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Original Research Article

Chemical Composition and Anti-Biofilm Activity of Burdock (*Arctium lappa* L *Asteraceae*) Leaf Fractions against *Staphylococcus aureus*

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Abstract

Purpose: *To determine the chemical composition and anti-biofilm activity of burdock leaf fractions against Staphylococcus aureus*
Methods: *The anti-biofilm activity of burdock leaf fractions obtained by column chromatography against S. aureus was determined by minimum inhibitory concentration (MIC). Scanning electron microscopy was employed to further investigate the inhibitory activity. Analysis of the chemical composition of the fractions was performed by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS).*
Results: *The 20 and 34 % ethanol fractions each inhibited the formation of biofilm by S. aureus, with half maximal inhibitory concentration (IC_{50}) ranging from 110 to 150 µg/ml. The 70 % ethanol elution fraction exhibited the strongest inhibitory activity against biofilm formation with IC_{50} of 13 µg/ml. The minimum inhibitory concentration of the 70 % ethanol fraction completely inhibited the formation of biofilm at a concentration of 0.5 mg/ml, which was lower than the MIC for the growth of the test bacterium (1.25 mg/ml). Scanning electron microscopy (SEM) showed that there was no biofilm formation for cultures treated with burdock leaf fraction, thus confirming the inhibitory efficiency of burdock leaf fraction against biofilm formation. UPLC-MS data identified five active compounds, namely, :caffeic acid, p-coumaric acid, cynarin, quercetin and luteolin.*
Conclusion: *The biofilm formation inhibitory effect of burdock leaf was not only due to its inhibitory effect on bacterial growth but appear to be influenced by its effect on bacterial surface hydrophobicity, and aggregation. Thus, the leaf fractions may be useful in the control of biofilms.*

Keywords: *Biofilm, Staphylococcus aureus, Arctium lappa, Burdock leaf, Scanning electron microscope, Ultra-performance liquid chromatography-mass spectrometry*

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INTRODUCTION

Biofilm is a stubborn contamination source in food industry sectors such as dairy processing, fresh agricultural product, poultry processing and red meat processing [1]. Once the contamination of food occurs, the source of the problem is often a biofilm [2,3]. Evidence indicates that biofilm mode of life results in growing resistance to antibacterial agents [4,5]. Compared to planktonic cells, biofilm is more resistant to antibacterial products. Searching for new strategies for the inhibition of biofilm is becoming highly necessary [6,7]. In recent years, the effect of plant constituents on biofilm inhibition has attracted increasing attention [8,9]. The extracts

and natural compounds of plants to replace existing synthetic antimicrobials are becoming more popular [10].

Arctium lappa L belongs to the family Asteraceae, and is a popular vegetable in China, Japan and many other Asian countries; its leaf exhibits high antibacterial activity against *Streptococcus sobrinus* and *Streptococcus mutans* [11,12]. However, the effect of this plant leaf on the formation of biofilm by *S. aureus* has not been studied before. Furthermore, if burdock leaf components exhibit inhibitory effect on biofilm formation, its mechanism including impacting bacterial surface hydrophobicity, aggregation, bacterial extracellular polysaccharides effect could be investigated in order to provide more information for inhibition of biofilm formation.

In the present work, a food-related bacterium *S. aureus* was chosen as the test microbe against which, the inhibitory activity of burdock leaf extract assessed for formation of biofilm. Visual analysis by scanning electron microscopy (SEM) was performed to further evaluate the effect of burdock leaf fractions on biofilm formation. Furthermore, the chemical composition of burdock leaf fraction with the strongest inhibitory activity against biofilm formation 70 % ethanol fraction was studied in the last part of this paper.

EXPERIMENTAL

Materials

Dry burdock (*Arctium lappa* L) leaves were obtained from Sideline Products Co, Ltd (Xuzhou, China). A voucher specimen JNU2013091501) has been deposited at Herbarium of Food Nutritional and Functional Factors Center of Jiangnan University, Wuxi, China. They were identified by Professor Jian Tang in Jiangnan University, Wuxi, China.

Alcohol (95 %), sodium chloride, glutaraldehyde, and ethanol were purchased from Sinopharm Chemical Reagent Co, Ltd (Shanghai, China). Caffeic acid, chlorogenic acid, vanilic acid, o-hydrobenzoic acid, ferulic acid, benzoic acid, quercetin, cynarin, quercitrin, luteolin, arctiin were obtained from Sigma (Shanghai, China). They were all of analytical grade with a purity of 98 %.

Extraction and fractionation of leaf material

After cleaning with tap water and then with double distilled water, the leaves were freeze-dried, crushed into powder in a grinder (ZSJD, Kaichuang Mechanism Co, Ltd, China) to a size range of 0.5 – 1.2 mm. The dried powder of burdock leaf (500 g) was extracted in a 12 L flask filled with 30 % aqueous ethanol and the ratio of solvent to solid was 20:1 (ml/g). The extraction was carried out at for stiring at 250 rpm using a electric stirrer (JB50-D, Shanghai Specimen model Factory, China) at 30 ^{o}C, for 10 h. The extracts were filtered and then concentrated using a rotary evaporator at 50 ^{o}C under vacuum. The crude extracts (100 g) were dissolved in water, then loaded on a glass column (6 cm × 100 cm) of macroporous resin (HPD-100, Cangzhou Bon Adsorber Technology Co, Ltd, China). In the macroporous resin column chromatography, water and ethanol (20, 34, and 70 %) was successively used to desorb target components at a successively flow of 1.5 BV/h, respectively. Each eluted fraction was collected and dried. Thereafter, the water as well as 20, 34 and 70 % ethanol elution fractions were obtained. The resulting extract was kept in a vacuum dryer in a dark place at room temperature until used.

Measurement of the effect of burdock leaf fractions on formation of biofilm by *S. aureus*

The effect of the fractions on biofilm formation was assessed in LB broth (trypsin: 10g/L, Yeast Extract: 5g/L, Nacl: 5g/L) according to the method of Figueiredo *et al* [13]. *S. aureus* 6538 was incubated overnight for 12 h in glass bottles at 37 ^{o}C. The bacterial suspension (10^{8} CFU/ml, 1.0 ml) was transferred into 9.0 ml of fresh broth (trypsin: 10g/L, yeast extract: 5g/L, NaCl: 5g/L) containing various concentrations of burdock leaf fractions dissolved in alcohol and sterilized. Then plastic coverslips (Q7-4735; Dow Corning) were added. *S. aureus* was allowed to form biofilms on plastic cover slips (Q7-4735; Dow Corning). After incubation for 24 h at 37 ^{o}C, the growth medium was removed and the cover slips rinsed with 0.9 % (w/v) NaCl. Each cover slip was transferred to 1 ml of saline normal in tubes. The tubes were subjected three times to 1 min of sonication (Ultrasonic cleaner, DL-360, 40 kHz, 90 W, Shanghai Zhixin Corp, China). Each suspension (100 µl) in tubes was coated on LB agar plates and incubated at 37 ^{o}C. Twenty four hours later, the colonies were counted and only plates containing between 30 and 300 counts for each dilutions series were counted. For all the assays, a positive control without burdock leaf fraction and a negative control without inoculation were prepared. IC_{50} was defined as the lowest agent concentration that showed 50 % of inhibition of the formation of biofilm. The procedure was performed in triplicate.

Determination of MIC

To determine the MIC of each spice extract, a broth micro-dilution method was performed according to the method of Jorgensen and Turnidge [14], Ninety-six-well culture plates were prepared, and serial two-fold dilutions of the extracts were dispensed into the plate wells. The volume of dispensed extract was 0.1 ml per well in the concentration range of 50 mg/ml to 5 mg/ml. The concentration values were expressed on the basis of lyophilized materials dissolved in water. The same volume (0.1 ml) of overnight bacterial culture at a density of 10^5 CFU/ml was added to the wells, and the culture plates were placed in an incubator set at 37 °C for 24 h. The lowest concentration of the plant extract required to inhibit visible growth of the tested microorganism was designated as the MIC.

Scanning electron microscopy

The 70 % ethanol eluted fraction exhibited the highest anti-biofilm activity. Thus, a scanning electron microscopy analysis [15] was used to further observe the inhibitory activity of 70 % ethanol elution fraction against the formation of biofilm by *S. aureus* on plastic coverslips.

Overnight culture of *S. aureus* {200 µl} was cultivated in small conical flasks containing 10ml of and plastic coverslips. After incubation at 37 ^oC for 5 h, burdock leaf fraction was added (1 mg/ml) to the conical flasks. After incubation for 18 h, the coverslips were rinsed three times with phosphate buffer and then fixed by glutaraldehyde (2.5 %, in 0.075 M phosphate buffer, pH 7.4) for 6 h [16]. The cultures on coverslips were rinsed with phosphate buffer at each interval. The cultures on coverslips were dehydrated in a gradient alcohol concentration (25, 50, 70, 80 90 and 100 %) for 15 min at each concentration [17], and the cultures dehydrated twice in 100 % ethanol. Lastly, they were covered with gold before examination.

Chemical composition analysis of burdock leaf fractions

UPLC-MS/MS analysis was carried out using an ultra performance liquid chromatography (UPLC) apparatus equipped with a Waters Acquity PDA detector (Waters, USA) and a BEH C18 column (50 mm × 2.1 mm, particle size 1.7 µm) (Waters, USA). The eluents were: A, water 0.1 % formic acid; B, acetonitrile/water 0.1% formic acid (20:80, v/v). UV-V absorption spectra were recorded on-line from 200 to 700 nm during the UPLC analysis.

Mass spectroscopic analysis of phenolic compounds in the 70 % ethanol eluted fraction was performed using a SYNAPT Mass Spectrometer (Waters, USA), equipped with an electrospray ionization source operating in negative ion mode which have better peak shape The effluent from UPLC was introduced into an electrospray source (source block temperature 100 ^oC, desolvation temperature 400 ^oC, capillary voltage 3000V, and cone voltage 20 V) [18]. Argon was used as collision gas (collision energy 6 V) and nitrogen as desolvation gas (500 l/h). The peak belonging to each compound was determined by using the software Masslynx (Waters, USA). Identification of the phenolic compounds from burdock leaf was achieved by comparison with retention times of standards.

Statistical analysis

Anti-biofilm and anti-bacterial activitiy data were obtained in triplicate and the results analyzed statistically by ANOVA using SPSS 17.0. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Effect of burdock leaf fractions on the formation of biofilm

The results are shown in Figure 1. The 20 % ethanol elution fraction and the 34 % ethanol elution fraction significantly inhibited the formation of biofilm with the IC_{50} of 0.15 and 0.11 mg/ml, respectively. The 70 % ethanol elution fraction exhibited the strongest inhibitory activity on biofilm formation with an IC_{50} of 13 µg/ml, while water elution fraction showed no significant inhibition on the formation of bacterial biofilms at the concentration of 5 mg/ml. Figure 1 showed that the 70 % elution fraction inhibited 40.9 – 100 % of biofilm formation. The inhibitory effect of the fraction against biofilm formation was related to the concentration. At a concentration of 0.1 mg/ml, the inhibition rate was 76.3 %. When the concentration of 70 % ethanol-eluting fraction was 0.5 mg/ml, 100 % inhibition of biofilm formation was achieved. The IC_{50} value of 70 % ethanol fraction for biofilm inhibiting was 0.013 mg/ml. The inhibition effect of 70 % ethanol elution of burdock leaf against the growth of bacteria was evaluated. The minimum concentration of burdock leaf component, which did not show any growth of tested bacteria was found to be 1.25 mg/ml.

The minimum inhibitory concentration (MIC) of 70 % ethanol fraction, which completely inhibited

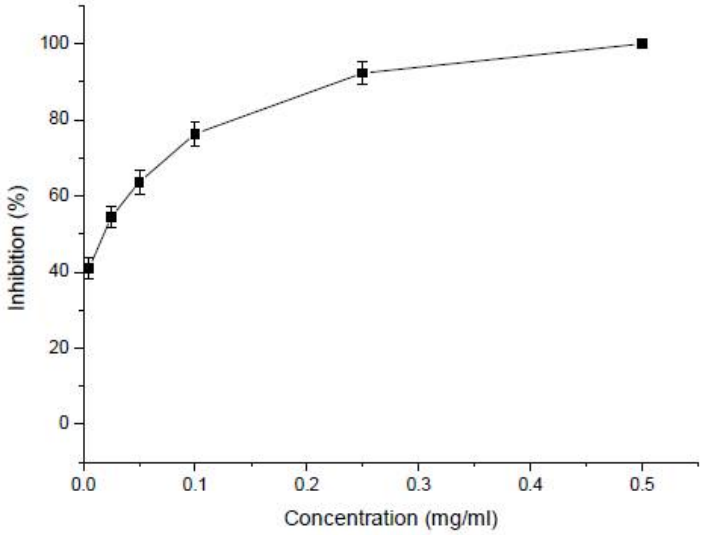
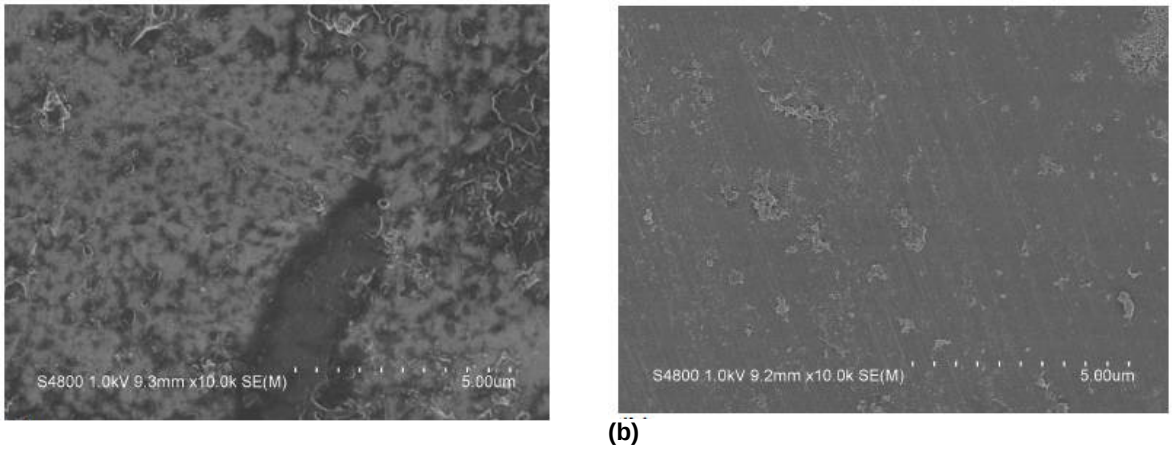


Figure 1: Effect of 70% ethanol elution fraction of burdock leaf on formation of biofilm by *S. aureus*



(a) **(b)**

Figure 2: SEM of bacterial biofilms of 70 % ethanol elution fraction (0.5 mg/ml) of (a) absence of burdock leaf fraction (b) treated with burdock leaf fraction

the formation of biofilm was 0.5 mg/ml which is lower than the MIC of 70 % ethanol fraction for the growth of the tested bacteria (1.25 mg/ml).

Scanning electron microscopy observation

The scanning electron microscopy (SEM) observation showed that there was no biofilm formation for cultures treated with burdock leaf fraction (Figure 2).

The biofilm formation in the absence (control) or presence of 70 % ethanol elution fraction illustrated in Figure 2. A clear picture of the superficial structure and morphology of biofilm can be seen. Figure 2 (a) showed the multilayer growths of bacterial biofilm without the treatment of burdock leaf fraction. However, incubation of *S. aureus* with burdock leaf fraction at a concentration of 0.5 mg/ml resulted in significant reduction of biofilm. There was no formation of biofilm in the sample treated with burdock leaf fraction, suggesting that burdock leaf fraction significantly inhibited the formation of *S. aureus* biofilm.

Chemical composition of burdock leaf fraction

The chromatogram of the 70 % ethanol fraction of burdock leaf was shown in Figure 3. Peak identification was performed by comparing retention times (t_R), UV-V spectra and mass spectra (Table 1) with those of reference standards and literature data. As is shown in Table 1, the first peak was identified as caffeic acid with λmax of 243 and t_R of 3.13. The [M-H]- peak of 179 (along with the fragment ions at 135)

was similar to those reported by in the literature [19,20]. Peak 2, with the t_R of 3.75, was identified as p-coumaric acid (λmax - 310) and the [M-H]- peak of p-coumaric acid was observed at m/z 163. Its characteristic fragment ions, such as m/z 119, were also identical with those of the standard and those reported by Olsen [20]. Peak 3, with the t_R of 4.33 and λmax of 295, was identified as cynarin and the [M-H]- peak of it was observed at m/z 515. Its characteristic fragment ions, such as m/z 191 and 349, were in consistent with those reported in the literature [21]. Peak 4 exhibiting an [M-H]- ion at m/z 301 with the λmax of 255 and a t_R of 5.44 (same as those of standard quercetin), was identified as quercetin. It also showed the release of the predominant fragment ion at m/z 179 and 245. These results are in agreement with those reported in the literature [22]. The fifth peak yielding [M-H]− at m/z 285 was identified as luteolin with the t_R of 6.19 and λmax of 255. Its fragment ions at 133 were found, similar to those reported elsewhere [23]. The high biofilm inhibition efficiency of burdock leaf fraction was probably due to the combination action of phenolic compounds present in the fraction.

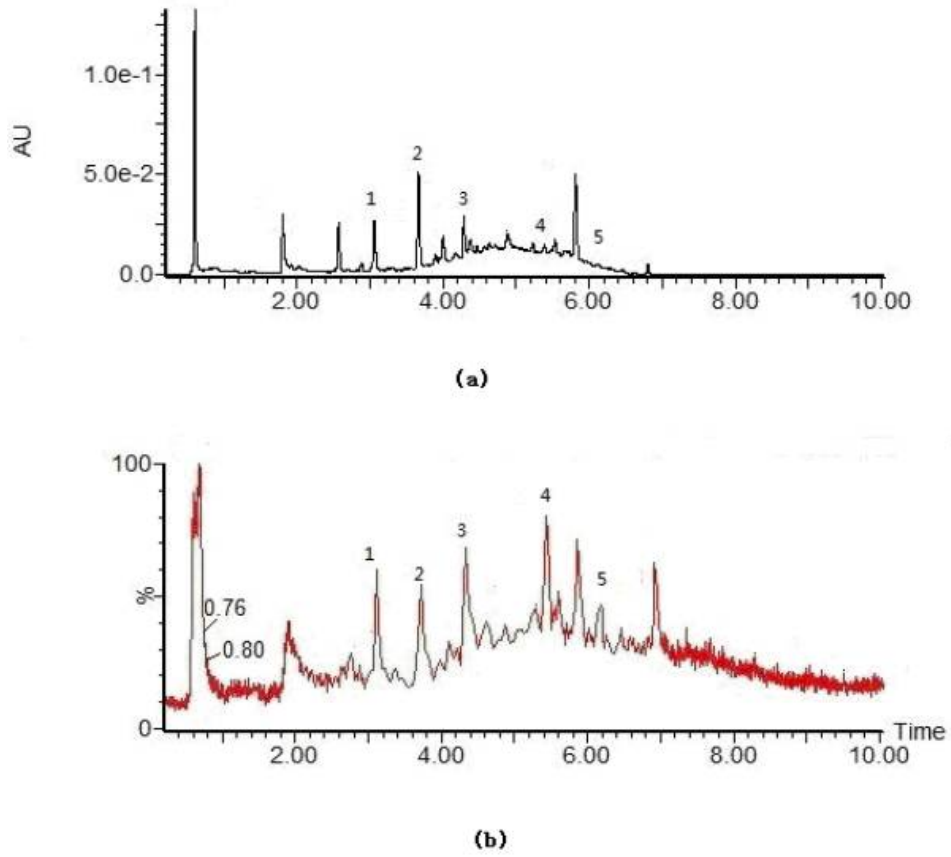


Figure 3: Chromatograms of 70 % ethanol fraction from burdock leaf; (a) UPLC–PAD chromatogram at 280 nm. (b) TIC chromatogram in negative ion mode

Table 1: Identification of compounds in the 70 % fraction using UPLC with Photo-Diode Array (PDA) and electrospray ionization-MS detection

Peak no.	T_R	Precursor ion [M–H]$^-$ (*m/z*)	Product ion (*m/z*)	MW	λmax (nm)	Identity
1	3.13	179	135	180	243	Caffeic acid
2	3.75	163	119	164	310	p-Coumaric acid
3	4.33	515	191 349	516	295	Cynarin
4	5.44	301	179 245	302	255	Quercetin
5	6.19	285	133	286	255	Luteolin

TR:Retention time MV:molecular weight

DISCUSSION

The results show that the lowest biofilm inhibition concentration of 70 % ethanol eluted fraction of burdock leaf was 0.5 mg/ml. Therefore, the minimum concentration of this burdock leaf fraction to completely inhibit biofilm formation was lower than the minimum concentration to inhibit the growth of bacteria. So it could be inferred that the biofilm formation inhibition effect of 70 % ethanol fraction of burdock leaf was not all due to its inhibition effect on bacterial growth, and there are other reasons. Thus, the anti-biofilm mechanism of burdock leaf component needs to be investigated in subsequent studies.

Scanning electron microscopy (SEM) observation showed that there was no biofilm formation for cultures treated with burdock leaf fraction. Micro-colonies were formed without burdock leaf fraction. One more complex three-dimensional structure can be seen. A small amount of mucus can be seen around the micro-colonies. But after treating with burdock leaf, micro-colonies could not be seen. The surface becomes smooth, it can be inferred that burdock leaf fraction significantly inhibited the formation of *S. aureus* biofilm. Caffeic acid, p-coumaric acid, cynarin, quercetin, luteolin were identified as five active compounds in burdock leaf. Caffeic acid is a natural fungicide. Some studies have reported that the sterilization mechanism may be related to its antioxidant activity. The antibacterial activity of caffeic acid depends on pH. It will significantly reduce the antimicrobial activity at lower or higher pH values. It will play a better bactericidal function when it is kept faintly acid [24].

Some studies suggest P-coumaric acid has a significant role in the anti-bacterial activity and interference quorum sensing system [25,26,27]. Quercetin was found to inhibit biofilm formation by *V. harveyi* BB120 and *E. coli* O157:H7 [28]. This three should be the anti-biofilm compounds in 70 % burdock leaf. These compounds can be separated to further verify the biofilm inhibiting activity and to study the synergy or antagonism between these compounds.

The component of burdock leaf was proved to exhibit anti-biofilm activity by pour plate method and scanning electron microscopy. Especially the 70 % ethanol eluted burdock leaf fraction, the inhibition activity reached 50 % at a concentration of 0.03 mg/l. The inhibition activity can reach 100 % at a concentration of 0.5 mg/ml. Burdock leaf is an edible vegetable and hence its fraction can be used as a safe and harmless bacterial biofilm inhibitor to inhibit biofilm formation in food. Currently, there is growing concern over food safety and health. The safety of food additives has received attention as well. Burdock leaf fractions, prepared as described in this study can be used as a natural inhibitor of biofilm and thus have a wide range of applications in the food industry.

CONCLUSION

Burdock leaf can inhibit the formation of biofilms, and this has great significance in efforts to improve food safety standards to protect the health of citizens. Since biofilm formation inhibition by burdock leaf is not entirely due to the inhibition of bacterial growth, the anti-biofilm mechanism of the burdock leaf components needs to be further investigated.

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