ANTIMICROBIAL EFFECTS OF *BOESENBERGIA PANDURATA* AND *PIPER SARMENTOSUM* LEAF EXTRACTS ON PLANKTONIC CELLS AND BIOFILM OF ORAL PATHOGENS

SUWIMOL TAWEECHAISUPAPONG, SUPAPORN SINGHARA, PILANTHANA LERTSATITTHANAKORN* AND WATCHAREE KHUNKITTI**

Faculty of Dentistry, Khon Kaen University, Khon Kaen 40002, Thailand
**Faculty of Pharmacy, Mahasarakham University, Mahasarakham 44150, Thailand*
***Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand*

ABSTRACT

Increasing awareness of hazards associated with the use of antibiotic and chemical agents has accelerated investigations into plants and their extracts as new sources of antimicrobial agents. Therefore, this study aimed at determining the effects of oil and 95% ethanol extracts of *Boesenbergia pandurata* rhizomes and *Piper sarmentosum* leaf against four oral pathogens which were *Streptococcus mutans, Lactobacillus sp., Aggregatibacter actinomycetemcomitans* and *Candida albicans.* Employing the disc diffusion and broth microdilution methods, the results showed that *B. pandurata* oil (BPO) was the most effective extract against *C. albicans*. Time-kill assay with the BPO demonstrated killing of *C. albicans* at concentrations equal to 2 and 2.5 times the MIC. The times required to reach the fungicidal endpoint at 2 and 2.5 times the MIC were 60 and 44 min, respectively. In addition, our results also demonstrated that the BPO possesses potent anti- Candida biofilm activity *in vitro*. Therefore, the BPO could be considered as a natural antifungal agent against Candida infections and has significant potential for further investigation.

Keywords: *Boesenbergia pandurata*, *Piper sarmentosum*, Antimicrobial activity, biofilm, *Candida albicans*, bacteria.

INTRODUCTION

The increasing prevalence of multidrug resistant strains of microorganism and the recent appearance of strains with reduced susceptibility to antibiotics raises the specter of untreatable microbial infections and adds urgency to the search for new infection-fighting strategies. Many efforts have been made to discover new antimicrobial compounds from various kinds of sources such as micro-organisms, animals, and plants. One of such resources is folk medicines. Systematic screening of them may result in the discovery of novel effective compounds. In this study, volatile oil and ethanolic extracts of 2 plants, *Boesenbergia pandurata* and *Piper sarmentosum*, were screened for their antimicrobial effects against a range of oral microorganisms. All microorganisms used in this study are the most common pathogens associated several oral diseases, i.e., dental caries, periodontal disease and candidiasis (Rindum *et al.*, 1994; Henderson *et al.*, 2003; Seminario *et al.*, 2005).

Boesenbergia rotunda (L.) Mansf. (*syn*: *Boesenbergia pandurata* (Roxb.) Schltr.; local name: krachai), is a perennial herb of the family Zingiberaceae. The fresh rhizome is used in cooking, also in folk medicine as an aphrodisiac, and for the treatment of colic disorder. Previous investigations of the rhizomes of *B. pandurata* dealt with the constituents of the essential oil (Lawrence *et al.*, 1971); boesenbergin, cardamonin, pinostrobin (Jaipetch *et al.*, 1983), 5, 7-dimethoxyflavone, 1,8-cineole and panduratin (Pancharoen *et al.*, 1987). Jantan *et al.* (2003) found that the essential oil of *B. pandurata* was effective against three filamentous fungi (*Aspergillus niger*, *Aspergillus fumigatus* and Mucor sp.) and five strains of yeast (*Saccharomyces cerevisiae, Cryptococcus neoformans, Candida albicans, Candida tropicalis and Torulopsis glabrata*).

Piper sarmentosum Roxb. (*syn*: Piper rostratum Roxb.), a terrestrial herb of the piperaceae family, has been known locally as 'Cha-plu' and is widely distributed throughout Thailand, in the tropical and subtropical region of the world. The leaves of Chaplu are used as food and traditional medicine in Thailand and various countries (Saralamp *et al.*, 1996). The water extract of the whole plant showed a hypoglycemic effect in rats (Peungvicha *et al.*, 1998) and the methanolic extract of the leaves was found to possess a marked neuromuscular blocking activity in rat phrenic nerve-hemidiaphragm preparation (Ridtitid *et al.*, 1998). It was also reported that the administration of the crude extract of *Piper sarmentosum* in mature onset diabetic patients caused a reduction in the blood glucose level (Pongmarutai, 1989). Chaplu has also been used as a carminative, expectorant and to relieve muscle pains and coughs (Li, 1980). The plant has been shown to have antiplatelet aggregation (Han *et al.*, 1992) and antibacterial effects (Masuda *et al.*, 1991). In

Corresponding author: Fax: 66-4320-2862, e-mail: suvi_taw@kku.ac.th

addition, the chloroform and methanol extracts of the leaves showed considerable antiplasmodial activity against *Plasmodium falciparum* and *Plasmodium berghei* parasites (Najib Nik *et al.*, 1999). Previously isolated constituents from the leaves of *Piper sarmentosum* were 1-allyl-2,6-dimethoxy-3,4-methylenedioxybenzene, 1-allyl-2,4,5-trimethoxybenzene, 1-(1-E-propenyl)-2,4,5-trimethoxybenzene and 1-allyl-2-methoxy-4,5-methylene-dioxybenzene (Masuda *et al.,* 1991).

However, there is no report regarding the antimicrobial effects of these plants against oral microorganisms, especially for dental caries and periodontal pathogens. Therefore, the aim of the present work was to evaluate the antimicrobial potentiality of oil and ethanolic extracts of *B. pandurata* rhizomes and leaves of *P. sarmentosum* against the growth of several oral pathogenic microbial strains. Then the most effective extract was selected to examine the killing patterns by time-kill assay and to determine its inhibitory effects on biofilm formation of the most susceptible microorganism.

MATERIALS AND METHODS

Plant materials and preparation of extracts

Yellow rhizomes of *B. pandurata* and leaves of *P. sarmentosum* were locally collected in Khon Kaen province and voucher specimens were deposited at the Department of Biology, Faculty of Science, Khon Kaen University, Thailand. Plant materials were washed, cut into small pieces and dried in an oven at 55^{o}C for 10 h.

For oil extraction, each plant material was subsequently submitted to hydro-distillation. The distillation unit consisted of a retort (boiling flask), a condenser and a decanter (receptive flask). Plant materials were immersed in double their volume of distilled water and boiled. The condensate was collected in the receptive flask, and the oil was removed with a Pasteur pipette and stored at a temperature of 4^{o}C in well-filled, tightly closed glass vials wrapped in aluminum foil to avoid exposure to light and oxygen (Guenther, 1949).

For ethanol extraction, each plant material was subsequently macerated in 95% ethanol, at the ratio of 1:5 and 1:20 for *B. pandurata* rhizomes and leaves of *P. sarmentosum*, respectively. The supernatants were collected after 7 days. Whole ethanol extract from each plant was filtered and evaporated to dryness under a low pressure with a rotary evaporator, at 55^{o}C. The extracts were then stored at 4^{o}C until use. Ethanol extraction of *B. pandurata* and *P. sarmentosum* gave 5.87 and 11.87% yield, respectively.

To prepare working solution of the ethanol extracts, 95% ethanol and distilled water were used as solvent and diluent, respectively. For the oils, they were dissolved in 95% ethanol to an initial concentration of 900 µl/ml and further diluted with the solution contained 5% ethanol and 5% Tween 80 to a desire concentration before used.

Microorganisms

Four isolates of oral microorganisms which were *Streptococcus mutans*, *Lactobacillus sp, Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* and *Candida albicans* were used in this study. *S. mutans*, *Lactobacillus sp, A. actinomycetemcomitans* and *C. albicans* were grown in Todd-Hewitt, rogosa, tryptic soy (BBL Microbiology Systems, Cockeysville, MD) and Sabouraud's dextrose broth (Pronadisa, Hispanlab, S.A.), respectively. All microorganisms were incubated at 37^{o}C, as static cultures for 18-24 h. Then their densities were adjusted to give an optical density (OD) at 600 nm of 0.1. These suspensions were used as inoculum for subsequent studies.

Screening for antimicrobial activity

Disc diffusion method

Antimicrobial effects of the oil and ethanolic extracts were determined by the disc diffusion method previously described (Barry, 1991). For comparison, various concentrations of chlorhexidine gluconate (Sigma, USA) (0.001-0.8%w/v) were used. The *S. mutans*, *Lactobacillus sp, A. actinomycetemcomitans* and *C. albicans* suspensions were spread on mitis salivarius bacitracin, rogosa, tryptic soy and Sabouraud's dextrose agar, respectively, using sterile glass L-rod. About 45 µl of each oil, ethanolic extract and chlorhexidine gluconate was applied in paper disc (8 mm diameter) and allowed to dry before being placed on the top layer of the agar plate. For *S. mutans*, *Lactobacillus sp* and *C. albicans*, the plates were incubated at 37°C for 24 h, while for *A. actinomycetemcomitans*, the plates were incubated at 37°C, 5% CO_2, for 48 h. Then the results were recorded by measuring the diameter of inhibition zones at the end of 24-48 h. All tests were repeated on three separate occasions for each microbial strain, with triplicate determinations on each occasion.

Broth microdilution method

For broth microdilution method (Barry, 1976), 50 µl of the oil and ethanolic extracts of each sample was two-fold serially diluted with appropriate broth in a microtitre plate. An equal volume of microbial suspension was added and mixed with the extract. The plates were incubated for 24 h, at 37^{o}C. Then microbial growth was examined and the lowest concentrations of the extracts which inhibited the visible growth of microorganisams were recorded as the minimum growth inhibitory concentration (MIC). The positive growth of each microorganism cultured in the broth without the extracts served as a positive control and the negative growth found in the mixture of broth and the extracts without microorganism served as a negative control.

Aliquots of the mixture of the extracts and micro-organisms which showed negative-visible growth after the first 24 h of incubation were inoculated onto the surface of appropriate agar. The lowest concentrations of the extracts giving negative growth of microorganisms were recorded as the minimum bactericidal concentration (MBC) or the minimum fungicidal concentration (MFC). The tests were repeated on three separate occasions for each microbial strain, with triplicate determinations on each occasion.

Time-kill curve studies

The results from the screening test showed that *B. pandurata* oil (BPO) exhibited the strongest effect against *C. albicans*. Therefore, we conducted time-kill studies with the BPO against *C. albicans* in an effort to characterize the relationships between concentration of the BPO and antifungal activity and compared its activity with nystatin. Time-kill procedures were conducted as previously described (Klepser *et al.*, 1997). The fungal suspensions was adjusted according to spectrophotometric methods to give an optical density (OD) at 600 nm of 0.1. A 1:10 dilution of this suspension was made by adding 1 ml of fungal suspension to 9 ml of Sabouraud's dextrose broth with or without (control) the desired amount of the BPO and nystatin. This dilution yielded a starting inoculum of approximately $1x10^5$ to $5x10^5$ CFU/ml. The resulting BPO and nystatin concentrations in test solutions were equal to 1, 1.5, 2 and 2.5 times the MICs for test isolate. Test solutions were incubated at 37°C. At predetermined time points (0, 2, 4, 8, 12, and 24 h for the test solutions containing 1xMIC and 1.5xMIC of the antifungal agents; 0, 30, 60, 90 and 120 min for the test solutions containing 2xMIC of the antifungal agents; 0, 15, 30, 45, 60, 90 and 120 min for the test solutions containing 2.5xMIC of the antifungal agents) following the introduction of the test isolate into the system, 100-µl aliquots were removed from each test solution. Ten fold serial dilutions were performed on samples, and a 10-µl aliquot from each dilution was streaked on a Sabouraud-dextrose agar (BBL Microbiology Systems, Cockeysville, MD) plate for colony count determination. Following incubation at 37°C for 24 h, the number of CFU on each plate was determined. All experiments were run in triplicate. A given concentration of the antifungal agents was considered fungicidal if it reduced the inoculum viable count by ≥ 3 $\log_{10}$ CFU/ml, or fungistatic if it reduced the inoculum viable count by <3 $\log_{10}$ CFU/ml.

Inhibitory effects of B. pandurata oils on Candida biofilm formation

To determine the effects of the BPO in inhibiting of biofilm formation, the *Candida* biofilm formation in wells of microtiter plates was performed as described previously (Ramage *et al.*, 2001). Briefly, 100 µl of the BPO was two-fold serially diluted with Sabouraud's dextrose broth in a microtitre plate. An equal volume of the *Candida* suspension (10^6 cells/ml) was added and mixed with the oils. The plates were incubated for 48 h at 37°C. After biofilm formation, the medium was aspirated, and nonadherent cells were removed by thoroughly washing the biofilms three times in sterile PBS. A series of the BPO-free wells and biofilm-free wells were also included to serve as positive and negative controls, respectively. The inhibitory effect of the BPO on biofilm formation was determined by using the (2,3)-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbo-nyl]-2*H*-tetrazolium hydroxide (XTT)-reduction assay described below. The percentage of inhibiting effects was calculated by using the formula [1 - (OD_{492} sample/OD_{492} control)] x 100%. Testing was performed in triplicate and repeated thrice.

Antifungal activity of B. pandurata oils against preformed C. albicans biofilm

Antifungal susceptibility testing of sessile cells was performed as described previously (Ramage *et al*., 2001). Briefly, *C. albicans* were grown in Sabouraud-dextrose broth (Pronadisa, Hispanlab, S.A.) for 18 h. Biofilms were formed on commercially available presterilized, polystyrene, flat-bottom 96-well microtiter plates (Corning Inc., Corning, N.Y.) by pipetting standardized cell suspensions (100 µl of the 10^6 cells/ml) into selected wells of the microtiter plate and incubating them for 48 h at 37°C. After biofilm formation, the medium was aspirated, and nonadherent cells were removed by thoroughly washing the biofilms three times in sterile PBS. Residual PBS was removed by blotting with paper towels before the addition of the BPO. The BPO was then added to the biofilms in serially double-diluted concentrations (32 to 0.25 mg/ml) and incubated for a further 48 h at 37°C. A series of the BPO-free wells and biofilm-free wells were also included to serve as positive and negative controls, respectively. Susceptibility of the sessile cells were determined by using the XTT-reduction assay described below. The percentage of inhibiting effects was calculated by using the formula [1 - (OD_{492} sample/OD_{492} control)] x 100%. The assay was prepared in triplicates and repeated thrice.

XTT-reduction assay

The XTT solution was prepared by dissolved 0.5 mg/ml of a water soluble tetrazolium salt, XTT (Sigma, St. Louis, MO, USA) and 40 µg/ml of coenzyme Q_0 (Sigma) in PBS and diluted 1:6 with PBS before used. A 100-µl aliquot of the XTT solution was then added to each prewashed biofilm and to negative control wells (for the measurement of background XTT-reduction levels). The plates were then incubated in the dark for 2 h at 37°C. A colorimetric change in the XTT-reduction assay, a direct correlation of the metabolic activity of the biofilm, was then measured in a microplate reader (Bio-tek instruments, Inc., Vermont, USA) at 492 nm.

Identification of B. pandurata oils components

Constituents of BPO were identified using Gas Chromatograph Mass Spectrometer (GCMS-QP 2010, Shimadzu®) equipped with autoinjector. The essential oils were diluted with hexane to give the suitable concentration that was 10 µl/ml. A capillary column used was Rtx®-5 (30 m x 0.25 mm id. x 0.25 µm film thickness). Working condition was described as followed; injector 250°C; transfer line to MSD 230°C; oven temperature was started with 80°C and hold for 2 min, increased to 280°C at 10°C/min and hold for 1 min; carrier gas used was helium at a flow rate 1.55 ml/min; split raito was 1:100; ionization 0 kV and scanned m/z over 35-550 amu at 1,111 amu/sec. Constituents of each BPO were identified by comparison their linear retention indices related to C_8-C_{40} n-alkanes with those obtained on methyl silicone column provided in the literature (Davies, 1990) and by comparison their mass spectra with the data provided by the NIST and WILEY mass spectral libraries.

RESULTS

Antimicrobial effect of the extracts

The results of the disc diffusion method showed that 0.63 mg of the BPO possessed inhibitory effects towards all microbial strains tested whereas 0.63 mg of *P. sarmentosum* leaf oil (PSO) did not have those effects. The 0.9 mg ethanolic extract of *B. pandurata* (BPE) has inhibitory effects on *S. mutans,* and *Lactobacillus sp*, but 0.9 mg of *P. sarmentosum* leaf extract (PSE) was only active against *C. albicans* (table 1). To compare the antimicrobial activity of the oil and ethanolic extracts with chlorhexidine, various concentrations of chlorhexidine were tested. The results revealed that inhibitory effects of 0.63 mg of the BPO against *A. actinomycetemcomitans*, *S. mutans, Lactobacillus sp*. and *C. albicans* were equivalent to 0.31, 0.0015, 0.04 and 0.47%w/v of chlorhexidine, respectively, while inhibitory effects of 0.9 mg of the BPE on *S. mutans* and *Lactobacillus sp*. were equivalent to 0.0037 and 0.17%w/v of chlorhexidine, respectively. The 0.9 mg of PSE has inhibitory effects on *C. albicans* equivalent to 0.06%w/v of chlorhexidine.

The MIC and MBC/MFC of the oil and ethanolic extracts of *B. pandurata* rhizomes and leaves of *P. sarmentosum* towards all tested microbial strains using broth microdilution method are presented in table 2. The BPO and BPE possessed killing effects towards all microbial strains tested, while the PSO has no antimicrobial activity. Among the four microbial isolates, *C. albicans* and *Lactobacillus sp*. appeared to be the most susceptible to the BPO and BPE, respectively. The PSE exhibited killing effects on both *A. actinomycetemcomitans* and *C. albicans* (table 2).

Time-kill curve studies demonstrated that the killing activities of the BPO against *C. albicans* were faster than nystatin at concentrations ranging from 1 to 2.5 times the MIC (fig. 1). Fungistatic activity was observed with the BPO at concentrations equal to 1 and 1.5 times the MIC, while the BPO at concentration equal to 2 and 2.5 times the MIC exhibited fungicidal activity, with a reduction in ≥ 3 $\log_{10}$ CFU/ml compared to the starting inoculum, after 60 and 44 min of incubation, respectively (fig. 1c, d).

Table 1: Antimicrobial activity of chlorhexidine, the oil and ethanolic extracts of the rhizomes of *B. pandurata* and leaves of *P. sarmentosum* by disc diffusion method

Tested materials	Microorganism / Inhibition zone (mm)(Mean ± SD;n = 9)			
	1	2	3	4
BPO (0.63 mg/disc)	15.17±1.09	10.77±0.58	10.46±0.29	15.84±0.76
PSO (0.63 mg/disc)	-	-	-	-
BPE (0.9 mg/disc)	-	15.14±0.13	14.57±0.31	-
PSE (0.9 mg/disc)	-	-	-	10.81±0.30
Chlorhexidine gluconate (%w/v)				
0.0015	-	-	10.32±0.39	-
0.0031	-	-	14.22±0.12	-
0.0062	-	-	16.75±0.19	-
0.0125	-	-	17.34±0.30	-
0.025	10.88±0.27	9.96±0.50	17.78±0.13	-
0.05	11.08±0.40	11.21±0.23	19.45±0.31	9.79±0.50
0.1	11.89±0.76	13.14±0.43	20.38±0.29	12.29±0.67
0.2	14.06±0.44	15.59±0.46	20.74±0.15	14.16±1.43
0.4	15.82±0.16	17.90±0.36	22.48±0.30	15.75±0.48
0.8	ND	ND	23.40±0.14	16.71±0.71

1: *A.actinomycetemcomitans* ATCC 43718, 2 : *Lactobacillus sp.,* 3 : *S. mutans*, 4 : *C. albicans*, BPO : *B. pandurata* oil, PSO : *P. sarmentosum* oil, BPE : ethanolic extract of *B. pandurata*, PSE : *P. sarmentosum* leaf ethanolic extract, - : No inhibition zone, ND: Not determine

Table 2: Antimicrobial activity of the oil and ethanolic extracts of the rhizomes of *B. pandurata* and leaves of *P. sarmentosum* by broth microdilution method

Microorganism	MIC (mg/ml)		MBC / MFC (mg/ml)			
	PSO	BPO	PSO	PSE	BPO	BPE
A. actinomycetemcomitans	-	0.5	-	2.5	1	2.5
S. mutans	-	2	-	-	2	1.25
Lactobacillus sp.	-	1	-	-	2	0.04
C. albicans	-	0.5	-	1.25	0.5	1.25

MIC: minimum growth inhibitory concentration, MBC: minimum bactericidal concentration, MFC: minimum fungicidal concentration, PSO: *P. sarmentosum* oil, PSE: *P. sarmentosum* leaf ethanolic extract, BPO : *B. pandurata* oil, BPE : ethanolic extract of *B. pandurata*, - : No inhibitory / killing effect.

Table 3: Chemical compositions of the essential oil from *B. pandurata*

Chemical composition	Retention time (min)	Area %	LRI
linalool (linalyl alcohol)	5.29	2.36	1115
Camphor	6.07	38.03	1160
α-terpineol	6.71	1.28	1198
Geraniol	7.58	56.68	1259
Not identified	6.13	1.65	1164

LRI : Linear Retention indices

Inhibitory effect of B. pandurata oils on Candida biofilm

Employing a formazan salt reduction assay for biofilm study, the results revealed that the inhibitory effect of the BPO on biofilm appeared to be dose-related (fig. 2). The BPO at concentrations between 4-32 µl/ml exhibited 63-98% inhibition on biofilm formation, while the same concentrations of the BPO showed less active against preformed biofilm of *C. albicans*. Lower concentrations of the BPO (< 2 µl/ml) had almost no effect on preformed biofilm.

Gas chromatographic and mass spectrometry (GC-MS) of B. pandurata oils

The BPO was identified its components by GC-MS and the results were shown in table 3. The major constituents found in BPO were geraniol (56.68%) and camphor (38.03%).

DISCUSSION

The antimicrobial effects of BPE on *A. actinomycetemcomitans* and *C. albicans*, and the PSE on *A. actinomycetemcomitans*, which were tested by the disc diffusion method showed no inhibitory zone. However, using broth microdilution method, the BPE and PSE exhibited killing effects on those microorganisms. This could be expected because it is well known that the antimicrobial property of an agent in disc diffusion method is directly related to its ability to diffuse in agar, so the concentration of the BPE and PSE in agar might not reach the inhibitory concentration for the tested microorganisms (*A. actinomycetemcomitans* and *C. albicans* for BPE and *A. actinomycetemcomitans* for PSE) and led to the result of non apparent inhibitory zone.

In this study, we compared the antimicrobial activity of the oil and ethanolic extracts with chlorhexidine because chlorhexidine has been used to treat or prevent dental caries and periodontal disease for over two decades as a 0.12-0.2% oral rinse. Moreover, it has also been reported to have antifungal effects when used topically in the oral cavity (Epstein *et al.*, 1992). We found that the BPO (0.63 mg), the BPE (0.9 mg) and the PSE (0.9 mg) possessed inhibitory effects against the tested microorganisms equivalent to 0.0015-0.47%w/v of chlorhexidine. Among the four extracts, only the BPO exhibited inhibitory effects against all the tested microorganisms. In addition, *C. albicans* appeared to be the most susceptible to the BPO. Antifungal activity of the BPO found in this study is consistent with previous report (Jantan *et al*., 2003). Therefore, the BPO was selected to examine the killing patterns by time-kill assay compared with nystatin and to determine its inhibitory effects on Candida biofilm.

The antibiofilm activity of essential oils has not been studied extensively. Recently, measurements of cellular mitochondrial dehydrogenase activity using tetrazolium salts have been employed with *Candida* cells for studies of biofilm formation on plastic (Hawser, 1996). In this study, we use the conventional 96-well microtiter plates coupled to a colorimetric method to assess the inhibitory effects of the BPO on Candida biofilm formation and against preformed biofilm. This method is rapid,

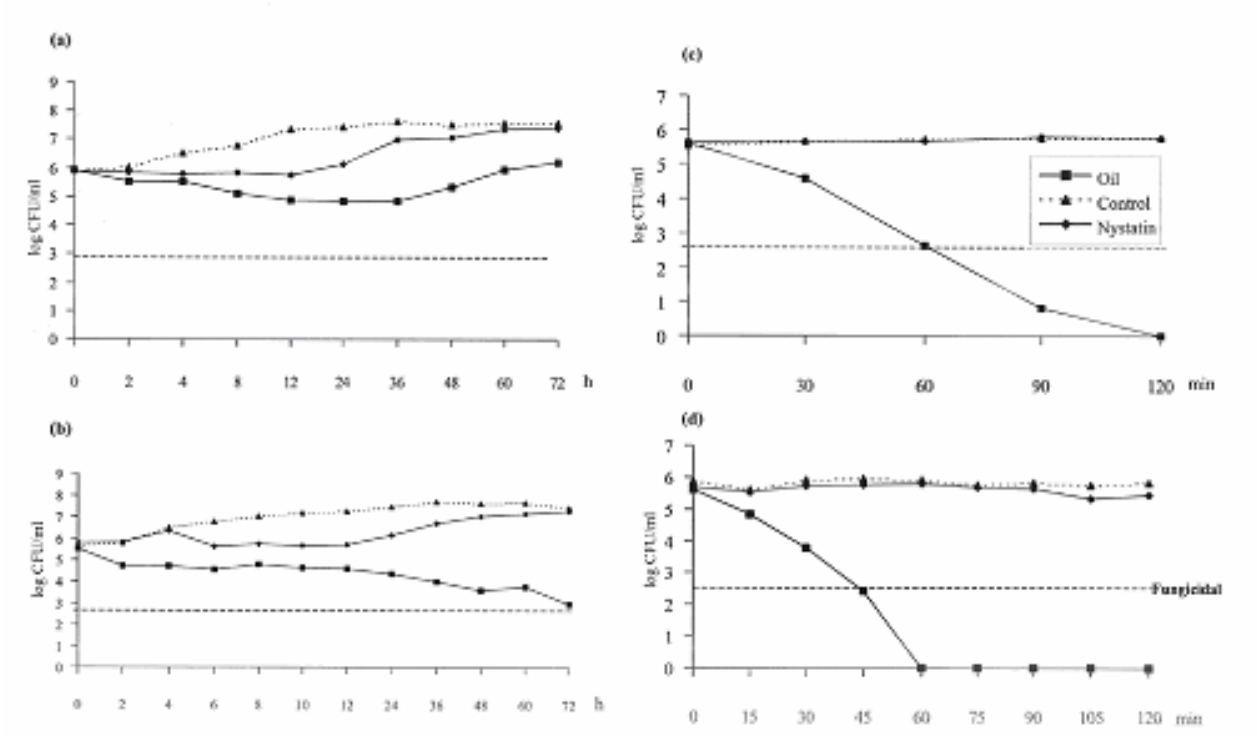

Fig. 1: Time-kill curves of *B. pandurata* oil (■) and nystatin (э) at concentration 1 MIC (a), 1.5 MIC (b), 2 MIC (c) and 2.5 MIC (d) against *C. albicans*. 4 = control. The fungicidal effect was defined as a $\geq 3\log_{10}$ reduction in CFU/ml compared with the initial inoculum.

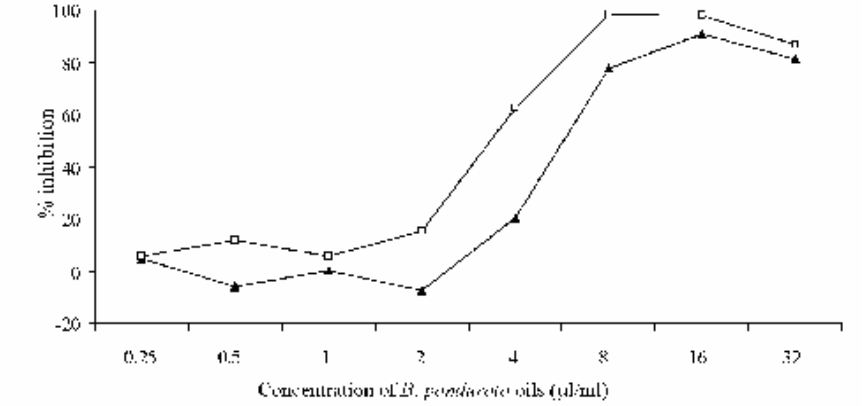

Fig. 2: Effects of different concentrations of *B. pandurata* oil on biofilm formation (□) and against preformed biofilm (▲) of *C. albicans*. The presented percentages of inhibition were calculated from the formula [1 - (OD_{492} sample/OD_{492} control)] x 100%. Results are from three experiments performed in triplicate (n=9).

inexpensive, easy to use, accurate, and reproducible methodology for antifungal susceptibility testing of *Candida* biofilms (Ramage *et al*., 2001). Our data demonstrated that the preformed Candidal biofilms were more resistant to the BPO than their planktonic cells. The observation in this study is consistent with previous reports that biofilm-associated *Candida* cells are resistant to antifungal agents relative to their planktonic counterparts (Bachmann *et al.*, 2002; Shuford *et al.*, 2007). Although adherent populations were not completely eradicated by treatment with the BPO, a 90% reduction in the metabolic activity of adherent cells was detected at the BPO concentrations 16 µl/ml. In addition, the BPO interfered with the starting phases of biofilm formation by reducing the amount of metabolically active yeast.

Identification of the BPO components in the present study revealed that the major constituent was geraniol, an olefinic terpene. Moreover several terpenic derivatives, i.e. linalool and α-terpineol, were also found. Geraniol was shown to alter cell permeability by penetrating between the fatty acyl chains making up the membrane lipid bilayers, disrupting lipid packing and changing *C. albicans* membrane fluidity (Bard *et al.*, 1988). These phenomena could led to major surface alterations, thereby interfere the adherence capacity of *C. albicans.* In addition, the strong antifungal activity of some major components of essential oils, i.e. terpenes, has been described in several studies (Manohar *et al.*, 2001; Hammer *et al.*, 2003; Dalleau *et al.*, 2008). Therefore, the antifungal and antibiofilm activities of the BPO found in this study could be attributed to the presence of various terpenic derivatives in the essential oil. Those constituents may synergistically increase the effect on the fungal cells.

The present work has shown that the BPO displayed broad spectrum of activity, being active against all of the microorganisms tested. In addition, time-kill curve and biofilm studies demonstrated efficacy of the BPO against both planktonic and biofilm cells of *C. albicans*. Since all microorganisms tested are the common pathogens associated with several oral diseases, i.e., dental caries, periodontal disease and candidiasis (Rindum *et al*., 1994; Henderson *et al.,* 2003; Corby *et al.*, 2005; Seminario *et al.,* 2005), the BPO may be potentially good sources of antimicrobial agents and that further investigation for development of BPO as a natural product for oral hygiene is worthwhile.

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