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Screening of New Bioactive Materials from Microbial Extracts of Soil Microorganism (I) Antimicrobial Activity from 200 Samples Using Microdilution Assay

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The microdilution assay recommended by NCCLS (National Committee for Clinical Laboratory Standards) is one of the standardized methods of antibiotic susceptibility test. This method has been widely used clinically to obtain MIC values of antibiotics on pathogenic microorganisms. It is more convinient, rapid and simple to test many samples than other test methods such as agar diffusion assay and broth macrodilution assay. The screening of antimicrobial agents from microbial extracts is too laborious in its process. Therefore, a number of screening methods having more simple procedure have been developed. In our laboratory, we applied microdilution assay for screening the antimicrobial agents. This assay showed dose-response results and was more sensitive than disc diffusion assay in our system. We tested 200 samples of microbial extracts originated from 100 microbial strains and selected several samples as potential candidates. In this report, we show that the microdilution assay is more convenient method in screeing of antibiotic susceptibility than those previously reported.

Key words : Antibiotics, Susceptibility test, Microdilution assay, Secondary Metabolites, Soil microorganisms

INTRODUCTION

Microbial metabolites are rich sources for new potential therapeutic drugs (Sanglier *et al.*, 1996). Since the discovery and development of penicillin and subsequent antibiotics, numerous novel useful microbial products have been isolated (Yarbrough *et al.*, 1993). Most of microbial products developed to therapeutic drugs are secondary metabolites. Secondary metabolites synthesized by micoorganisms are not required for growth (Sanglier *et al.*, 1996). Although it is not known why microorganisms produce these, it is desirable to introduce as much metabolite diversity into a screening program as possible (Yarbrough *et al.*, 1993).

In the past, large number of organisms, mainly *Actinomycetes* and fungi, have been isolated and screened for valuable products (Vandamme, 1994). To date, over 7000 compounds have been detected from this source and over 1000 secondary metabolites were characterised from *Actinomycetales* during the period of 1990-1994, including derivatives of novel and known metabolites (Sanglier *et al.*, 1996). So soil samples are remained as the main source of *Actinomycetes* for screening, and interesting compounds have been isolated from *actinomyctes* strains sampled from the sea and sea environments (Franco *et al.*, 1991).

There are several reasons for the expanding the intensive research activities in this field. The need for less toxic, more potent antibiotics as well as non-antiinfectives, the evolving resistance to existing antibiotics, continued malarial resistance, and emergence of new viral diseases are some of the medical area that are going to pose a challenge to the therapeutics of the 1990s (Franco *et al.*, 1991). Especially, the resistance of pathogenic bacteria and fungi to antibiotics is serious problem in immunocompromised hosts. Some of the most important emerging pathogens having a serious potential to cause disease in the immunocompromised host include *Enterococcus* spp., *Streptococcus viridans*, Gram-positive bacilli, resistant Gram-negative organisms, *Stentrophomonas maltophilia*, resistant *Candida* spp., *Fusarium* spp., and resistant *Mycobacterium tuberculosis* (Bodey, 1995). The development of new drugs should be continued based on these re-

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quirements.

We tried to screen the antimicrobial substances from soil microorganisms. The several methods have been developed to test antimicrobial activity. In general, these methods can be categorized into three groups i.e. disc diffusion assay, dilution plate and dilution broth assay (Hindler, 1992). Recently the new method using fluorescein diacetate (Chand *et al.,* 1994) and flow cytometer (Ordonez *et al.*, 1993) have been introduced with simplicity and rapidity of handling in their process, and to generate more accurate and reproducible data. Also some commercial MIC systems, such as ET(E test), FAS (fastidious antimicrobial susceptibility panel), FOX (fox fastidious panel), BACTEC 460 have been widely used (Karen *et al.*, 1994 and Collins *et al.,* 1997).

Usually broth macrodilution assay and disc diffusion assay are the most general methods for antimicrobial susceptibility test. However, these methods are too laborious, unsuitable for volatile or unstable antimicrobial agents and only comparable among antimicrobial agents with similar physical properties (Chand *et al.*, 1994).

In broth dilution assay, the microdilution assay which is one of the standard methods recommended by NCCLS (National Committee for Clinical Laboratory Standards) using 96-well tray is simple, rapid and powerful method for susceptibility of antimicrobial agent and widely used to determine MIC (minimum inhibition concentration) or IC_{50} (50% inhibition concentration) value of antibiotics on pathogenic bacteria (Weber *et al.*, 1991, Jorgensen *et al.*, 1994) and pathogenic yeast (Sewell *et al.,* 1994). The correspondence of the results of this assay with other methods was reported previously in antimicrobial or antifungal susceptibility (Dowzicky *et al.*, 1994, Krisher *et al.*, 1994, Sewell *et al.*, 1994). Therefore, this microdilution assay was applied for screening the antimicrobial agents from microbial extracts of soil microorganisms because of its simplicity, rapidity and reproducibility.

We isolated microorganisms from soil samples and prepared assay samples by solvent extraction for screening the bioactive secondary metabolites. In this experiment, 200 samples were tested and several potent candidates against bacteria or yeasts found.

MATERIALS AND METHODS

Reagents

The standard antibiotics, ampicillin, gentamicin, streptomycin and amphotericin B were purchased from Sigma Chemical Co. (USA). The Ketoconazole was kindly donated from Dong-Il Pharm. Co., Ltd (Korea). Dimethylsulfoxide was obtained from Sigma Chemical Co. (USA). The microbial culture media were purchased from Difco Laboratories (USA). All other chemicals and reagents were reagent grade.

Organisms and Culture

The standard strains of microorganisms used for antimicrobial susceptibility test were *Escherichia coli* (KCCM 11234, ATCC 25922), *Staphylococcus aureus* (KCCM 12255, ATCC 29737) and *Candida albicans* (KCCM 11282, ATCC 10231) and were obtained from KCCM (Korean Culture Center of Microorganisms). The culture media used for growth of type strains were Müeller Hinton Broth (MHB) for bacteria and Sabouraud Dextrose broth for yeast. Agar medium was prepared by the addition of 1.5% or 2% agar to broth medium. The strains were cultured at 37°C for 24 or 48 hours. The stocks of standard strains were stored at -20°C in 20% glycerol solution.

Isolation of microorganisms from soil samples

The soil samples were collected from several sites of Korea, and isolated as shown in Table I. One gram of soil samples were heat-treated at 70~75°C for 30 min, dispersed in 10 ml of distilled water and mixed well for 10 min with vortex mixer. The solution was stood at room temperature for 10 min and diluted to 1/10 with distilled water. The 100 ml of diluted solution was inoculated on Actinomycetes Isolation agar (Difco Co., Cat. No. 0957-17-4) and incubated at 30°C for 7 days. After incubation the microbial colony were transferred to half strength of YEME agar medium (yeast extracts 2 g, malt extracts 5 g, glucose 2 g, distilled water 1000 ml, pH 7.0~7.4) and incubated at 30°C for 7 days. And then pure isolates were suspended in 20% glycerol solution and stored at -20 °C for further experiments.

Preparation of assay samples

The microorganisms isolated from soil were cultured in 50 ml of fermentation broth (glucose 15 g, soybean meal 15 g, NaCl 5 g, yeast extract 1 g, $CaCO_3$ 1 g, glycerol 2.5 ml, distilled water 1000 ml , pH 6.8~7.2) on a rotary shaker at 30°C, 150 rpm for 7 days. After incubation the whole broth was divided into broth and mycelium by centrifugation at 3,000 rpm for 15 min. The mycelium was extracted with 20 ml of methanol and also the 20 ml of culture supernatant was mixed with the same volume of ethylacetate with vortex mixer and centrifugated at 3,000 rpm for 5 min. The methanol layer from mycelium and the ethylacetate layer from broth were dried at 40 °C under reduced pressure with rotary evaporator. The dried extracts were dissolved in dimethylsulfoxide and

Table I. The sampling sites of soil and isolated strains

Sample number	Sampling site	isolates number	selected number
S003	Seo-shin in Kyunggi	36	31
S009	Chae-suk-kang in Kyuk-po, Chunbuk	15	2
S010	Pu-an in Chunbuk	60	31
S011	Mo-ak mountain provincial park	47	5
S014	Bi-dong in Chungnam	38	15
S016	Chil-gap mountain provincial park	18	1
S017	Chi-ak mountain national park	3	3
S020	So-bak mountain national park	5	5
S045	Boog-myun in Cheonan city	7	7
total		229	100

stored at -20°C.

Inoculum preparation

The inoculum preparation of *C. albicans* was followed by the method of Odds *et al.* (1995) with minor modification. The colonies of *C. albicans* were suspended in 50 ml of sabouraud dextrose broth and incubated for 12 hrs at 37°C by shaking at 150 rpm and the culture broth was dispensed to sterilized microtube by 300 μl as inoculum stock. The inoculum stocks were preserved at -20°C for further use. For two bacterial strains of *Escherichia coli* and *Staphylococcus aureus*, inoculum preparation was the same as above except using MHB as culture broth (Hindler, 1992).

To obtain the pre-culture time and the constant cell concentration, the growth curve of type strains were examined. The inoculum stock solution (200 μl) was inoculated in 20 ml of sabouraud dextrose broth (MHB in *E. coli* and *S. aureus*) and incubated for 24 hrs at 37°C by shaking at 150 rpm. The culture broth (500 μl) was harvested every 3 hrs and measured the optical density at 650 nm by ELISA reader (Molecular Dynamics. Co.).

Susceptibilities of antibiotics on standard strains by disc diffusion assay and microdilution assay

The susceptibilities of standard antibiotics on type strains were tested as following. In disc diffusion assay, the culture broth of test strains were diluted to 1/10 with fresh culture broth and further diluted to 1/10 with sterilized molten agar solution (0.5%) maintained at 45°C. The diluted solution (2.5 ml) was overlaid on an agar plate of the same culture medium. After the agar solution was solidified, the filter disc (9 mm) absorbed with standard antibiotics was loaded. The plate was incubated at 37°C for 24 hrs and the inhibition zone was measured.

In microdilution assay, the culture broth of *C. albicans* was diluted to 1/100 with RPMI 1640 (2% glucose, buffered with 0.165 M MOPS buffer, pH 7.0, without serum) (Odds *et al.*, 1995). The culture broth of *E. coli* and *S. aureus* were diluted to 1/10 with MHB and further diluted to 1/10 with CAMHB (cation-adjusted MHB, containing 1% phenol red in gram-negative bacteria and 1.6% bromocresol purple in gram-positive bacteria) (Hindler, 1992). The concentrations of calcium and magnesium in CAMHB were adjusted to 22.5 mg/L and 11.25 mg/L, respectively. The diluted inoculum solution (200 μl) was added to 96 well microplate and added standard antibiotics. The micro-plate was incubated at 37°C for 24 hrs and the absorbance at 650 nm was measured by ELISA reader.

Antimicrobial activity of soil microbial extracts with microdilution assay

In the case of bacteria the inoculum of standard strains were prepared by pre-incubation for 12 hrs in MHB as described above and diluted to 2/100 with 2x CAMHB. For assay, 100 μl of MHB was added to each wells of 96 well microplate and then assay samples were added and serially diluted to 1/8. The 100 μl of inoculum were added to each well and incubated at 37°C for 18~24 hrs. After incubation the O.D (optical density) value was measured by ELISA reader at 650 nm.

In the case of yeast, the inoculum of standard strain was prepared by pre-incubation for 12 hrs in sabouraud dextrose broth and diluted to 2/100 with RPMI 1640 medium. The RPMI 1640 medium (100 μl) was added to each wells of 96 well microplate. The assay samples were added and serially diluted to 1/8. Then 100 μl of inoculum was added in each well and incubated at 37°C for 18~24 hrs. After incubation the O.D (optical density) value was measured by ELISA reader at 650 nm. The results were represented by inhibition rate.

RESULTS

Isolation of microorganisms and preparation of assay samples

The *Actinomycetales* are good source for screening of antibiotic agents because about two-thirds of the naturally discovered antibiotics were produced by these strains (Franco *et al.*, 1991, Sanglier *et al.*, 1996). Since Waksman introduced *Actinomycetes*, specially *Streptomycetes*, into screening of new antibiotics in the early 1940s, the *Actinomycetes* recognized as the largest group of microbial producers of antibiotics. Also the *Streptomycetes* are still dominant microbial producers as well as *Actinomycetes* (Franco *et al.*, 1991; Okami *et al.*, 1988, Sanglier *et al.*, 1996). Therefore, the *Actinomycetes* group selected as the target family from soil in this study. We collected 11 soil samples from 9 sites in Korea. The total isolates of mi-

croorganisms were 206 strains (Table I). We selected 100 strains randomly from these isolates, cultured in fermentation broth and prepared assay samples as described above. Total 200 assay samples originated from 100 strains were prepared for screening of antimicrobial activity.

The growth curve and the cell concentration of test strains for inoculum preparation

To determine the pre-culture time, the growth curve of standard strains were obtained and calculated the cell number by CFU (colony forming unit). The optimal pre-culture time of standard strains observed in the growth curve of each strain and cell number calculated by CFU as shown in Fig. 1. The growth of standard strains were reached in logarithmic phase at 12 hrs and the cell number (CFU/ml) of *C. albicans, S. aureus* and *E. coli* were 5.6×10^8, 6.7×10^9 and 1.5×10^8, respectively. These cell concentrations are sufficient to test susceptibility by microdilution assay.

There are several variations in antibiotic susceptibility test. The end point definition, inoculum size, medium composition and length of incubation has significant effects on test results (Hindler, 1992, Peng *et al.*, 1993, Odds *et al.*, 1995, Pujol *et al.*, 1997). In this respect, Peng *et al.* (1993) emphasized that test parameters had significant effects included end point definition, starting inoculum size, medium composition, different buffer and length of incubation in broth macrodilution susceptibility test. But the pH of medium and incubation temperature had little or no systematic effect. In the respect of end point definition, when the starting yeast inoculumn was varied from 10^2 to 10^5 cells/ml, MIC results were increased according to their inoculum size. For MIC_{90} results, the differences were as much as >75,000 fold. But IC_{50} result showed very little differences. According to the test results reported by Odds *et al.* (Odds *et al.*, 1995), the lowest (10^3 cells/ml) and highest (10^6 cells/ml) initial cell concentrations were not suitable for susceptibility test because of their poor end point reproducibilities. But initial cell concentrations of 10^4 and 10^5 yeasts per ml both gave a resonably broad spread of susceptibility endpoints for all antifungal agents except ampotericin B.

Based on these results, the preculture time was determined to 12 hrs and initial cell concentration adjusted to 10^6 to 10^7 cells/ml.

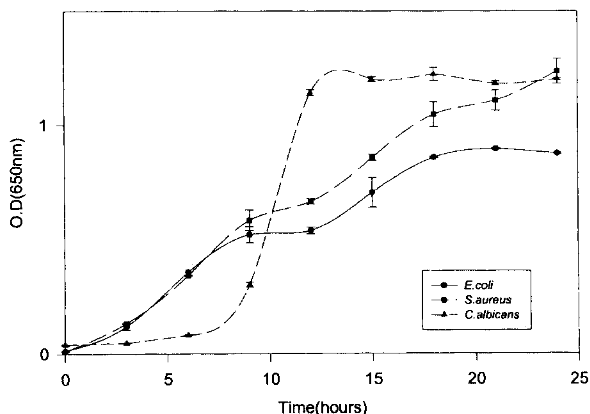

Fig. 1. The growth curve of tested strains.

Comparison of susceptibilities of antibiotics on standard strains between disc diffusion assay and microdilution assay

To compare the susceptibility between disc diffusion assay and microdilution assay, the dose-response activity test was performed using standard antibiotics in three strains of *C. albicans* (Table II), *E. coli* (Table III) and *S. aureus* (Table III). As shown in Table II, the inhibition zone of amphotericin B and ketoconazole in disc diffusion assay using *C. albicans* revealed 1 μg and 100 μg, respectively. However, in microdilution assay, the inhibition rate was 95% at 0.1 μg/ml of amphotericin B and 43% at 0.01 μg/ml of ketoconazole. It can be explained that the microdilution assay is more sensitive than disc diffusion assay by 10 or 1000 folds. Moreover, the same tendency of sensitivity was obtained in type strain of *E. coli* (Table III) and *S. aureus* (Table III). From these results, the microdilution assay is more sensitive and useful tool for the susceptibility test than others.

Antimicrobial activity of microbial extracts by microdilution assay

The overall test results are summarized in Table IV. No significant antimicrobial activity was observed in the tester strain of *E. coli,* a typical gram negative strain, except methanol extract of mycelium of RJ0252 and ethylacetate extract of culture broth of RJ0210, RJ 0230, RJ0231 and RJ0238.

However, the significant antimicrobial activity against the tester strain of *S. aureus,* a typical gram positive strain, was observed. The IC_{50} value of mycelial methanol extracts of RJ0002, RJ0172, RJ0189, RJ0193, RJ

Table II. The growth inhibition of *C. albicans* by filter disc assay and microdilution assay

dose (μg or μg/ml)	Amphotericin B		Ketoconazole	
	filter disc* assay	microdilution** assay	filter disc assay	microdilution assay
100	13.6	90	10.7	92
10	10.7	97	-	43
1	6.0	97	-	43
0.1	-	95	-	51
0.01	-	7.5	-	43
0.001	-	0	-	0

*the diameter of inhibition zone (mm).
**the inhibition rate (%)

Table III. The growth inhibition of *E. coli* and *S. aureus* by filter disc assay and microdilution assay

dose (mg or mg/ml)	Gentamicin		Ampicillin		Streptomycin	
	filter disc* assay	microdilution** assay	filter disc assay	microdilution assay	filter disc assay	microdilution assay
E. coli	9.6	92.8	22	100	6	95.6
1	5	94.9	16.5	100	2	95.4
0.1	1	94.1	11	100	-	76.1
0.01	-	85.6	2.5	100	-	5.7
0.001	-	5.1	-	66.9	-	0
0.0001	-	0	-	12.2	-	0.8
0.00001	18	80.4	32	95.9	15.5	81.5
S. aureus	13	81.5	29	97.9	11.5	74.4
1	8.5	76.2	23	98.4	5.5	81.0
0.1	3	69.6	15.5	77.2	1	35.1
0.01	-	4.2	3.5	39.9	-	14.3
0.001	-	0.6	-	25.9	-	32.1

*the diameter of inhibition zone (mm).
**the inhibition rate (%).

Table IV. The IC_{50} values (mg/ml) of microbiological extracts on standard strains

strain No.	IC_{50} value on *E. coli*		IC_{50} value on *S. aureus*		IC_{50} value on *C. albicans*	
	Methanol extracts	Ethylacetate extracts	Methanol extracts	Ethylacetate extracts	Methanol extracts	Ethylacetate extracts
RJ0001	0	0	0	0	<0.625	>2
RJ0002	0	0	<0.13	0	<0.625	<0.625
RJ0003	0	0	0	0	0	0
RJ0004	0	0	0	0	1.09	>2.5
RJ0005	0	0	0	0	0	0
RJ0006	0	0	0	0	0	0
RJ0007	0	0	0	0	0	>0.5
RJ0167	0	0	0	0	0	0
RJ0168	0	0	0	0	0	0
RJ0169	0	0	0	0	0	0
RJ0170	0	0	0	0	0	>0.25
RJ0171	0	0	0	0	0	0
RJ0172	0	0	0.12	<0.0631	>2.5	0.25
RJ0173	0	0	0	0	0	0
RJ0174	0	0	0	0	>5	<1
RJ0175	0	0	0	0	0	>0.5
RJ0176	0	0	0	0	0	0
RJ0177	0	0	10	0	0	>1
RJ0178	0	0	0	0	0	>5
RJ0179	0	0	0	0	2.5	<0.625
RJ0180	0	0	0	0	0	>0.5
RJ0181	0	0	0.2	0	0	0
RJ0182	0	0	>0.25	0	<0.625	>1
RJ0183	0	0	0	0	0	>1.25
RJ0184	0	0	0	0	0	>0.5
RJ0185	0	0	0	0	0	0
RJ0186	0	0	0	0	>10	>1
RJ0187	0	0	0	0	2.5	>0.1
RJ0188	0	0	0	0	0	0
RJ0189	0	0	<0.125	<0.016	<0.625	<0.063
RJ0190	0	0	0	0	>5	>1.5

Table IV. Continued

strain No.	IC_{50} value on *E. coli*		IC_{50} value on *S. aureus*		IC_{50} value on *C. albicans*	
	Methanol extracts	Ethylacetate extracts	Methanol extracts	Ethylacetate extracts	Methanol extracts	Ethylacetate extracts
RJ0191	0	0	0	0	<0.625	0.5
RJ0192	0	0	0	0	0.93	0
RJ0193	0	0	<0.125	<0.031	1.25	>1
RJ0194	0	0	0	0	0.625	0
RJ0195	0	0	0	0	0	0
RJ0196	0	0	0	0	2.5	>1
RJ0197	0	0	0	0	0.313	1
RJ0198	0	0	0	0	0	0
RJ0199	0	0	0	0	0	0
RJ0200	0	0	0	1.25	0	>5
RJ0201	0	0	0	0	0	0
RJ0202	0	0	0	0	0	>2.5
RJ0203	0	0	0	0	0	0
RJ0204	0	0	0	0	>2.5	0
RJ0205	0	0	0	1.25	0	>5
RJ0206	0	0	0	0	0	0
RJ0207	0	0	0	0	0	>1.5
RJ0208	0	0	0	0	0	>2.5
RJ0209	0	0	0	0	0	0
RJ0210	0	0.71	0	0	0	0
RJ0211	0	0	0	0	0	>1
RJ0212	0	0	0	0	0	>2.5
RJ0213	0	0	0	0	0	>0.0125
RJ0214	0	0	1.25	0	0	0
RJ0215	0	0	0	0	0	>0.25
RJ0216	0	0	0	0	0	>0.125
RJ0217	0	0	0	0	0	0
RJ0218	0	0	0	0	0	0
RJ0219	0	0	0	0	0	0
RJ0220	0	0	0	0	0	0
RJ0221	0	0	0	0	0	>0.125
RJ0222	0	0	0	0	0	0
RJ0223	0	0	0	0	>1	0
RJ0224	0	0	0	0	>0.325	0
RJ0225	0	0	0	0	0	0
RJ0226	0	0	0	0	0	0
RJ0227	0	0	0	0	0	0
RJ0228	0	0	0	0	<0.125	>0.85
RJ0229	0	0	0	0	0	0
RJ0230	0	>1.5	0.625	<0.187	<0.313	0
RJ0231	0	>1.25	0	0	0	0
RJ0232	0	0	0	0	>1.25	0
RJ0233	0	0	0	0	0	0
RJ0234	0	0	0	0	>0.5	0
RJ0235	0	0	0.625	1.5	0.038	>3
RJ0236	0	0	0	0	0	0
RJ0237	0	0	0	0	0	0

Table IV. Continued

strain No.	IC_{50} value on *E. coli*		IC_{50} value on *S. aureus*		IC_{50} value on *C. albicans*	
	Methanol extracts	Ethylacetate extracts	Methanol extracts	Ethylacetate extracts	Methanol extracts	Ethylacetate extracts
RJ0238	0	>1.25	0	0	0	0
RJ0239	0	0	0	0	0	0
RJ0240	0	>0.1	0	0	0	0
RJ0241	0	0	0	0	0	0
RJ0242	0	0	0	0	0	>1
RJ0243	0	0	0	0	0	0
RJ0244	0	0	0	0	0	0
RJ0245	0	0	0	0	0	0
RJ0246	0	0	0	0	0	0
RJ0247	0	0	>1.75	0	0	0
RJ0248	0	0	0	0	0	0
RJ0249	0	0	0	0	0	0
RJ0250	0	0	0.625	0	0	0
RJ0251	0	0	0	0	0	0
RJ0252	<0.313	0	<0.313	0	<0.313	0
RJ0253	0	0	0	0	0	0
RJ0254	0	0	0	0	0	0
RJ0255	0	0	0	0	0	0
RJ0256	0	0	0	0	0	0
RJ0257	0	0	0	0	0	0
RJ0258	0	0	0	0	0	0
RJ0259	0	0	0	0	>0.75	>0.5

0230, RJ0235, RJ0250 and RJ0252 showed lower than 1 mg/ml. The ethylacetate extracts from culture broth of RJ0172, RJ0189, RJ0193 and RJ0230 showed strong inhibitory activity with low IC_{50} value. Especially, the strong inhibitory activity i.e. below 0.016 mg/ml of IC_{50} was observed in both methanol and ethylacetate extracts of RJ0172, RJ0189, RJ0193 and RJ0230.

The tester strain *C. albicans* is one of the important pathogenic fungi. It has been reported that there is a relationship with immunodeficient syndrome such as AIDS (Georgopapadakou *et al.,* 1996). The mycelial methanol extracts of RJ0001, RJ0002, RJ0182, RJ0189, RJ0191, RJ0194, RJ0197, RJ0228, RJ0230, RJ0235 and RJ0252, and the ethylacetate extracts from culture broth of RJ0002, RJ0172, RJ0179 and RJ0189 showed strong antifungal activity against *C. albicans.* Especially, RJ0002 and RJ0189 showed very strong antifungal activity in both methanol and ethylacetate extracts against *C. albicans.*

DISCUSSION

The effective screening system should have sensitivity, selectivity and high efficiency (Yarbrough *et al.,* 1993). There are many assay systems for screening of antimicrobial agents from natural products but disc diffusion assay or broth macrodilution assay is the general test method.

The microdilution assay was adopted in this study as a screening method because it is very simple and rapid to test many samples, and convenient to read the result using ELISA reader in our laboratory. Also the amount of microbial extracts for the microdilution assay are small quantity compared with other assay systems. Because the amount of samples originated from microorganism is small, it is not sufficient to test in several assay system at the same time.

In this experiment, the results of microdilution assay is more sensitive than the disc diffusion assay. In general, disc diffusion assay can be affected by other factors such as inoculum size, growth rate, medium formulation, pH, incubation time, drug diffusion rate and agar depth (Hindler, 1991). So disc diffusion assay has the possibility to mislead and to loss an active ingredients by these factors. Therefore, it is suggested that the microdilution assay is more useful method for screening of antimicrobial metabolites than other methods.

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