6	66.8
	OTC	62.9	32.9	0.3	4.0	37.2
cotyledon	0.5 MIC	89.9	7.6	0.4	2.1	10.1
	MIC	88.5	7.2	1.2	3.2	11.6
	2 MIC	84.6	7.7	2.3	5.4	15.4
pericarp	0.5 MIC	87.7	6.2	4.6	1.5	12.3
	MIC	90.4	2.6	5.6	1.4	9.6
Treatments		**Response pattern of *S. aureus* to both extracts**				
		Non-responder (viable)	**Membrane-damaged cells**	**Injured cells (lost granularity)**	**Dead cells**	**Total of responder cells**
0 h	10 %DMSO	99.6	0.2	0.1	0.0	0.3
	Ethanol	35.5	61.9	0.1	2.5	64.5
	OTC	95.6	3.5	0.2	0.7	4.4
3 h	10 %DMSO	99.6	0.4	0.0	0.0	0.4
	Ethanol	44.3	53.7	0.0	2.0	55.7
	OTC	79.1	19.9	0.2	0.9	21.0
pericarp	0.5 MIC	94.1	0.3	5.1	0.5	5.9
	MIC	81.0	0.7	16.9	1.5	19.1
	2 MIC	84.0	0.4	14.8	0.9	16.1
6 h	10 %DMSO	99.3	0.6	0.0	0.0	0.6
	Ethanol	35.5	61.9	0.1	2.5	64.5
	OTC	81.9	16.7	0.3	1.1	18.1
pericarp	0.5 MIC	85.5	0.7	12.6	1.3	14.6
	MIC	55.8	2.4	33.9	8.0	44.3
	2 MIC	68.4	1.9	24.6	5.1	31.6

Conclusions

The present study demonstrated the FACS method in determining patterns of bacterial responses, including intracellular organelle interference and membrane loss by select plant extracts. The FACS analysis appears to be a simple and appropriate technique to observe the physiological responses of individual bacteria, giving counts across a population sample. The study also corroborated the antibacterial efficacy of *V. diospyroides* fruit extracts, and encourages future development of antimicrobial therapies based on these extracts.

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