

Original Research Paper

Antibacterial Activity of Lotus Leaves (*Nelumbo Nucifera*) Against Food-Borne Pathogens

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Abstract: The antibacterial activity of different solvent extracts from lotus leaves against four food-borne bacteria (*Escherichiacoli*, *Salmonellatyphimurium*, *Staphylococcus aureus*, *Bacillus subtilis*) was analyzed by agar diffusion method and the ethanol extract of the plant was found to have the highest antimicrobial activity with the diameter of inhibition zones ranging from 17.2 to 17.8 mm. Then the ethanol extract of lotus leaves were further separated by MCI-gel column and the antibacterial activity were investigated via microdilution broth method. Furthermore, preliminary phytochemical test were also carried out. Fraction 3 eluted from MCI-gel was found to have highest antibacterial activity with the minimum inhibitory concentration and minimum bactericidal concentration values in the ranges of 0.0313~0.125 g mL⁻¹ and 0.0626~0.25 g mL⁻¹, respectively. Antifungal activity analysis showed that ethanol extract of lotus leaves had a low inhibitory activity against all fungi. The result of the phytochemical analysis of the extract showed the presence of phenolic compounds, flavones and alkaloids which may be responsible for its antibacterial activity. Furthermore, lotus leaves extract was observed to have a better antiseptic capacity than sodium benzoate in apple juice (preservative used in food). Therefore, lotus leaves may be used as a botanical natural food preservative against food-borne pathogens.

Keywords: Lotus Leaves, Antibacterial Natural Product, Food Preservative, Phytochemicals

Introduction

Spoilage of food products is due to chemical, enzymatic or microbial activities. One-fourth of the world's food supply is lost through microbial activity alone (Ghaly *et al.*, 2010). Although the chemical food additives have a good effect on microorganism control and have been widely used in food preservation, it is reported that synthetic chemicals like paraben, have been found to have harmful effects (Ishiwatari *et al.*, 2007). Besides, a series of food safety issues has aroused consumers' increasing concerns and their wish of pursuing more natural and safer antimicrobial compounds. Some natural substances of plant origin, phenolic compounds and secondary metabolites such as flavonoids, tannins, alkaloids, organic acid and essential oils, have been

intensively reported to be biologically active, especially endowed with antimicrobial properties (Cowan, 1999).

Lotus (*Nelumbo nucifera*) is an angiosperm and classified in floating leaved plants among aquatic plants. It distributes widely in Hunan, Hubei, Zhejiang and Jiangsu Provinces of China. China is rich in lotus leaves resources. Lotus leaves has been included in the second batch of "both food and medicine" list by the Ministry of Health of the People's Republic of China in 1999, which is considered safe in traditional food processing. Many bioactive and pharmacologically important compounds have been found in *Nymphaeas* species and used in medicine. It has been reported that the quercetin extracted from lotus leaves may be a potential antibacterial agent for periodontitis (Li and Xu, 2008). Furthermore, the flavonoids, alkaloids and volatile oils from lotus leaves has been reported have

strong inhibition on bacteria but no distinct inhibition on yeast and mold (Yihong, 2007). Besides the antibacterial activity, lotus leaves extracted by methanol and ethanol was reported to possess antioxidant activities (Choe *et al.*, 2010). With the above information, the lotus leaves were tested for antibacterial activity against some food-borne pathogens. The aim of the research was to evaluate the antimicrobial activity and qualitative analysis phytochemicals of lotus leaves.

Materials and Methods

Microorganism Strains

The tested microorganisms including Gram-negative bacteria: *Escherichia coli* (*E. coli*), *Salmonella typhimurium* (*S. typhimurium*) and Gram-positive bacteria: *Staphylococcus aureus* (*S. aureus*), *Bacillus subtilis* (*B. subtilis*); fungi: *Saccharomyces cerevisiae* (*S. cerevisiae*), *Aspergillus niger* (*A. niger*), *Penicillium citrinum* (*P. citrinum*). The bacterial were maintained on Luria-Bertani agar culture medium at 4°C and cultured on nutrient agar culture medium at 37°C for 24 h. The fungi were cultured on Potato Dextrose Agar (PDA) at 28°C for 24 to 72 h.

Reagents and Equipment

Lotus leaves were collected from local ponds at Wuxi, China in October, 2012. Petroleum ether, chloroform, ethyl acetate, n-butyl alcohol Dimethylsulphoxide (DMSO), sodium benzoate, oxford plant were purchased from Sino harm Chemical Reagent Co. Ltd. MCI-gel column (CHP20/P120) was purchased from Mitsubishi Chemical Holdings Corp.

Extract Preparation

Lotus leaves were dried at room temperature and crushed into powders with a grinder (DFT-50, xinnuo, Co, Ltd, Shanghai, China). The powders (20 g) were extracted by maceration in different solvents, petroleum ether, chloroform, ethyl acetate, n-butyl alcohol, ethanol and aqueous successively for 12 h. Thereafter, the extracts were filtered through Whatman No. 1 filter paper and condensed by rotary evaporator at 45°C, the dried extractions were dissolved in 100% Dimethyl Sulfoxide (DMSO) to a final concentration of 1 g mL⁻¹ (defined as 20 g lotus leaves/20 mL DMSO) and stored in 4°C for further analysis.

Inhibition Zone Test

The inhibition zones were determined by agar diffusion method (CLSI, 2012) with some modifications. Tested bacterial and fungi were cultured in nutrient broth at 37 or 28°C for 24 h, then

100 µL of suspension bacteria and fungi spores were coated on the plats. Holes (6 mm in diameter) were made by Oxford cup and impregnated with 100 µL of different solvent extracts. The DMSO was used as negative control. Sodium benzoate (100 mg mL⁻¹) was used as positive control. All the plates were incubated at 37°C for 24 h. Each assay was replicated three times.

Determination of Minimum Inhibitory Concentration (MIC), Minimum Bactericide Concentration (MBC) and Minimum Fungicidal Concentration (MFC)

MIC is the lowest concentration that completely inhibits growth of the organism in the tubes as detected by the unaided eye. The MIC were determined by macrodilution broth method (Wikler, 2009). The tested lotus leaves extract added to tubes contains autoclaved nutrient broth to keep the final concentrations of extract in the range of 31.3~500 mg mL⁻¹. All the bacteria and yeast cultures were adjusted to 0.5 McFarland standard contain approximately 1 to 2×10⁸ CFU mL⁻¹, then the adjusted in oculum suspension in broth was diluted to keep the final bacteria concentration of 5×10⁵ CFU mL⁻¹. Hemocytometer was used to control the final fungi spores concentration of 0.4 to 5×10⁴ cells mL⁻¹ (CLSI, 2002). MIC values were recorded after incubated for 16 to 20 h.

The inoculated tubes showing no increases in turbidity were coated on plats. The plates were incubated for 24 h at 37°C for bacterial strains and for 24 to 72 h at 28°C for fungal strains. The corresponding concentration of the extracts on which the growth of the microorganism was totally inhibited was determined as the MBC or MFC values.

Isolation of the Antibacterial Compounds

The lotus leaves powders (10 g) were added to 200 mL of ethanol with stirring at 37°C for 24 h, the extract was filtered and condensed and dissolved in water (10 mL), then separated by MCI-gel column with a methanol-water gradient to give five fractions. The elution volumes were as follows: Fraction 1:10% methanol-water (500 mL), fraction 2:30% methanol-water (500 mL), fraction 3:50% methanol-water (500 mL), fraction 4:70% methanol-water (500 mL), fraction 5: Methanol (500 mL). The eluent were condensed and dissolved in 10 mL 10% DMSO defined as 1 g mL⁻¹ for further analysis.

Phytochemical Screening

The antibacterial fractions isolated by MCI-gel column were subjected to phytochemical tests as

described by (Morsy, 2014) to determine the presence of various chemical compounds.

Antiseptic Capacity in Apple Juice

The fresh apple juice was used to evaluate the antiseptic capacity of lotus leaves extract in food system. The ethanol extract of lotus leaves dissolved in water was added to the apple juice with the final concentration of 0.05% (w/v). The same concentration of sodium benzoate was used as the positive control. Then, the apple juice was stored at 37°C. The total counts of bacteria were determined by diluting the apple juice with water to a suitable concentration and 1 mL of the sample was coated on the surface of Luria-Bertaniagar culture medium. Colony counting after 24 h of incubation at 37°C was performed at 0, 3, 6, 9, 12 and 15 days. All procedures were performed under sterile conditions.

Statistical Analysis

Descriptive statistical analyses for calculating the means and the standard deviation of the mean were performed using the Statistical Package for Social Sciences (SPSS) version 19 program. A probability value at $p < 0.05$ was considered statistically significant.

Results

In Vitro Antibacterial Assay

The antibacterial activity of the plant extract can be judged according to the size of the inhibition zone. As shown in Table 1, the petroleum ether and aqueous extracts of lotus leaves were not effective against the growth of bacteria tested. Comparatively, the ethanol extract exhibited the highest inhibitory effect on four tested bacteria with the diameter of inhibition zones ranging from 17.2±0.6 to 17.8±0.3 mm.

The inhibitory effect of ethanol extract was higher than the positive control sodium benzoate, which is effective food preservative of bacteria spoilage. The inhibitory effects of other solvent extractions are relatively weak with the inhibition zone less than 17 mm.

In Vitro Antifungal Assay

The ethanol extract of lotus leaves was also tested for its antifungal activity, as shown in Table 2, the lotus extract exhibited comparative inhibitory effect to sodium benzoate against *S.cerevisiae* with inhibition zone of 13.4 mm, where as neither lotus leaves extract nor sodium benzoate had any antifungal activity (inhibition zone) against the mycelium of *A.niger* and *P.citrinum*. Ethanol extract of lotus leaves showed MIC/MFC values of 0.25/0.25 (g mL⁻¹) against *S.cerevisiae*, 0.5/1 (g mL⁻¹) against the spore of *A.niger* and *P.citrinum*. Sodium benzoate showed MIC/MFC value of 0.0625/0.125 (g mL⁻¹) against *S. cerevisiae*, 0.125/0.25 (g mL⁻¹) against *A.niger* and *P.citrinum*.

Antibacterial Assay of MCI Gel Separated Samples

The antibacterial activity of five fractions separated by MCI-gel was assessed by the inhibition zones and MIC/MBC values. As shown in Fig. 1, all fractions except fraction 1 and 5 showed different degrees of antibacterial activity with the fraction 3 being found to be most effective against the tested bacteria with diameter of inhibition zones ranging from 13.5~15.3 mm, while as positive control the diameter of inhibition zones of sodium benzoate ranging from 12.7~14.1 mm. The result indicated that *S.typhimurium* was the most sensitive bacterium to the fraction 2, 3, 4 with the inhibition zone of 11.4, 15.3 and 14.2 mm, respectively.

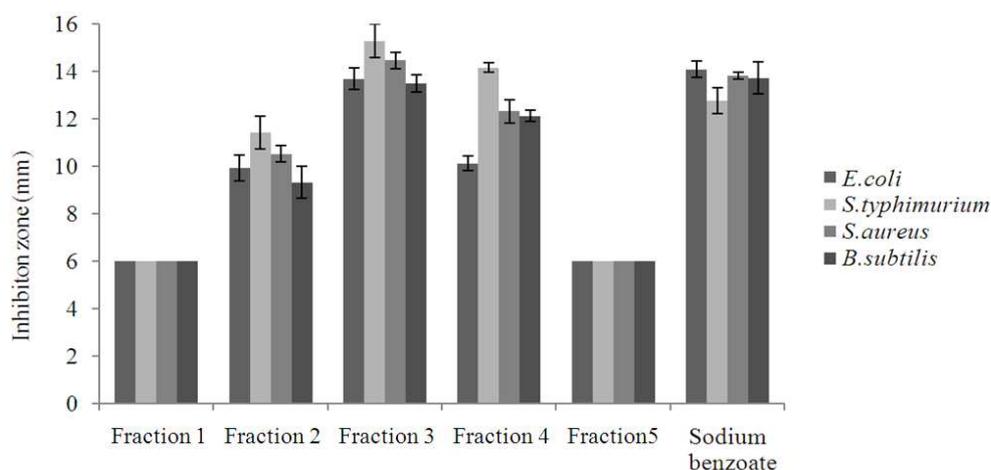


Fig. 1. Antibacterial effect of five fractions by MCI-gel column chromatographic

Table 1. Antibacterial activity (inhibition zone, mm) of various solvent extracts of lotus leaves against food-borne pathogens

Microorganisms	<i>E. coli</i>	<i>S. typhimurium</i>	<i>S. aureus</i>	<i>B. subtilis</i>
Petroleum ether	NI ^a	NI ^a	NI ^a	NI ^a
Chloroform	11.9±0.4 ^b	12.2±0.4 ^b	12.8±1.0 ^c	10.9±1.0 ^b
Ethyl acetate	13.9±0.7 ^c	13.3±1.5 ^b	12.8±0.5 ^c	13.7±0.3 ^c
n-butyl alcohol	16.3±0.5 ^d	16.9±0.6 ^c	15.8±0.3 ^d	15.5±0.2 ^d
Ethanol	17.8±0.3 ^e	17.2±0.6 ^c	17.6±0.6 ^e	17.6±0.3 ^e
Aqueous	NI ^a	NI ^a	NI ^a	NI ^a
Positive control	16.5±0.2 ^d	16.8±0.3 ^c	17.2±0.3 ^e	13.7±0.2 ^c
Negative control	NI ^a	NI ^a	NI ^a	NI ^a

*NI means no inhibition zone

Each value is expressed as mean ± SD (n = 3)

100 mg mL⁻¹ sodium benzoate was used as positive control

DMSO was used as negative control

a, b, c, d and e: Different letters within same column means significant different at p<0.05

Table 2. Antifungal activity of ethanol lotus leaves extract against food-borne pathogens

Microorganisms	Inhibition zone (mm) (Mycelium)		MIC/MFC (g/mL) (Spore)	
	Lotus leaves extract	Sodium benzoate	Lotus leaves extract	Sodium benzoate
<i>S. cerevisiae</i>	13.4±0.5	15.4±0.7	0.25/0.25	0.0625/0.125
<i>A. niger</i>	NI	NI	0.5/1	0.125/0.25
<i>P. citrinum</i>	NI	NI	0.5/1	0.125/0.25

Table 3. MIC/MBC of five-fractions against four bacterial (g/mL)

Microorganisms	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5
<i>E. coli</i>	>1	0.25/0.5	0.125/0.25	0.25/0.5	>1
<i>S. typhimurium</i>	>1	0.125/0.25	0.0313/0.0626	0.125/0.25	>1
<i>S. aureus</i>	>1	0.25/0.25	0.125/0.25	0.25/0.25	>1
<i>B. subtilis</i>	>1	0.25/0.5	0.125/0.125	0.25/0.5	>1

Table 4. Qualitative analysis of the phytochemical screening of the bioactive fractions

Phytochemical test	Fraction 2	Fraction 3	Fraction 4
1 Phenolic compounds: FeCl ₃ test	+	+	+
2 Flavones: AlCl ₃ test	+	+	+
3 Tannins: Gelatin test	-	-	-
4 Alkaloids: I-KI test	-	+	-
5 Organic acid: Methyl red test	-	-	-
6 Amino acids: Ninhydrin test	-	-	-
7 Proteins: Biuret test	-	-	-

“+” means the test proved positive; “-” means the test proved negative

The MIC and MBC values of different fractions against the tested bacteria were shown in Table 3. Fraction 3 had the highest antibacterial activity which is consistent with the results of inhibition zones test, the MIC value of fraction 3 against the tested bacteria ranged from 0.0313 to 0.125 g mL⁻¹ and MBC from 0.0626 to 0.25 g mL⁻¹, respectively. As a control, the solvent (10% DMSO) did not show any inhibition on the growth of the tested bacteria.

Phytochemical Screening

Results of the different phytochemical screening for the bioactive fractions of lotus leaves (*Nelumbo nucifera*) are shown in Table 4. Fraction 2, fraction 3,

fraction 4 were positive for the presence of phenolic compounds and flavones. Fraction 3, but not fraction 2 and fraction 4, was also positive for alkaloids. In addition, all fractions were negative for tannins, organic acid, amino acids and proteins.

Antiseptic Capacity in Apple Juice

We used apple juice to evaluate the preservation effect of lotus leaves extract by monitoring the total counts of bacteria. Since lotus leaves showed significant *in vitro* antibacterial activity, we proposed that lotus leaves might possess a potential antiseptic capacity in a food system. As shown in Fig. 2, lotus leaves extract had a better antiseptic capacity than sodium benzoate in apple juice.

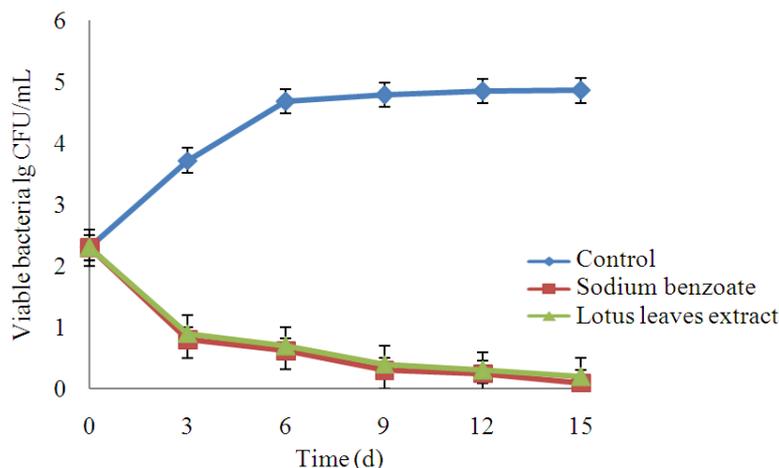


Fig. 2. Antiseptic capacity of lotus leaves extract in apple juice

Discussion

In vitro antibacterial assay results are in agreements with the findings of (Dubey *et al.*, 2012) who reported that ethanol extract of most plants had effective antimicrobial activity against all the isolated multidrug resistant bacteria. Furthermore, the extracts (ethanol, n-butyl alcohol) of leaves showed significant activity against Gram-negative bacteria and Gram-positive bacteria. While antifungal activity analysis showed that ethanol extract of lotus leaves had a low inhibitory activity against all fungi.

According to some reports the presence of secondary metabolites in plants such as alkaloids (Gurudeeban *et al.*, 2013; Budeyri *et al.*, 2012) and flavones (Islam *et al.*, 2002; Li *et al.*, 2012) had antimicrobial activities. This may explain why fraction 3 had the best antimicrobial activity, it indicated that the alkaloids and flavones may have synergistic effect against bacteria growth. While some alkaloids such as colchicine, aconitine, scopolamine, strychnine are toxic even they are isolated from natural product, there is no reports about the toxicological evaluation of lotus leaves alkaloids. It is indispensable for performing toxicological evaluation of lotus leaves alkaloids in the future for its safety applied in food.

Conclusion

Based on this study it may be concluded that the antibacterial activity of the lotus extract could be related to the presence of alkaloids and flavones components. Results of present study suggest that the lotus leaves extract possess antibacterial compounds that may be used as a botanical natural food preservative against food-borne pathogens.

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Author's Contributions

Xiaotian Chen and Changyin Wang do all the experiments. Jianxin Chen and Yuanda Song provided the ideas and guidance. Gbago Onivogui responsible for the modification of the article

Ethics

The authors guarantee this acritical don't have multiple submissions, academic fraud action.

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