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Antimicrobial activities of *Nitellopsis obtusa* (Desvaux) Groves and *Chara vulgaris* L.

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Summary

The extracts of *Nitellopsis obtusa* (Desvaux) Groves and *Chara vulgaris* L. were investigated for antimicrobial activities against the following common microorganisms using an agar diffusion method: *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Proteus vulgaris*, *Saccharomyces cerevisiae*, and *Candida albicans*. The extracts strongly inhibited the growth of Gram-positive bacteria, but did not show inhibitory activity against Gram-negative bacteria and yeast. In this study, novel methods for extraction of antimicrobial substances from *N. obtusa* and *C. vulgaris* were reported. The optimum extraction conditions were investigated using single factor experimental design and $L_{27}3^{(13)}$ orthogonal experimental design. Results showed that the optimum extraction conditions for *N. obtusa* are: solid to liquid ratio 1:20, temperature 85°C, ethanol concentration 50%, extraction time 6h; and the optimum extraction conditions for *C. vulgaris* are: solid to liquid ratio 1:15, temperature 85°C, ethanol concentration 70%, extraction time 10h. Stability study demonstrated neuter and alkaline conditions enhanced the antimicrobial activities from *N. obtusa* and *C. vulgaris*, and both extracts were stable under ultraviolet (UV). These data suggest that extracts from both *N. obtusa* and *C. vulgaris* might be of potential use as bactericidal agents.

Introduction

For many years, antibiotics have been used to treat bacterial diseases. However, microorganism resistance to antibiotics has increased in parallel (Bronzwaer et al., 2002). Several studies have been conducted using synthetic antimicrobials, which often cause undesirable side effects (Levy and Marshall, 2004). In order to prolong the storage stability of foods, synthetic antimicrobials are widely used in industrial processing. However, according to toxicologists and nutritionists, there are side effects associated with the use of some synthetic antimicrobials in food processing (Kajiwara et al., 2006; Nguyen et al., 2008). Plant diseases caused by microorganisms are the major problem in world agriculture because they cause tremendous losses in crop yield (Fletcher et al., 2006). Synthetic antimicrobials are also widely used in the control of plant diseases. However, the use of synthetic antimicrobials cause hazards to human health and may directly increase environmental pollution (Fletcher et al., 2006). Because of these associated problems, the need for new antimicrobial agents has lead to the search of new sources of potential antimicrobials (Carson and Riley, 2003). In the last several decades, various plant extracts have been the focus of great interest from researchers all over the world because they represent natural resources (Sivakumar et al., 2008; Cai et al., 2009; Dan et al., 2010; Hashem et al., 2010). Plants produce a wide variety of physiologically active substances. These secondary metabolites have various functions, including antimicrobial activity (Yazaki et al., 2008).

Charophytes are one of the most structurally complex green algae with a worldwide distribution (Zaneveld, 1940; Wood and Imahori, 1965). China has a wealth of charophyte resources in both fresh-waters and estuarine systems (Han and Li, 1994; Ling et al., 2000). They are commonly used as fertilizers of farmland and diets of aquatic animals (Zaneveld, 1940). In recent years, research of charophytes has focused on their physiological activities, ecological characters, and taxonomy (Lan et al., 2003; Kotta et al., 2004; Vouilloud et al., 2005). However, there are fewer reports in the area of antimicrobial activity, and only *Chara zeylanica*, *Chara conirari*, and *Nitella hyaline* were reported to have antimicrobial activities (Ghazala and Shameel, 2005; Khalid et al., 2010). Therefore, the objectives of the present study are (1) to perform assessment of the antimicrobial activities in eight solvent extracts from *Nitellopsis obtusa* (Desvaux) Groves and *Chara vulgaris* L., for the first time, against six microorganisms; (2) to test and optimize extraction that can give maximal antibacterial activities; (3) to evaluate the stability of the extracts under different pH and UV.

Materials and methods

Plant materials and microorganisms

Nitellopsis obtusa (Desvaux) Groves and *Chara vulgaris* L. were collected from Jinci park, Taiyuan, Shanxi Province, China, in July 2010. Taxonomic identification was performed by Prof. Xie Shulian, Shanxi University. Microorganisms: *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922), *Proteus vulgaris* (ATCC13315), *Saccharomyces cerevisiae* (ATCC 2601), and *Candida albicans* (ATCC 10231). All microorganisms were from the American Type Culture Collection (ATCC).

Preparation of plant extracts

Extraction was performed as previously described (Khalid et al., 2010) using eight solvents with different polarities. *N. obtusa* and *C. vulgaris* were cut into small pieces (2-4cm) respectively. Each were washed several times with running tap water, then with sterile water and dried at 40°C (Abdel-Monaim et al., 2011). Dry materials were grounded to fine powders in a grinder, then 10g of each powders blended in 50mL of 80% methanol, 80% ethanol, 80% acetone, 80% chloroform, 80% ethyl acetate, 80% butanol, 80% benzene, 80% petroleum ether solutions at room for 48h (Zhang et al., 2008). After filtration, the extracts were concentrated to 1 mg/mL by using rotary evaporation. The 1 mg/mL of extracts were evaluated for their antimicrobial activities as described below.

Antimicrobial assays

Determination of antimicrobial activity was accomplished by agar diffusion method (ADM; Michielin et al., 2009). The agar surface was perforated with 6 mm diameter holes, aseptically cut and filled with 50µL of extracts. 80% methanol, 80% ethanol, 80% acetone, 80% chloroform, 80% ethyl acetate, 80% butanol, 80% benzene and 80% petroleum ether solutions were used as negative controls. After diffusion of the solution in each hole, the plates were inverted and incubated at 37°C for 24 hours for bacteria and 28°C for 48 hours for yeast. Antimicrobial activities were determined by measuring the radius of the zone of inhibition around the hole. Each treatment was replicated nine times.

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Single factor experiment

The four factors including solid to liquid ratio (g:mL), extraction temperature (°C), ethanol concentration (%), extraction time (h) could affect extraction efficiency. Single factor experiments were applied to decide appropriate levels. For each single factor, five different levels were designed, with other factors being kept constant. For each experiment, a total of 12g sample was added to corresponding volume of ethanol and extracted as described in Tab. 1. Four replicates were used for each extraction. After filtration, the extracts were concentrated to 0.5 mg/mL by using rotary evaporation. *S. aureus* was used as a test organism by inoculating it into separate mixtures at a concentration of 10^7 CFU/mL. Then, antimicrobial activity of extracts from each sample was analyzed by ADM to choose three reasonable levels of four factors for orthogonal experimental design. Each treatment was replicated nine times.

Orthogonal experimental design

On the basis of single factor experiments, three levels of four factors were selected as described in Tab. 2. Then orthogonal array $L_{27}(3^{13})$ matrix was used to determine the optimum extraction conditions of antimicrobial substances from *N. obtusa* and *C. vulgaris*, with the consideration of the interactions between the parameters (Jia et al., 2010; Li et al., 2010; Zhou et al., 2010). For each experiment, a total of 12g sample was added to corresponding volume of ethanol and extracted as described in (Tab. 3). Four replicates were used for each extraction. The extracts were concentrated to 0.5 mg/mL by using rotary evaporation. *S. aureus* was used as a test organism by inoculating it into separate mixtures at a concentration of 10^7 CFU/mL. Then, antimicrobial activity of extracts from each sample was analyzed by ADM. Each treatment was replicated nine times.

Stability assays

The effect of pH on antimicrobial activity in extracts was examined by pH stability assays (Cheikhyoussef et al., 2009; Wu et al., 2008). Tests were conducted in two sets: test sets of extracts from *N. obtusa* and *C. vulgaris* were adjusted with 5M NaOH or 5M HCl to different pH values ranging from 5 to 9. Control sets were prepared using the same method with 80% ethanol except that no extracts was added. To test the impact of UV, each extract was incubated under UV (256rim, 6W, 5cm) for a period ranging from 1h to 5h (Jia et al., 2010; Chen and Dai, 2012). Then, antimicrobial activity was analyzed by ADM. Each treatment was replicated nine times.

Statistical analysis

Each data was presented as mean ± standard error (n = 9). ANOVA (One-way analysis of variance) and Duncan's multiple range test were carried out to determine significant ($P < 0.05$) differences between the means. The analyses were carried out using SPSS package software (Version 17.0).

Tab. 1: Single factor experimental design

Factors	Conditions	Levels				
		1	2	3	4	5
Solid to liquid ratio (g:mL)	Temperature 50°C	1:5	1:10	1:15	1:20	1:25
	Ethanol concentration 80%					
	Extraction time 10h					
Temperature (°C)	Solid to liquid ratio 1:5	40	55	70	85	97
	Ethanol concentration 80%					
	Extraction time 10h					
Ethanol concentration (%)	Solid to liquid ratio 1:5	40	50	60	70	80
	Temperature 50°C					
	Extraction time 10h					
Extraction time (h)	Solid to liquid ratio 1:5	4	6	8	10	12
	Temperature 50°C					
	Ethanol concentration 80%					

Tab. 2: Factors and levels of orthogonal experiment of *N. obtusa* extraction

Levels	Factors			
	Solid to liquid ratio (A)	Temperature (B)	Ethanol concentration (C)	Extraction time (D)
1	1:5	40°C	40%	4h
2	1:10	70°C	50%	6h
3	1:20	85°C	60%	8h

Tab. 3: Factors and levels of orthogonal experiments for *C. vulgaris* extraction

Levels	Factors			
	Solid to liquid ratio (A)	Temperature (B)	Ethanol concentration (C)	Extraction time (D)
1	1:5	70°C	40%	4h
2	1:15	85°C	50%	6h
3	1:20	97°C	70%	10h

Results

Antimicrobial activity

Initial antimicrobial screening tests using extracts obtained by eight solvents from *N. obtusa* and *C. vulgaris* indicated that there was almost no antimicrobial activity against Gram-negative bacteria (*E. coli* and *P. vulgaris*) and yeast (*S. cerevisiae* and *C. albicans*). Extracts obtained by methanol, ethanol, and acetone had greater activity against Gram-positive bacteria (*S. aureus* and *B. subtilis*) than other solvents, with an inhibition zone ranging from 7 mm and 15 mm. The ethanol extracts had the highest inhibition zone values and were significantly ($P < 0.05$) different from other solvents (Fig. 1).

Single factor experiments

As can be seen from Fig. 2 (A), antimicrobial activities of *N. obtusa* and *C. vulgaris* increased with an increasing solid to liquid ratio. Maximum extraction yields of antimicrobial substances were achieved at 1:10 ratio for *N. obtusa* and 1:20 ratio for *C. vulgaris*, then antimicrobial activities decreased with increasing ratio. For *N. obtuse* extracts, ANOVA shows that the best solid to liquid ratios with significant ($P<0.05$) difference (selected one level which was more resource saving, when there was no significant difference between two or more levels), were Level 1 (1:5), Level 2 (1:10), Level 4 (1:20), and selected them for orthogonal experimental design in Tab. 2. For *C. vulgaris* extracts, best solid to liquid ratios with significant ($P<0.05$) difference (selected one level which was more resource saving, when there was no significant difference between two or more levels) were Level 1 (1:5), Level 3 (1:15), Level 4 (1:20), and selected them for orthogonal experimental design in Tab. 3. Increase in temperature led to greater antimicrobial activities in extracts (Fig. 2 (B)), and the highest antimicrobial activities with significant ($P<0.05$) difference were observed at 85°C. However, increasing temperature did not improve the antibacterial activity at 97°C. For *N. obtuse* extracts, ANOVA shows that the best extraction temperatures with significant ($P<0.05$) difference (selected one level which was more resource saving, when there was no significant difference between two or more levels), were Level 1 (40°C), Level 3 (70°C), Level 4 (85°C), and selected them for orthogonal experimental design in Tab. 2. For *C. vulgaris* extracts, best extraction temperatures with significant ($P<0.05$) difference (selected one level which was more resource saving, when there was no significant difference between two or more levels), were Level 3 (70°C), Level 4 (85°C), Level 5 (97°C), and selected them for orthogonal experimental design in Tab. 3. Fig. 2 (C) showed the effect of ethanol concentration on the antimicrobial activities in extracts from *N. obtusa* and *C. vulgaris*. Antimicrobial activities of *N. obtusa* and *C. vulgaris* increased significantly ($P<0.05$) with increasing concentration until the equilibriums (60% for *N. obtuse* and 70% for *C. vulgaris*) were reached. For *N. obtuse* extracts, ANOVA shows that the best extraction concentrations with significant ($P<0.05$) difference (selected one level which was more resource saving, when there was no significant difference between two or more levels), were Level 1 (40%), Level 2 (50%), Level 3 (60%), and selected them for orthogonal experimental design in Tab. 2. For *C. vulgaris* extracts, best extraction concentrations with significant ($P<0.05$) difference were Level 1 (40%), Level 2 (50%), Level 4 (70%), and selected them for orthogonal experimental design in Tab. 3. Fig. 2 (D) depicted the effect of different extraction time on the antimicrobial activities in extracts from *N. obtusa* and *C. vulgaris*. For *N. obtuse*, the antimicrobial activity increased significantly ($P<0.05$) with extraction time extended, and equilibrium reached at 8h. ANOVA shows that the best extraction times with significant ($P<0.05$) difference (selected one level which was more resource saving, when there was no significant difference between two or more levels), were Level 1 (4h), Level 2 (6h), Level 3 (8h), and selected them for orthogonal experimental design in Tab. 2. For *C. vulgaris*, the highest inhibition zone value was observed at 10h, thereafter, inhibitory activity decreased gradually. ANOVA shows that the best extraction times with significant ($P<0.05$) difference (selected one level which was more resource saving, when there was no significant difference between two or more levels), were Level 1 (4h), Level 2 (6h), Level 4 (10h), and selected them for orthogonal experimental design in Tab. 3.

Optimization of extraction condition

Orthogonal experimental design, the main method of fractional factorial design, can effectively screen out key variables by several representative experiments (Antony, 2006; Kilickap, 2010). From experimental results, it was inferred that antimicrobial activities of *N. obtusa* and *C. vulgaris* were influenced by both different factors at different levels and their interactions. The term $L_{27}(3^{13})$ of an orthogonal array implies 27 groups of experiments (Tab. 4). This array handles up to four factors at three levels each. The subscripts 1, 2, and 3 represent the value of a designed factor at levels 1, 2, and 3 respectively. In other words, these subscripts designate each special trial run of the experiment. For example, in the first row of Tab. 4 (following the indicated subscripts), the factor level of factor A (which is assigned to the first column of the array) is 1, and the level of factors B, C and D are 1 as well. The first trial run of this experiment will be designed as a level set {1, 1, 1,1} for factors A, B, C and D according to Tab. 2 and Tab. 3. Therefore, the first experiment, for *N. obtuse* extracts, was carried under solid to liquid ratio 1:5 (g:mL), temperature 40°C, extraction concentration 40%, extraction time 4h conditions. For *C. vulgaris* extracts, the first experiment was carried under solid to liquid ratio 1:5 (g:mL), temperature 70°C, extraction concentration 40%, extraction time 4h conditions. The other experiments will perform in the same way, and the experimental results of the orthogonal design were shown in Tab. 4. Factors that influence antimicrobial activity of *N. obtusa* were listed in a decreasing order as follow: B > A > C > D. The individual levels within each factor were ranked as in Fig. 3: A: 3 > 2 > 1; B: 3 > 2 > 1; C: 2 > 3 > 1; D: 2 > 3> 1. Factors that influence antimicrobial activity of *C.vulgaris* were listed in a decreasing order as follow:

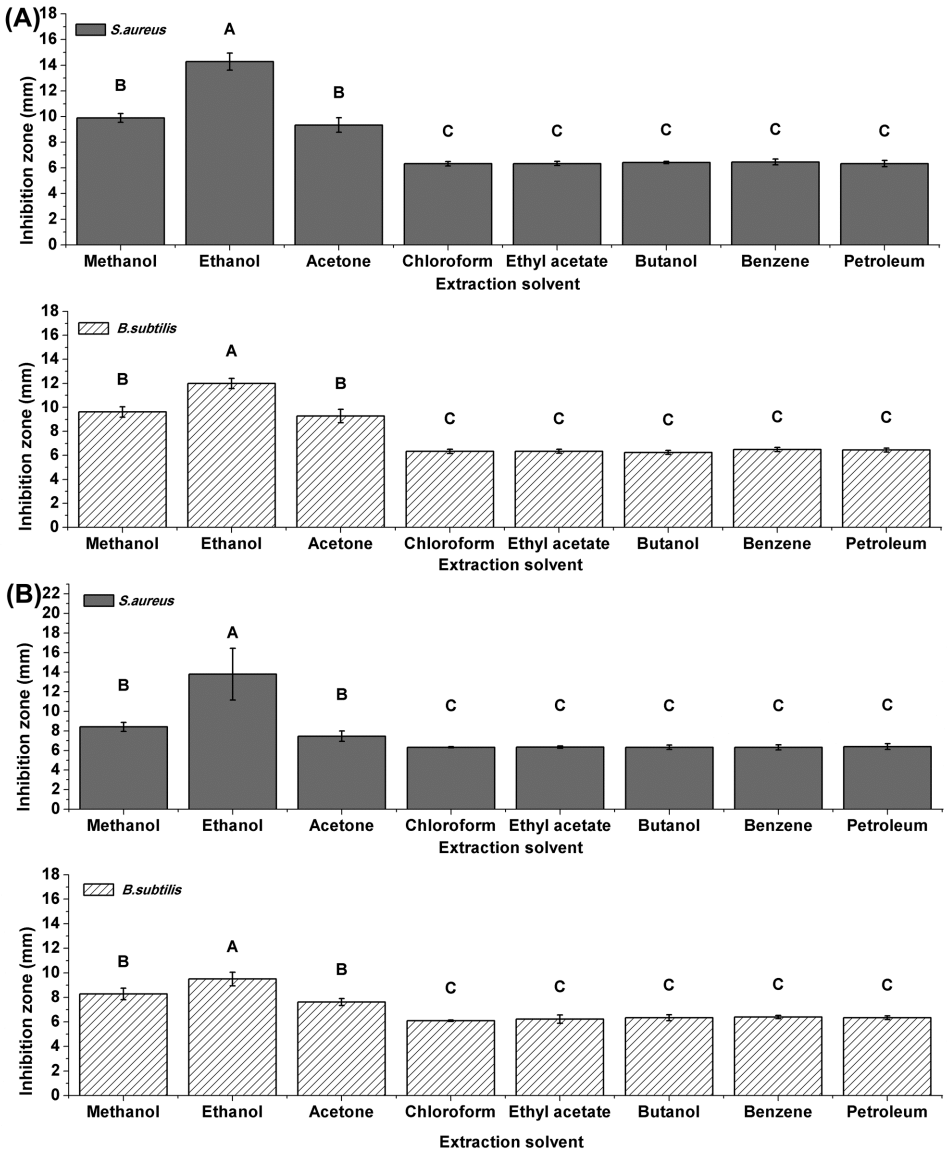

Fig. 1: Antimicrobial activities in extracts from *N. obtuse* (A) and *C. vulgaris* (B) against *S.aureus* and *B.subtilis*. A negative result was defined as an inhibition zone of 6mm. Greater than 6mm indicated positive result of the presence of antibacterial substance. Inhibition zones of controls were all 6mm. Different letters indicated significant differences ($p<0.05$; one-way analysis of variance (ANOVA) and Duncan's multiple range test). Bars represent the means ± standard deviation. Each was replicated nine times.

C > A > D > B. The individual levels within each factor were ranked as in Fig. 3: A: 2 > 3 > 1; B: 2 > 1 > 3; C: 3 > 2 > 1; D: 3 > 2 > 1. Because interactions between factors are complex, only low-order interactions were analyzed while high-order (three-, four-, and five-order) interactions were neglected. Tab. 5 and Tab. 6 summarize the analysis of variance (ANOVA) of factors and their second-order interactions that affect antimicrobial activity. The term ''interaction'', indicated by inserting the ''×'' symbol between the two interacting factors, is used to describe the condition in which the effect of one factor's influence upon the result is dependent on the condition of the other factor. In Tab. 5 and Tab. 6, F-ratio is defined as F = MSF/MSE, where MSF and MSE represent respectively mean square of factors or interactions, mean square of errors. *df*, SS and MS respectively represent degree of freedom, sum of squares and mean square. If the calculated value F is greater than critical value F_α [e.g. $F_{0.05}(2,6) = 5.14$], then that factor or interaction is statistically significant. In Tab. 5, if significant level $\alpha = 0.01$, then A (Solid to liquid ratio) and B (Temperature) were statistically significant factors that affect antimicrobial activity of *N. obtusa*. When significant level $\alpha = 0.05$, interaction A × B was statistically significant. Therefore, factors A, B and interaction A × B were regarded as dependent factors and interaction in extraction of antimicrobial substances. C (Ethanol concentration), D (Extraction time) and interactions A × C, B × C were regarded as independent factors and interactions. Optimum values of these factors for extraction of antimicrobial substances from *N. obtusa* were $A_3B_3C_2D_2$, solid to liquid ratio 1:20, temperature 85°C, ethanol concentration 50%, extraction time 6h (Tab. 4). In Tab. 6, if significant level $\alpha = 0.05$, then C (Ethanol concentration) was the statistically significant factor that affect antimicrobial activity of *C. vulgaris*. Therefore, factor C was regarded as dependent factor in antimicrobial activity. A (Solid to liquid ratio), B (Temperature), D (Extraction time) and interactions A × B, A × C, B × C were regarded as independent factors and interactions. Optimum values of these factors for extraction of antimicrobial substances from *C. vulgaris* were $A_2B_2C_3D_3$, solid to liquid ratio 1:15, temperature 85°C, ethanol concentration 70%, extraction time 10h (Tab. 4).

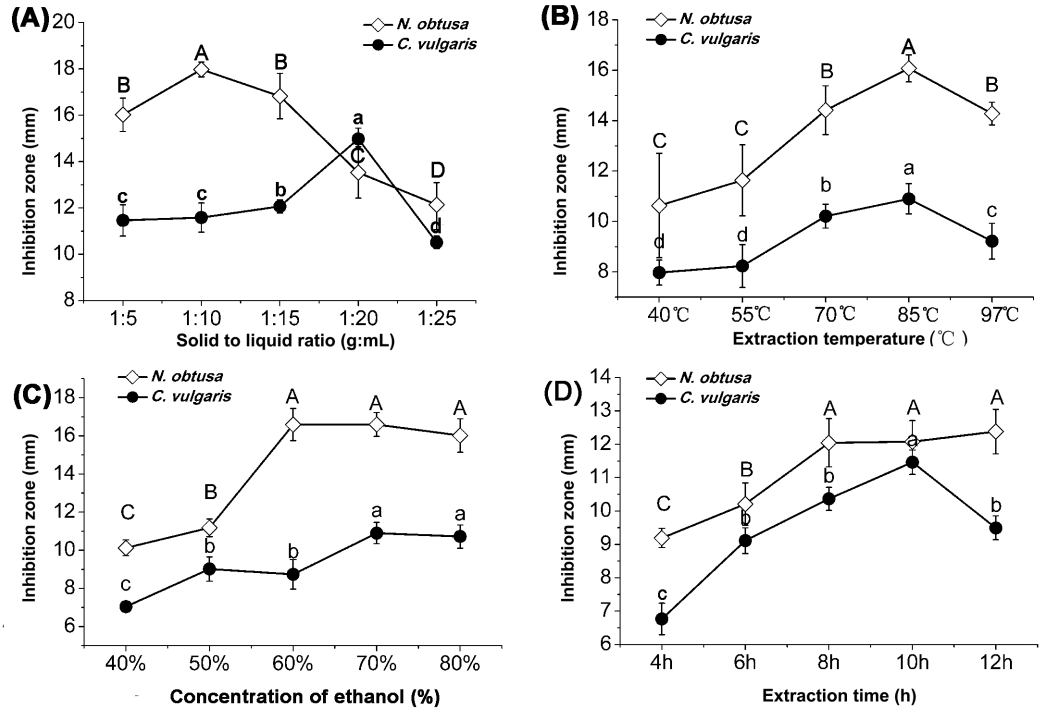

Fig. 2: Effect of solid to liquid ratio (A), extraction temperature (B), ethanol concentration (C), and extraction time (D) on antimicrobial activities in extracts from *N. obtusa* and *C. vulgaris* against *S.aureus*. Different letters indicated significant differences ($p<0.05$; one-way analysis of variance (ANOVA) and Duncan's multiple range test). Bars represent the means ± standard deviation. Each was replicated nine times.

Effect of pH and UV on the antimicrobial activity

Effect of pH was tested over a wide range (pH 5 to pH 9). As shown in Fig. 4 (A), maximum efficiency of antibacterial activity from *N. obtuse* extracts was observed when pH was 8 and 9, whereas the extract from *C. vulgaris* exhibited pH stability in the range from 6 to 9. Data from pH stability suggest that antimicrobial agents in extracts from *N. obtusa* and *C. vulgaris* are different. To test the UV stability of extracts, we investigated the antimicrobial activities of different treatments. There are no statistically significant ($P<0.05$) differences between samples that were exposed to UV light for different times (Fig. 4 (B)), implying that these extracts are not impacted by exposure to UV.

Discussion

In the present study, we evaluated the antimicrobial activities in extracts from *N. obtusa* and *C. vulgaris* against 6 microorganisms. Like most of antimicrobial activity results from algae (Reichelt and Borowitzka, 1984), extracts from *N. obtusa* and *C. vulgaris* were highly effective in inhibiting the growth of *S. aureus* and *B. subtilis* (Gram-positive bacteria). However, they showed no antimicrobial activities against *E. coli*, *P. vulgaris* (Gram-negative bacteria), and *S. cerevisiae*, *C. albicans* (yeast). These results might be attributed to the differences in the outer membrane structure and permeability between Gram positive bacteria, Gram negative bacteria and yeast. Bacterial membrane has peptidoglycan layer, while yeast membrane consists of dextran and mannan. Gram positive and Gram negative bacteria are reported to have further differences in cell wall composition (Ventosa et al., 1998). Gram positive cells contained more teichoic acid in cell walls that might be more sensitive to the extracts from *N. obtusea* and *C. vulgaris*. The extracts might inhibit synthesis of teichoic acid, and thus the biosynthesis of cell walls thereby. To our knowledge, *S. aureus* and *B. subtilis* are resistant to various antibiotics (Adedapo et al., 2008). Our results indicate that there is a possible alternative therapy for these antibiotics resistant strains. This therapy is very important because these types of microorganisms are difficult to treat and often require alternative therapy (Vila et al., 2010).

Solvent played a key role in extraction of antimicrobial substances from *N. obtusa* and *C. vulgaris*. Several extraction methods have been reported using solvents with different polarities, such as methanol, ethanol, chloroform, ethyl acetate, acetone, and petroleum ether (Cheung et al., 2003). In this study, the ethanol extracts were found to be highly effective against microorganisms (Fig. 1). Ethanol has a high dielectric constant and cohesive energy, as compared with other solvents, which provides strong bounding between solvent molecules and polar compounds from the solutes, causing their dissolution (Xie and Lu, 2004). Moreover, the results revealed that ethanol caused damages to cell walls and the cell membranes of *N. obtusa* and *C. vulgaris*, and thus enabled the solvent penetrate more effectively into cellular tissue and antimicrobial substances to be released rapidly. In addition, Ethanol has several advantages such as low toxicity, economical, and lower boiling point (Xie and Lu, 2004). Thus, ethanol was considered and also demonstrated as an ideal extraction solvent in our studies.

There are many factors affecting the extraction. Among them, the solid to liquid ratio, extraction temperature, the concentration and extraction time are key factors (Xiong et al., 2007). Single factor experiment was performed by one factor varied with different levels while other factors being fixed. When the solid to liquid ratio was too low, the contact between antimicrobial substances and solvents was not sufficient enough, and it was not conducive to extract maximal amount of antimicrobial substances. When the solid to liquid ratio was too high, concentration time would be long and antimicrobial components might be decomposed (Fig. 2 (A); Yang et al., 2010). Increasing temperature enhanced diffusivity and thus the yields of antimicrobial activities in extracts were increased with higher temperature (Liu et al., 2002). When temperature was too high, ethanol

Tab. 4: Result analysis of orthogonal experiments $L_{27}(3^{13})$

Experiment NO.	Factors				Inhibition zone (mm)	
	Solid to liquid ratio (A)	Extraction temperature (B)	Extraction concentration (C)	Extraction time (D)	*N. obtusa* [a]	*C. vulgaris* [a]
1	1	1	1	1	8.4	7.75
2	1	1	2	2	9.09	7.9
3	1	1	3	3	9.40	12.33
4	1	2	1	2	10.77	7.38
5	1	2	2	3	9.43	10.89
6	1	2	3	1	9.58	10.28
7	1	3	1	3	11.19	10.04
8	1	3	2	1	11.94	7.67
9	1	3	3	2	12.16	12.3
10	2	1	1	2	9.65	11.08
11	2	1	2	3	10.02	9.87
12	2	1	3	1	9.95	11.03
13	2	2	1	3	11.54	10.37
14	2	2	2	1	11.04	11.25
15	2	2	3	2	11.79	13.23
16	2	3	1	1	13.95	8.75
17	2	3	2	2	14.27	10.44
18	2	3	3	3	14.64	11.7
19	3	1	1	3	9.06	9.22
20	3	1	2	1	11.01	11.58
21	3	1	3	2	10.77	8.77
22	3	2	1	1	12.04	10.46
23	3	2	2	2	13.02	10.4
24	3	2	3	3	12.98	8.97
25	3	3	1	2	14.77	8.91
26	3	3	2	3	16.05	9.17
27	3	3	3	1	14.35	10.26
K_{1j} [b]	10.22	9.71	11.26	11.36		
K_{2j}	11.87	11.35	11.76	11.81		
K_{3j}	12.67	13.70	11.74	11.59		
k_{1j} [c]	9.62	9.95	9.33	9.89		
k_{2j}	10.86	10.36	9.91	10.05		
k_{3j}	9.75	9.92	10.99	10.28		
R_K [d]	2.45	3.99	0.5	0.45		
r_k [e]	1.24	0.41	1.66	0.52		
O_K [f]	A_3	B_3	C_2	D_2		
o_k [g]	A_2	B_2	C_3	D_3		

[a] 80% ethanol was used as control. A negative result was defined as an inhibition zone of 6mm. Greater than 6mm indicated positive result of the presence of antibacterial substance. Each value was means of nine determinations.

[b] $K_{ij} = (1 / 9) \sum$ mean inhibition zone of *N. obtusa* at factor *j* (*j* = A, B, C, D).

[c] $k_{ij} = (1 / 9) \sum$ mean inhibition zone of *C. vulgaris* at factor *j* (*j* = A, B, C, D).

[d] $R_{ij} = \max \{K_{ij}\} - \min \{K_{ij}\}$, *j* and *i* mean factor and setting level here, respectively.

[e] $r_{ij} = \max \{k_{ij}\} - \min \{k_{ij}\}$, *j* and *i* mean factor and setting level here, respectively.

[f] O means the optimum combination of conditions for *N. obtusa*, is $A_3B_3C_2D_2$.

[g] o means the optimum combination of conditions for *C. vulgaris*, is $A_2B_2C_3D_3$.

Tab. 5: Results of variance (ANOVA) analysis for *N. obtuse*

Source	SS	*df*	MS	*F* [a]	Significance [b]
A	28.2044	2	14.1022	69.54	**
B	72.6127	2	36.3064	179.03	**
C	1.4213	2	0.7107	3.50	
D	0.9023	2	0.4512	2.22	
A×B	3.747	4	0.9368	4.62	*
A×C	1.9536	4	0.4884	2.41	
B×C	1.6777	4	0.4194	2.07	
Error	1.2167	6	0.2028		
Total	111.7357	26			

[a] Significant parameter, $F_{0.05}(2, 6) = 5.14$, $F_{0.01}(2, 6) = 10.92$, $F_{0.05}(4, 6) = 4.534$, $F_{0.01}(4, 6) = 9.148$.
[b] *, ** and blank indicate significant different, more significant different and no significant different, respectively.

Tab. 6: Results of variance (ANOVA) analysis for *C. vulgaris*

Source	SS	*df*	MS	*F* [a]	Significance [b]
A	8.3716	2	4.1858	2.9805	
C	12.7238	2	6.3619	4.53	*
A×C	13.848	4	3.462	2.4651	
Error	25.2785	18	1.4044		
Total	60.2219	26			

[a] Significant parameter, $F_{0.05}(2,18) = 3.55$, $F_{0.01}(2,18) = 6.01$, $F_{0.05}(4,18) = 2.93$, $F_{0.01}(4,18) = 4.58$.
[b] *, ** and blank indicate significant different, more significant different and no significant different, respectively.

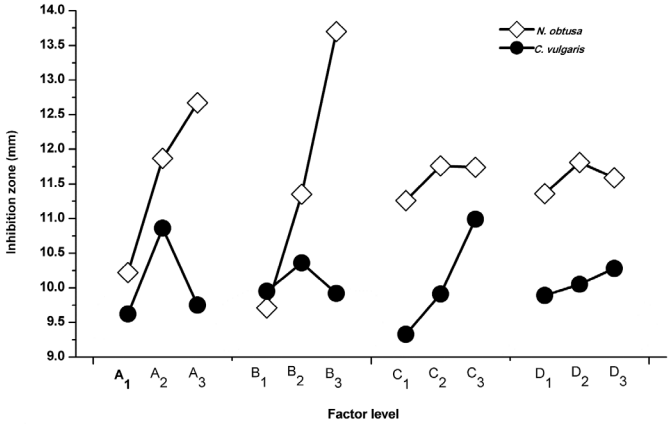

Fig. 3: Effect of each parameter on antimicrobial activities of *N. obtusa* and *C. vulgaris*. Parameters of *N. obtusa*: A, solid to liquid ratio (g:mL): A_1: 1:5, A_2: 1:10, A_3: 1:20; B, temperature: B_1: 40°C, B_2: 70°C, B_3: 85°C; C, ethanol concentration: C_1: 40%, C_2: 50%, C_3: 60%; D, extraction time: D_1: 4h, D_2: 6h, D_3: 8h. Parameters of *C. vulgaris*: A, solid to liquid ratio (g:mL): A_1: 1:5, A_2: 1:15, A_3: 1:20; B, temperature: B_1: 70°C, B_2: 85°C, B_3: 97°C; C, ethanol concentration: C_1: 40%, C_2: 50%, C_3: 70%; D, extraction time: D_1: 4h, D_2: 6h, D_3: 10h.

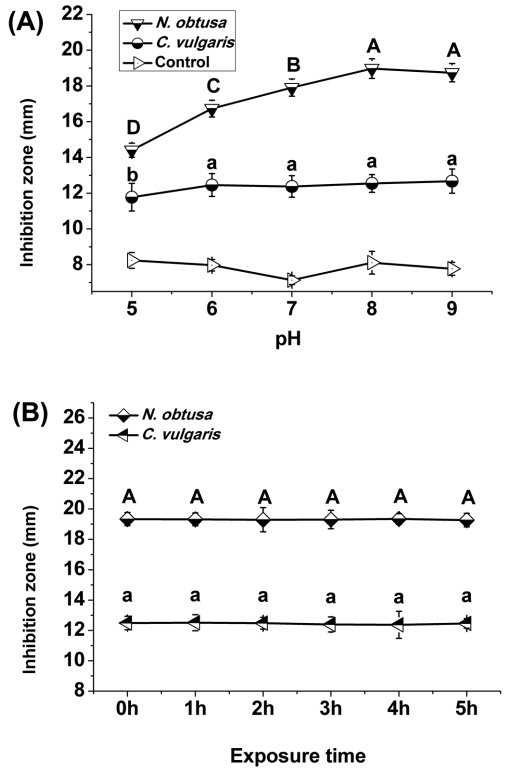

Fig. 4: Effect of pH (A) and UV (B) on antimicrobial activities in extracts from *N. obtusa* and *C. vulgaris* against *S.aureus*. For pH effect, the control sets were prepared using 80% ethanol that no extract was added. Different letters indicated significant differences ($p<0.05$; one-way analysis of variance (ANOVA) and Duncan's multiple range test). Bars represent the means ± standard deviation. Each was replicated nine times.

volatilization was accelerated and the solid to liquid ratio was lowed, and thus the yields of antimicrobial activities were decreased (Fig. 2 (B)). Shorter extraction times and lower ethanol concentration would result in incomplete extraction, longer extraction times and higher concentration would lead to waste of time and energy (Fig. 2 (C and D); Bernardo-Gil et al., 2009).

The orthogonal experimental design was used to study optimization of parameters for efficient extraction of antimicrobial substances from *N. obtusa* and *C. vulgaris*. The advantage of orthogonal experimental design is that it is economical for characterizing a complicated process in fewer experiments. However, it requires a specialized experimental design to properly set up the test and specialized statistics to analyze data (Díez et al., 2008). The results (Tab. 4, 5 and 6) revealed that factor A (Solid to liquid ratio), factor B (Temperature), and interaction A × B had significant effects on the antimicrobial activity of *N. obtusa*. For *C. vulgaris*, ethanol concentration had significant effect on the antimicrobial activity, while the other factors and interactions were identified as insignificant factors and interactions under the selected conditions based on ANOVA. We concluded that solid to liquid ratio and temperature were the two major factors affecting extraction of *N. obtusa*, and ethanol concentration was the major factor affecting extraction of *C. vulgaris*. Thus, we should pay more attention to these factors in extraction. In summary, the optimum extraction condition for *N. obtuse* was defined as below: solid to liquid ratio: 1:20, temperature: 85°C, ethanol concentration: 50%, extraction time: 6h, and the best combination of extraction parameters for *C. vulgaris* was: solid to liquid ratio: 1:15, temperature: 85°C, ethanol concentration: 70%, extraction time: 10h. Compared with conventional extraction condi-

tions, our optimum extraction conditions in this study are economic, convenient and efficient (Li et al., 2010). Further, this extraction method meets the actual needs and is also compliant with environmental regulations.

Environmental factors often influence the efficacy of bactericides (Qasem and Abu-Blam, 1995). In this study, we tested whether pH and UV could influence the antimicrobial activity. The pH of extraction is a significant factor, which may affect the extraction procedure. Maximum efficiency of antibacterial activity from *N. obtuse* extracts was observed when pH was 8 and 9, whereas the extract from *C. vulgaris* exhibited pH stability in the range from 6 to 9 (Fig. 4 (A)). It was observed that greater antimicrobial activities were obtained under neuter and alkaline conditions. As exposure time changed, no statistically significant ($P<0.05$) differences were observed between different UV treatments. The result (Fig. 4 (B)) showed that extracts were stable following exposure to UV light. These results indicate that *N. obtusa* and *C. vulgaris* have a great potential for the extraction of antibacterial substances. The antibacterial activities against Gram-positive bacteria should be applied to plants in the field, food process, and medicine.

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