RAPID SCREENING OF THE ANTIMICROBIAL ACTIVITY
OF EXTRACTS AND NATURAL PRODUCTS

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A spectrophotometric method has been developed for the rapid measurement of the antimicrobial activity of natural products, including crude extracts or pure materials. The assay depends on the measurement of non-specific esterase activity using fluorescein diacetate (FDA) hydrolysis in broth cultures of microbes after they have been treated with test compounds. The assay is accurate, reproducible and economical in both time and materials. The speed and economy of the method make it suitable for the rapid screening of many samples and the bioassay directed purification of antimicrobial substances. The assay can also be used with a wide variety of micro-organisms since most micro-organisms are FDA positive. Applications are described in the fields of marine natural products chemistry and essential oils research.

The objective of this study was to develop a simple, rapid, inexpensive, accurate and reproducible method for the determination of the minimum inhibitory concentration (MIC) of large numbers of test samples ranging from crude extracts or fractions of natural products to essential oils or pure substances.

Methods used for the determination of the MIC are varied although they can be grouped into either a single disc diffusion assay (BAUER et al., 1966) or the dilution plate/broth methods (HALTALIN et al., 1973). The former relates the zone of inhibition produced to the varying concentration of a test compound applied on to a paper disc for the determination of the MIC. There are several problems associated with the disc diffusion method for comparing the relative antimicrobial activity of different compounds. The assay does not always give a sharp demarcation between bacterial growth and inhibition. The time taken for incubation (18~24 hours) may render it unsuitable for volatile or unstable antimicrobial agents. The zones of inhibition may only be compared among antimicrobial agents with similar physical properties such as diffusion rates in agar, volatility or solubilities in aqueous solutions.

The dilution plate/broth group of methods measure the bacteriocidal/bacteriostatic effect of a potential antibiotic at a series of concentrations on a given microbial population. While being accurate, these methods are laborious due to the inclusion of viability counts in the assay and are liable to contamination of either the broth or the viable count agar plates or from test samples that often cannot easily be sterilised. Again, the time period involved (1~2 days) can be a major drawback.

The method described here is ideal for implementation in a chemistry laboratory by non-microbiologists, can be run under non-aseptic conditions, requires only small amounts of materials and gives results in two hours.

Non-specific esterases occur widely amongst microbes (LUNDGREN, 1981) and have been shown to hydrolyse colourless fluorescein diacetate (FDA) to fluorescein, a yellowish green compound, which absorbs strongly in the visible (λ_{max} 498 nm; ε 1 × 10^5, methanol-potassium hydroxide; GRASSELLI and RITCHEY, 1975). FDA hydrolysis/staining has found application in the determination of microbial populations

growing on plant tissues (SWISHER and CARROLL, 1980), microbial cultures (SUGAR *et al.*, 1983), textiles (McCARTHY, 1987), soil profiles (FEDERLE *et al.*, 1990) and sea water (GILBERT *et al.*, 1992).

SWISHER and CARROLL (1980) demonstrated, by spectrophotometric means, that the amount of fluorescein produced by hydrolysis of FDA provided a good approximation of the cumulative biomass of fungi, bacteria and algae growing on Douglas fir (*Pseudotsuga minziesii*) foliage. The fluorescein absorbance was directly proportional to the microbial population and a standardised method was developed to determine microbial biomass. SUGAR *et al.* (1983) used fluorescein diacetate staining of live cells to estimate standard colony forming units (viable counts) in pure microbial cultures. Similarly, FEDERLE *et al.* (1990) while investigating the vertical distribution of microbial biomass in soil profiles, found that the FDA hydrolysis was as accurate as the incorporation of radio-labeled thymidine into microbial DNA in estimating total microbial biomass. GILBERT *et al.* (1992) have recently shown that fluorescein is released quantitatively as a function of the number of cells of the marine micro-alga, *Tetraselmis suecica*. A rapid ecotoxicological test method was developed to measure the effect of various environmental toxins (*e.g.* insecticides, heavy metals, *etc.*) based on the measurement of algal esterase activity using FDA hydrolysis.

The widespread acceptance of FDA hydrolysis as a measurement of microbial viability suggested to us the possibility that FDA hydrolysis by microbes could be used as a sensitive bioassay method for the determination of MIC's on pure microbial cultures.

Materials and Methods

Chemicals and Materials

Fluorescein diacetate (FDA) and penicillin G (potassium salt) were obtained from Sigma Chemicals. Hospital grade hibitane from ICI Operations, Australia and chloramphenicol from Boehringer Mannheim, Germany. Tetracycline, glucose, peptone, tryptone, agar, yeast extract and 12.7 mm sterile paper discs (SCHLEICHER and SHUELL) were purchased from Bacto Laboratories Ltd., Australia. High cineole *Eucalyptus* oil was purchased from Felton and Bickland Ltd. Moorabbin, Australia and the tea-tree oil were a blend of commercial *Melaleuca alternifolia* essential oils obtained from Bronson and Jacobs, Sydney, Australia. Aplyroseol-1 was isolated from the marine sponge *Aplysilla rosea* (KARUSO *et al.*, 1986). Petri dishes (85 mm diameter) were purchased from Hardie Health Care Products, Australia. Disposable 96 well ELISA trays were purchased from Disposable Products Ltd., South Australia. Microbiological culture medium was obtained from Oxoid limited, Basingstoke, UK.

Test solutions of penicillin G, hibitane and tetracycline were prepared in sterilised milli-Q water. Chloramphenicol was dissolved in 5% aqueous ethanol, aplyroseol-1 was dissolved in 90% aqueous acetone and the essential oils were emulsified with polyoxyethylenesorbitan monooleate (Tween 80; Sigma) in a ratio of 5:1.

Organisms and Culture

The microorganisms used were *Escherichia coli* (NCTC 8196), *Staphylococcus aureus* (NCTC 4163), *Pseudomonas aeruginosa* (NCTC 6749) and *Candida albicans* (NCTC 3642). These particular strains were selected because they are used routinely in the testing of disinfectants (OLSEN, 1979). Stock cultures of these microorganisms were maintained at $-78°C$ in glycerol (15%) (JONES *et al.*, 1984). Cultures were prepared from stock cultures by streaking onto plate count agar (PCA) for bacteria and Sabouraud dextrose agar (SDA) for the yeast *C. albicans*. After an overnight incubation, a single colony was used to inoculate sterile broth. For the bacteria, this broth consisted of glucose (1 g), yeast extract (2.5 g) and tryptone (5 g) dissolved in distilled water (1 liter). For *C. albicans*, the broth consisted of peptone (10 g) and glucose (40 g) in water (1 liter). Broths were transferred into 25 ml McCartney bottles and sterilised in the autoclave (121°C; 16 minutes). Inoculated broths were placed on an orbital shaker (150 rev/minute) and incubated

overnight (30°C) by which time the microbial culture had attained an absorbance of 0.8 ~ 1.1 at 600 nm. The microbial cultures were diluted to an absorbance of 0.11 ~ 0.12 for the bacteria and 0.20 for *C. albicans*, corresponding to an approximate concentration of $10^6 \sim 10^7$ colony forming units (CFU)/ml and incubated for a further 15 minutes to permit the test microorganisms to enter into early exponential growth phase.

MIC Determinations

(i) Disc Diffusion Method

Disc diffusion assays were carried out using the method of BAUER *et al.* (1966) as modified by WILKINS *et al.* (1972). Briefly, a lawn of microorganisms was prepared by pipetting and evenly spreading 50 μl of overnight cultures (concentration $10^6 \sim 10^7$ CFU/ml) onto agar set in petri dishes. PCA agar was used for the bacteria, made by adding 15 g or agar to the liquid broth and SDA was used for the yeast, made in a similar fashion (WILKINS *et al.* 1972). A 12.7 mm sterile filter paper disc was placed at the centre of this agar plate to which 20 μl of the test compound dissolved in solvent was added. The plates were inverted and incubated for 18 hours, after which the diameter of the zone of inhibition around the discs were measured. Control experiments were performed where only equivalent volumes (20 μl) of solvents without added test compounds were applied to the paper discs. The MIC corresponded to the lowest concentration of the compound that produced a measurable zone of inhibition (BAUER *et al.*, 1966).

(ii) Broth Dilution Method

MIC determination using the broth dilution method was performed by a modified version of the method of HALTALIN (1973). To 175 μl of cultures (containing $10^6 \sim 10^7$ CFU/ml) contained in microcentrifuge tubes (1.9 ml; Eppendorf) 20 μl of the test compounds was added. The microcentrifuge tubes were incubated at 37°C on an orbital shaker (150 rpm). After incubation for 18 hours, CFU's were determined by preparing a 10-fold dilution series and spreading 20 μl of appropriate dilutions (four replicates) onto PCA for bacteria and SDA for *C. albicans*. The agar plates were incubated overnight (37°C) and CFU counted using a Gallenkamp colony counter. The MIC corresponded to the minimum concentration of the compound that caused 99% cell inhibition with respect to the CFU's in a control which contained microbial cultures and sterile distilled water or solvent replacing the test compound.

(iii) FDA Method

Each well of a 96 well ELISA tray was filled with 175 μl of an exponentially growing culture ($10^6 \sim 10^7$ CFU/ml). To each well, 20 μl solution of each concentration of the test substance, or the appropriate solvent as control, was added. The ELISA trays were incubated for 40 minutes before FDA (5 μl of a 0.2% w/v solution in acetone) was added. Incubation was continued for a further 90 minutes and the resulting green colour from the hydrolysis of FDA was measured at 490 nm (referenced to 630 nm) and blanked against control wells containing microbial cultures only, using an MR7000 automatic ELISA tray reader from Dynatech Laboratories.

To determine the percentage of cells killed under our assay conditions, we serially diluted cultures on both sides of the MIC that had been treated with varying amounts of penicillin G, aplyroseol-1 or tea-tree oil. The diluted cultures were plated on agar and after incubation, counts of visible growing colonies were performed (colony forming units).

The FDA method was standardised for cell concentrations of $10^6 \sim 10^7$ CFU/ml. The optimum FDA concentration (*i.e.* 2 mg/ml) was determined by treating 175 μl cultures of *E. coli* and *S. aureus* (OD 0.11) with 5 μl of FDA in acetone where the FDA concentration in acetone varied from 1 ~ 10 mg/ml. These solutions were incubated and their absorbance measured at ten minute intervals.

The first incubation time (*i.e.* 40 minutes) was determined for *E. coli* and *S. aureus* by administering 20 μl of lethal doses of penicillin G (100 μg/ml), aplyroseol-1 (1,000 μg/ml) or tea-tree oil (10,000 μg/ml) to 175 μl of cultures followed by the addition of FDA (5 μl; 2 mg/ml) at various times. Incubation was continued for a total time of 90 minutes after which absorbance of fluorescein was measured.

The second incubation time (90 minutes) was optimised by measuring the hydrolysis rate of FDA (5 μl of 2 mg/ml solution) by cultures of *E. coli*, *S. aureus*, *P. aeruginosa* and *C. albicans*. Cultures (175 μl) were treated with acetone only (20 μl) and absorbance of fluorescein produced from FDA measured and compared with those of microbial cultures devoid of acetone; to ensure there was no effect of the solvent on bacterial growth. Absorbance readings were taken at 490 nm (fluorescein absorption maxima) and were referenced to absorbance at 630 nm. This procedure eliminated the absorbance due to an increase in cell

numbers during the course of the experiment.

All experiments were run in triplicate. The data shown in Figs. 1~4 are the means with standard deviation of mean as error bars.

Results and Discussion

Protocol Development
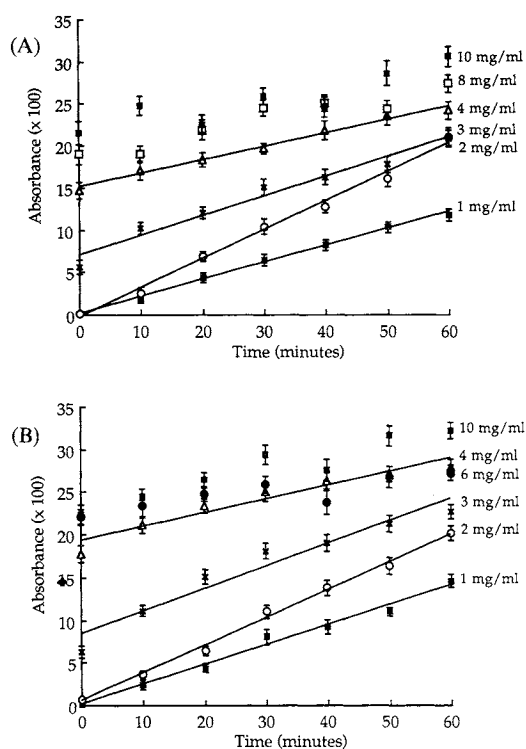
(a) FDA Concentration

For a particular concentration of microorganism, there will be an optimum concentration of FDA that will produce the maximum rate of fluorescein production. Knowing this concentration would allow us to keep the assay time to a minimum. The FDA concentration was optimised by studying the hydrolysis rate of varying concentrations (1 ~ 10 mg/ml) of FDA in 5 μl by cultures of E. coli (Fig. 1A) and S. aureus (Fig. 1B). For FDA concentrations of 1, 2, 3 and 4 mg/ml, the rate of hydrolysis was linear for both E. coli and S. aureus. These results are in agreement with previous findings on the hydrolysis of FDA by other microorganisms (SWISHER and CAROLL, 1980; GILBERT et al., 1992). However, 6, 8 and 10 mg/ml concentrations of FDA gave non linear results over time (Figs. 1A and 1B). In addition, at t = 0, the time just after the addition of FDA, 3 ~ 10 mg/ml concentrations of FDA gave absorbance of greater than 0.05. The non-zero values at t = 0 and the non linear absorbance at higher concentrations of FDA (6, 8 and 10 mg/ml) were related to the insolubility of FDA in the culture broth as experiments using cultures with final concentration of FDA above 0.050 mg/ml were visibly turbid. This turbidity resulted in poor transmittance of light (hence non-zero readings at t = 0). The 2 mg/ml solution of FDA (final concentration of 0.050 mg/ml) gave the steepest slope; 0.35 for E. coli and 0.33 for S. aureus, corresponding to the greatest rate of fluorescein production. The lower concentration (1 mg/ml) resulted in a slower production of fluorescein (slope = 0.23 for E. coli and 0.22 for S. aureus).

All further experiments were conducted by adding 5 μl of a 2 mg/ml solution of FDA in acetone to 175 μl of culture.

(b) First Incubation Time

The first incubation time refers to the time allowed between the addition of the test compound (or mixture) to the culture and the addition of FDA.

Fig. 1. Hydrolysis of different concentrations of FDA by E. coli (A) and S. aureus (B) over time.

Cell concentrations were approximately $10^6 \sim 10^7$ cells/ml at the start of the assay. 5 μl of FDA solution in acetone was added at t = 0 and the absorbance at 490 nm (ref. 630 nm) was measured over time. Data points for concentrations over 4 mg/ml are not connected due to the non-linear results. {linear regression Fig. 1A 1 mg/ml, 0.996; 2 mg/ml, 0.994; 3 mg/ml, 0.969 and 4 mg/ml, 0.988; Fig. 1B 1 mg/ml, 0.987; 2 mg/ml, 0.996; 3 mg/ml, 0.941 and 4 mg/ml, 0.967}.

Fig. 2. Effect of a lethal dose of penicillin G (100 μg/ml), tea tree oil (10,000 μg/ml) and aplyroseol-1 (1,000 μg/ml) on esterase activity.

(×) Penicillin G, (○) tea-tree oil, (■) aplyroseol-1.

(A)

(B)
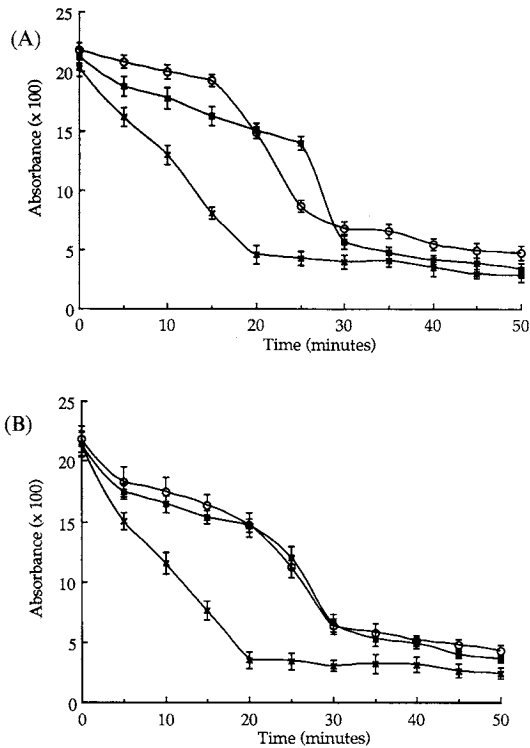
The time axis shows the time at which the FDA was added to cultures of E. coli (A) and S. aureus (B). The recorded absorbance readings were taken after 90 minutes of incubation from the addition of FDA.

Fig. 3. Rate of FDA hydrolysis at the optimised concentration of FDA (5 μl at 2 mg/ml) by cultures of E. coli, S. aureus, P. aeruginosa and C. albicans as measured by absorption at 490 nm.

(×) C. albicans, (○) P. aeruginosa, (□) E. coli, (●) S. aureus.

During this time, the potential antibiotic acts upon the cells and, if they are killed, residual esterase activity gradually disappears. The optimum time for addition of FDA would be just after residual esterase activity had dropped to insignificant levels. This time was estimated for E. coli and S. aureus by administering a lethal dose of penicillin G followed by the addition of FDA at various times. Incubation was continued for a total time of 90 minutes after which absorbance of fluorescein was measured. The results are shown in Figs. 2A and 2B. For both organisms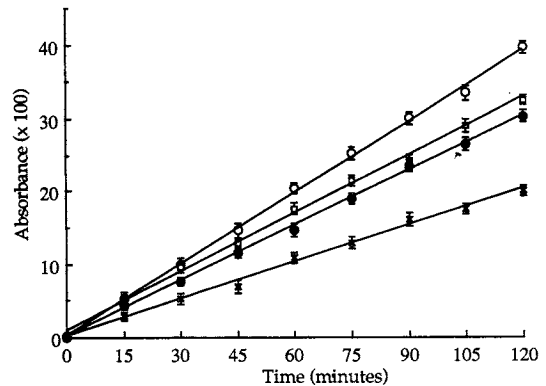, using penicillin G, esterase activity dropped quickly over the first 20 minutes. This experiment was repeated for a less active antibiotic (aplyroseol-1) and a weak antimicrobial (tea-tree oil). In both cases (Figs. 2A and 2B), a similar result was obtained as for penicillin except the weaker antimicrobial took longer to act. This resulted in an induction period of 15~25 minutes followed by a sharp decline in esterase activity. The lack of an induction period for penicillin G was probably due to the concentration of penicillin G used, which was greater than 50 times the lethal dose (HALTALIN et al., 1973). In comparison, aplyroseol-1 was used at approximately double the lethal dose and tea-tree oil which was used at 5 ~ 10 times the lethal dose (WALSH and LONGSTAFF, 1987). The slope of the decline would approximate the natural decay of esterase activity after all the cells had been killed. After a lethal dose of antimicrobial, both organisms studied had lost all their esterase activity after 30 minutes. To allow for some margin of error, a first incubation time of 40 minutes was chosen as optimal for all further experiments.

(c) Second Incubation Time

The time between addition of FDA and the final measurement of fluorescein absorption was optimised

Table 1. A comparison of MIC values (μg/ml) for various antibiotics and natural products by (i) FDA spectrophotometric method, (ii) broth dilution method and (iii) disc diffusion method.

Antibiotics	Minimum Inhibition Concentrations (μg/ml)											
	E. coli			S. aureus			P. aeruginosa			C. albicans		
	FDA	Broth	Disc	FDA	Broth	Disc	FDA	Broth	Disc	FDA	Broth	Disc
Penicillin G	2	2	4	2	2	4	5	5	7	—*	—*	—*
Tetracycline	6	5	8	5	4	6	6	5	9	20	15	30
Chloramphenicol	15	10	15	10	10	15	15	15	20	30	30	40
Hibitane (%)	0.2	0.15	0.3	0.2	0.15	0.3	0.3	0.3	1.2	0.15	0.15	0.5
Aplyroseol-1	690	690	950	690	500	895	895	895	1,500	—*	—*	—*
Tea tree oil	1,500	1,000	5,000	1,500	1,000	5,000	7,000	6,000	8,000	300	500	1,000
Eucalyptus oil	4,500	2,000	5,000	1,500	1,000	4,000	7,000	5,000	9,000	1,500	1,000	7,000

—* Denotes no effect.

for a minimum time yet provide significant fluorescein absorption in unaffected cultures. Fig. 3 shows the linear increase in absorption over time for the four organisms used in this study. At 90 minutes (chosen as the second incubation time), the absorbance range was $0.15 \sim 0.30$; an order of magnitude above the background (0.015) absorbance for cells not treated with FDA. In addition, the linear increase in fluorescein absorbance (linear regression (r^2) = 0.993 for C. albicans; 0.995 for S. aureus; 0.995 for E. coli and 0.994 for P. aeruginosa) with time and absorbance of 0 at $t = 0$ indicated that the chosen FDA concentration (5 μl at 2 mg/ml) was optimal for all four microorganisms.

Minimum Inhibitory Concentration

The MICs obtained by the FDA spectrophotometric method were compared to MICs as obtained by the disc diffusion assay method and the broth dilution method. MICs for the antibiotics, antiseptics and the natural products active against E. coli, S. aureus, P. aeruginosa and C. albicans as determined by all the three methods are given in Table 1.

Comparison of Disc Diffusion, Broth Dilution and FDA
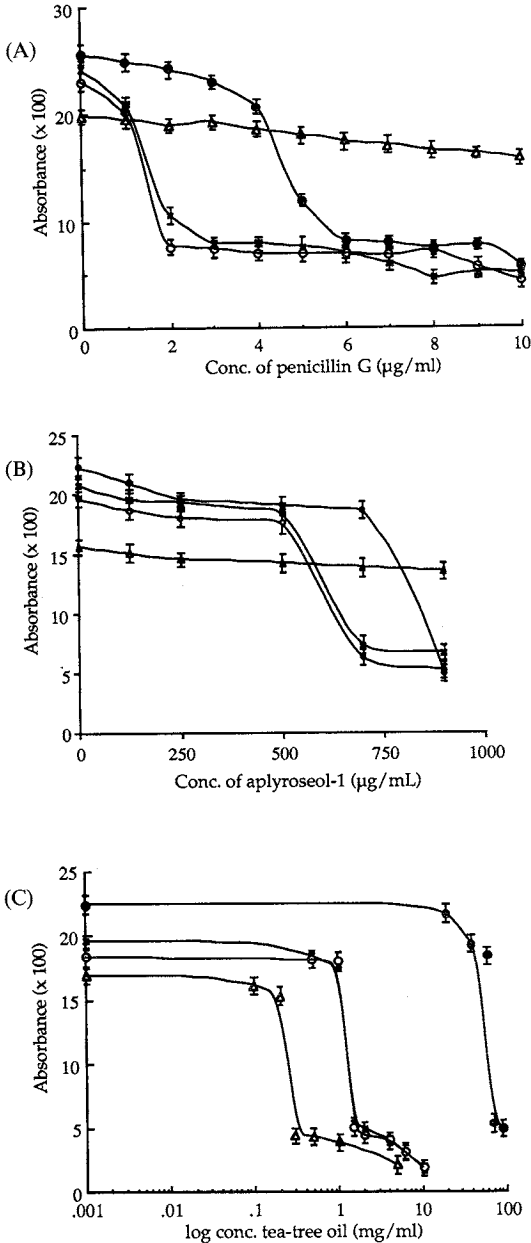### Methods for Determining MIC

Accurate determinations of MICs require a sharp cut off point between microbial growth and inhibition. With both the broth dilution (Fig. 5) and the FDA hydrolysis method (Fig. 4) a sharp cut off point is evident allowing for a precise determination of the MIC. At this cut off point there is at least a 99% loss of viability, typically greater than 99.9%. With the disc diffusion assay it is often difficult to determine the exact MIC. This is because with many compounds, particularly volatile compounds, there is no clear cut off point between inhibition and growth on the agar plate. This can lead to subjective interpretations.

The broth dilution technique can be regarded as the standard method for determining MICs against which all other methods should be compared. The disc diffusion assay is frequently used since it is simpler, cheaper, and more convenient than the broth dilution technique. With commercially available antimicrobials (penicillin G, tetracycline, chloramphenicol and hibitane) similar MIC values were obtained by the disc diffusion, broth dilution and FDA methods (Table 1). It should be noted however, that the FDA method gave consistently more reliable MIC results than the disc diffusion method using the broth dilution technique as a standard.

With the natural products (aplyroseol-1, tea-tree oil and eucalyptus oil) tested considerably more variation was observed in the MICs obtained using different methods (Table 1). With these products the

Fig. 4. Determination of MIC of penicillin G (A), aplyroseol-1 (B) and tea-tree oil (C) against *E. coli*, *S. aureus*, *P. aeruginosa* and *C. albicans*.

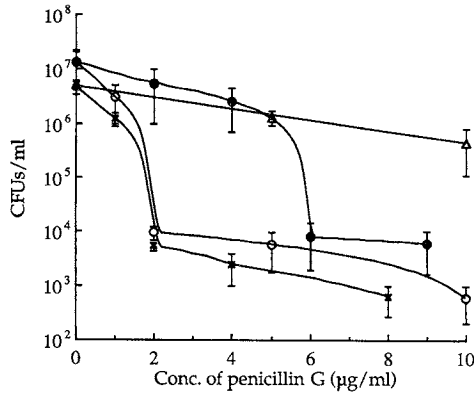(×) *E. coli*, (○) *S. aureus*, (●) *P. aeruginosa*, (△) *C. albicans*.

Fig. 5. Correlation of the number of viable cells or CFUs (colony forming units) for *E. coli*, *S. aureus*, *P. aeruginosa* and *C. albicans* when treated with varying concentrations of penicillin G.

(×) *E. coli*, (△) *C. albicans*, (○) *S. aureus*, (●) *P. aeruginosa*.

The close correlation with Fig. 4A is taken as evidence of the equivalence of our FDA assay and the standard broth dilution method.

MIC is defined here as the concentration at which there is a sharp decline in absorbance value.

disc diffusion assay consistently over estimated the MIC required compared to the broth dilution assay and FDA assay. This overestimation was as high as five fold for tea tree oil. The reasons for this over-estimation probably reflect the various physical properties of the antimicrobial being tested, for example, its solubility, diffusibility in agar and volatility. With volatile antimicrobial agents much of the activity may be rapidly lost. The lack of a sharp cut off point between inhibition and growth on the disc diffusion plates also made the interpretation of the zones of inhibition rather subjective.

The MIC values obtained using the FDA method described are comparable with literature values. For example HALTALIN *et al.* (1973) found the MIC for penicillin G against *Staphylococcus* spp. (for $10^4 \sim 10^5$ viable cells) to be 1.25 units/ml, which would approximate $0.8 \mu g/ml$. The present study gave MIC values for penicillin G against *S. aureus* as $2 \mu g/ml$. The discrepancy can be attributed to two factors; firstly, we were using two orders of magnitude higher concentration of microbes ($10^6 \sim 10^7$; to facilitate fluorescein production) and, secondly, that a different strain of *S. aureus* was used in the present study.

MICs for various essential oils have been reported (BEYLIER, 1979; WALSH and LONGSTAFF, 1987) in the literature. The composition of *Melaleuca alternifolia* and *Eucalyptus* species essential oils used by BEYLIER (1979) were only partially defined. WALSH *et al.* (1987) did not mention the quantity nor composition of *Melaleuca alternifolia* essential oil in the Melasol (*M. alternifolia* oil and isopropyl alcohol blend) used. Therefore, MIC results obtained from our study could not be rigorously compared to these literature values. However, our results are similar to published values. For the essential oils, the disc diffusion assay method showed considerable variability in the MIC values obtained when compared to our FDA assay method or the agar/broth dilution methods (Table 1). This could be attributed to; (i) the effect of minor components in these essential oils, (ii) the volatility of the essential oils at 37°C (iii) diffusion rates in agar and (iv) the problem of the disc retaining some of the compounds. Due to these problems, the disc diffusion method is not suitable for quantitative antimicrobial testing of essential oils. In comparison, our FDA method consistently gave results comparable to the much more elaborate broth dilution method.

For the marine natural product (aplyroseol-1) the MIC, as determined by the FDA and the broth dilution methods, was the same for *E. coli* and *S. aureus* but differed by 1 dilution for *P. aeruginosa* (Table 1). Lack of activity against *C. albicans* (Fig. 4B) suggested a specific activity against prokaryotic cells. In comparison, hibitane, chloramphenicol and the essential oils affected all the organisms. Further studies on the mode of action of aplyroseol-1 and related compounds revealed by this work are under way in our laboratory.

The results obtained using our FDA method are consistent with the observation that penicillin G acts specifically on prokaryotic cells but has little effect on eukaryotic cells such as the yeast, *C. albicans* (Table 1). The results obtained with the FDA method also demonstrate that *P. aeruginosa* is more resistant to the antimicrobials used than the other bacteria tested. *P. aeruginosa* is generally more resistant to many antimicrobial agents due to the structure of its cell envelope (SAKAGAMI *et al.*, 1989).

Comparing the time taken for the assay to observe the MIC: The FDA method required 2 hours, the disc diffusion method required 18 hours and the broth dilution method required up to 36 hours. Furthermore, the use of a microtitre-plate reader and the 96 well ELISA disposable trays in the FDA method allowed for a large number of samples to be tested at a time. This resulted in considerable time savings and made the inclusion of controls (both positive and negative) and duplicates less of a chore.

The amount of antimicrobial substances required for the FDA assay is small. This can be particularly important if the antimicrobial is scarce; as is the case for many natural products, for example ant secretions (VEAL *et al.*, 1992). The disc diffusion and the broth dilution methods were found to be laborious in comparison and required more materials than the FDA assay method. The disadvantages associated with the disc diffusion and the broth dilution methods make them unsuitable to test large numbers of sample or where results are required in a matter of hours. Furthermore, the disc diffusion method is not suitable for MIC determination because zones of inhibition depend not only on the antimicrobial activity but also on the diffusion and stability characteristics of the test compound.

The speed, capacity and simplicity of this assay method allows chemists to assay many fractions on the same day without the usual 1~2 days delay encountered with other methods. The method is also robust and not subject to problems of contamination sometimes experienced with the broth dilution method.

We now use this method routinely for bioassay directed isolation of active components from marine invertebrates, glandular secretions of Australian social insects and African medicinal plants. The method is also being used to estimate the anti-fungal activity (against *C. albicans*) of tea-tree oils obtained from

new cultivars before commercial blends are formulated. Finally, accurate MIC determinations can be performed on the pure isolates which are directly comparable to standard broth/agar dilution methods and literature values.

One problem experienced with this method was related to the observation that the antibiotic chloramphenicol hydrolysed FDA. Thus, in the presence of chloramphenicol, samples of FDA slowly became green even in the absence of microorganisms. It was, however, possible to measure the MIC for chloramphenicol (Table 1) by subtracting the absorbance of fluorescein produced by controls devoid of cells. A second, more serious caveat is if the test substance or extract absorbs strongly at the same wavelength as fluorescein. This was encountered with some anthracyline compounds and no simple solution was found. However, even this problem could be overcome by using fluorescence instead of absorption in reading the assay. The use of fluorescence has the added advantage of increased sensitivity allowing even shorter incubation times and more accurate readings.

In conclusion, this method was found to be economical in both time and resources and it was possible to perform the assay on a large number of samples. It could also be used with a wide range of different microorganisms since most microorganisms will hydrolyse FDA (CHRZNOWASKI et al., 1984; LUNDGREN, 1981). The liquid sample preparations used allowed for maximum exposure of bacteria to both nutrients and the test compound(s). The FDA method displayed greater reproducibility than the disc diffusion assay method where there is always a danger of flooding the paper disc (TOAMA et al., 1979) or compounds evaporating or diffusing at different rates. In addition, the FDA hydrolysis assay technique could be adjusted to a micro-assay when very little of the antimicrobial is available or large numbers of samples need to be screened. The method could prove useful to both biologists and chemists but especially natural products chemists faced with assaying hundreds of column fractions in the bioassay directed isolation of natural products. Each could be simultaneously assayed for biological activity, within 2 hours. Accurate MIC values of pure isolated compounds could also be established by this method that are comparable to other methods and literature values.

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