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Study on the Antimicrobial Effects of Aqueous Extracts from *Portulaca oleracea* L

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***Portulaca oleracea* L. is distributed across China and used a long time ago in clinic for the treatment of microbial infections, as a traditional Chinese herbal medicine. This work was performed in order to evaluate the antimicrobial activities of aqueous extracts from *Portulaca oleracea* L.. The MIC and MBC determinations combined with confocal laser scanning analysis were used to study the antimicrobial activities of aqueous extracts, against the common pathogens of *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Escherichia coli*. The microorganisms were incubated on the biofilm-grown and planktonic stages, respectively.It was found that the extracts had the great potential inhibiting activities against all 3 microorganisms, with the MIC value of 0.25 %. As to these microorganisms, *Staphylococcus aureus* has the lowest MBC value (0.125 %). Further, the CLSM data reveal that the antimicrobial effects of the extracts are with the concentration- and time-dependent relationships, and there is no linear relationship between bacterial biofilm inhibition activities and concentration of the extracts. Therefore, the aqueous extracts from *Portulaca oleracea* L. might be considered as a potential antimicrobial agent.**

Key words: *Portulaca oleracea L,* Aqueous extracts, Antimicrobial, MIC, MBC, Confocal laser scanning.

Common purslane (*Portulaca oleracea* L., also known as Verdolaga) belongs to genus Portulaca and the family of *Portulacaceae*. It is an annual succulent and has a cosmopolitan distribution including north of China, with the properties of fast growing and long viability[1]. Although in some regions it is considered as an invasive weed, it can be eaten as a leaf vegetable with mucilaginous quality, throughout Europe and Asia[2]. In China, purslane has a long history of use for medicinal purposes[3, 4].

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Previous reports indicated that purslane offers better nourishment than the major cultivated vegetables, such as a richer source of α-linolenic acid (LNA)[3, 4]. In particular, it was well known as Traditional Chinese Medicine, being clinically effective treatment for oral lichen planus, bacillary dysentery, diarrhea, hemorrhoids, postpartum or intestinal bleeding, hypotensive and antidiabetic patients[5]. Further studies reveal that it contains many compounds, including noradrenaline, calcium salts, alkaloids, malic acid, citric acid, nicotinic acid, flavonoids, polysaccharide and cardiac glycosides[6]. Among them, the flavonoids show in vitro cytotoxic activities towards some types of human cancer cells, and purslane is always used for pregnant woman with cold and weak digestion[7, 8]. Besides, it was found that the extracts

from various parts of *Portulaca oleracea* L. exhibit antimicrobial activities with the immunity enhancing[9]. Currently, the applicability of herbal medicine has been largely expanded as their antimicrobial, antibiotic, anticarcinogenic, and sedative properties, with less toxicity and side effect[10-12]. Indeed, their antimicrobial activities have ever been reported and some of them are already used in topical applications against bacteria and fungi infections[12-14]. However, the antimicrobial activities of aqueous extracts from *Portulaca oleracea* L. have not been studied objectively under the microorganism biofilms.

It has become clear that bacterial biofilms are communities of unicellular organisms attached to the surface and the properties of biofilm-grown and planktonic cells are distinct significantly, one of which is an increased resistance to antimicrobial agents[15]. Recent work has indicated that there are great alterations on the structure of exopolysaccharides or other aspects (rpoS, multiple drug resistance pumps and et. ac.) of biofilm architecture, with a biofilm-specific biocide-resistant phenotype[15]. Thus, great efforts have been devoted to the exploration of novel antimicrobial agents directed on the bacterial biofilms[16-18].

To the best of our knowledge, the antimicrobial activities of aqueous extracts from *Portulaca oleracea* L. in the biofilms have not been evaluated yet. Therefore, in this work, the extracts will be extracted by water extraction method and the activities will be evaluated by the MIC and MBC determinations, as well as the exposure of biofilms to extracts. Subsequently, the physiological active of cells in biofilms will be visualized and studied via the confocal laser scanning. We anticipate that the investigation will be of value in the development of antimicrobial agents against the bacterial biofilms.

MATERIALS AND METHODS

Extraction of *Portulaca oleracea* L.

Portulaca oleracea L. was collected in July from the forest farm of Northeast Forestry University, Harbin, China, and was authenticated by Prof. Shao-Quan Nie from the Key Laboratory of Forest Plant Ecology, Ministry of Education. Voucher specimens were deposited in the herbarium of laboratory. Fresh and intact *Portulaca oleracea* L. was picked to shade dried as experimental material. Then, the extracts were filtered and concentrated via the water extraction method.

Microbial strains and culture conditions

The microorganisms used for testing antimicrobial sensitivity included Staphylococcus aureus ATCC 6538, *Staphylococcus* epidermidis ATCC 49134 and *Escherichia coli* ATCC 11229. They were obtained from the Center for Microbiology Research, Jiamusi Medical Research Institute. The strains were cultured in Luria-Bertani (LB) at 37 °C[12, 19].

MIC and MBC determination

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were measured by the broth micro-dilution method according to the National Committee for Clinical Laboratory Standards[20]. The aqueous extracts were dissolved in sterilized physiological saline solution (0.9 % w/v) supplemented with Tween 80 (Sigma)[12, 19]. Serial doubling dilutions of the aqueous extracts were prepared in a 96-well microtiter plate in the range of 5.000 % to 0.039 %. The final concentration of each strain was adjusted to 1.0×10^5 CFU/ml. All microtiter plates against all microorganisms were incubated at 37 °C for 24 h[12, 19]. After activation, the MICs and MBCs were determined, with the positive control of Amphotericin B (Tianjin Chemical Reagents Co., Tianjin, China). Each experiment was repeated in triplicate.

Cultivation of biofilms

The conditions and incubation period for the production of the bacterial biofilms were established according to previous reports[21-23]. Bacterial biofilms were prepared by aliquotting 200 ml of the bacterial suspension containing 1.0×10^5 CFU/ml into the wells of white walled, clear bottom, tissue culture-treated 96-well microtitre plates. Four wells in the last column of each plate were left blank to serve as bioluminescence negative controls. Suspensions of *Staphylococcus aureus, Staphylococcus epidermidis* and *Escherichia coli.* were prepared in the LB supplement. Microtitre plates containing bacterial suspensions were incubated in air at 37 °C for 48 h.

Exposure of biofilms to aqueous extracts

Mature biofilms were exposed to the aqueous extracts from *Portulaca oleracea* L.,

under the final concentrations of 1.25, 2.50, 5.00 and 10.00 % (v/v), respectively. The saline (0.9 % w/v) and ethanol solutions (5.0 % v/v) were treated as blank and positive control, respectively. In these experiments, the spatial distribution of dead and live cells was observed, after 24 h of exposure at 37 °C and washed three times with PBS (pH 7.4).

Confocal laser scanning microscopy

Image acquisition was performed with an Olympus FV1000 confocal laser scanning microscope (Olympus, Japan) equipped with an argon and a NeHe laser and detectors and filter sets for simultaneous monitoring (excitation, 488 nm). Before observation, the specimens were stained with propidium iodide (PI, sigma) and fluorescein diacetate (FDA, sigma) refereed to previously literatures[24-26]. The alive cells will be stained with the FDA dye and visualized with a diffusely distributed green fluorescence, whereas those with damaged membranes (dead) will be stained with PI and with fluorescent red. Thus, the viability of cells could be assessed by this way. Each assay was performed in quadruplicate and repeated at three times.

Table 1. Antimicrobial activity of the aqueous extract from *Portulaca oleracea* L.

Bacterial strain	MIC (g/ml)	MBC (%)
Staphylococcus aureus ATCC 6538	0.25	0.25
Staphylococcus epidermidis ATCC 49134	0.25	0.25
Escherichia coli ATCC 11229	0.25	0.25

RESULTS AND DISCUSSION

MICs and MBCs

Results of the MIC and MBC studies are presented in Table 1. It was found that the aqueous extracts from *Portulaca oleracea* L. exhibited significant antibiosis activities, especially for Staphylococcus aureus, with the MIC and MBC values of 0.25 % and 0.125 %, compared with the results of Franzblau *et. al*.[9]. Besides, the aqueous extracts exhibited rather high inhibitory effects on *Staphylococcus epidermidis* and *Escherichia coli.* As to the two testing microorganisms, growth inhibition was observed with the MICs and MBCs equaling 0.25 %. The MBCs were similar or even higher than the corresponding MIC values. Confirmed by both MICs and MBCs data, it was indicated that the gram-positive instead of gram-negative bacteria were sensitive to the aqueous extracts from *Portulaca oleracea* L.

Inhibitory effects on bacteria biofilm

It has been confirmed that a number of human infections diseases are associated with the corresponding bacteria in nature living in spatially distinct communities, also as bacterial bioflims[16, 27, 28]. Therefore, consistent efforts have been devoted to new potential antimicrobial therapeutics, such as aqueous extracts[16, 27, 28]. As the MICs and MBCs, the aqueous extracts from *Portulaca oleracea* L. are attracting increasing interest as a novel potential agent. Thus, the 3 microorganisms were further used to evaluate the bioactivities of extracts in the bioflims, followed by the observations via confocal laser scanning microscopy (CLSM).

CLSM image analysis demonstrated that the aqueous extracts inhibited the gram-positive

Table 2. The ratios of fluorescence intensity with the treatment of various concentration of the aqueous extract derived from *Portulaca oleracea* L.

(Alive /Dead)	Negative Control	5% C_2H_5OH	Essential oil 1.25 %	2.50 %	5.00 %	10.00 %
Staphylococcus aureus, ATCC 6538	11.75± 1.17	1.43± 0.09	3.96± 1.31	0.11± 0.02	0.04± 0.01	0.62± 0.42
Staphylococcus epidermidis, ATCC 49134	0.46± 0.01	2.65± 1.10	0.21± 0.04	0.27± 0.05	0.48± 0.15	0.33± 0.25
Escherichia coli, ATCC 11229	44.21± 2.29	30.16± 3.32	1.99± 0.24	0.59± 0.04	0.43± 0.07	0.01± 0.002

bacteria bioflims at each concentration in the range of 1.25 -10.00 % (v/v), Fig. 1 and 2. Confirming the results from Fig. 1 and 2, surface rather than other planes bacteria are relatively sensitive to the aqueous extracts, as the density of dead bacteria in the middle layer is smaller than the surface one, under the same concentrations (Fig. 1 and 2). There are a large number of dead bacteria at the surfaces of *Staphylococcus aureus* and *Staphylococcus epidermidis* bioflims with the treatment of 2.50 % aqueous extracts, associated as the fluorescence intensity (Alive /Dead) ratios of 0.11 ± 0.02 and 0.27 ± 0.05, respectively (Table 2). With the concentration increasing, the significant inactivation of *Staphylococcus aureus* and *Staphylococcus epidermidis* was observed, such as those of 5.00% (Table 2). Reaching 10.00 % aqueous extracts, a certain number of bacteria in the middle layer were still survived, with the fluorescence intensity (Alive /Dead) ratios of 0.62 ± 0.42 and 0.33 ± 0.25, respectively. We thought that it could be attributed to the limitation by permeation rate and lack concentration of the effective antibacterial ingredient[15]. Regarding *Escherichia coli*, there were statistically apparent dead bacteria since the treatment of 1.25 % aqueous extracts, which was 1.99 ± 0.24 for the fluorescence intensity ratio (Fig. 3 and Table 2). As to 10.00 % aqueous extracts, however, there is much less growth of Escherichia coli, with the value of 0.01 ± 0.002. It is consistent with the results of MIC and MBC, and further supported that the bacteria were rather sensitive to the aqueous extracts from *Portulaca oleracea* L. Furthermore, it was indicated that there might be concentration- and time-dependent relationships. With the aid of

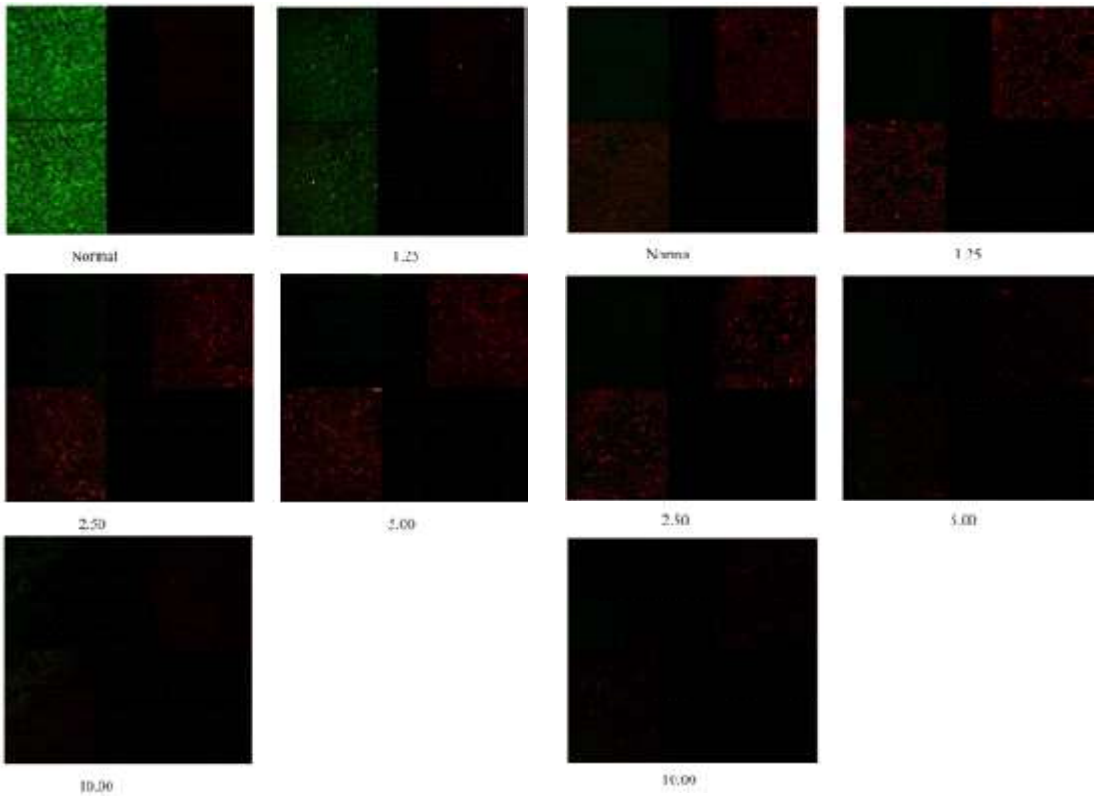

Fig. 1. *Staphylococcus aureus* biofilms after the treatment ,The 5.00 % (v/v) C_2H_5OH was treated as the positive control group. The confocal laser scanning micrographs show the section in the xz plane. Live cells ppear green because of fluorescein diacetate; dead cells in red, staining with propidium iodide

Fig. 2. *Staphylococcus epidermidis* biofilms after the treatment ,The 5.00 % (v/v) C2H5OH was treated as the positive control group. The confocal laser scanning micrographs show the section in the xz plane. Live cells appear green because of fluorescein diacetate; dead cells in red, staining with propidium iodide

CLSM image analysis, it was found that the proportions of dead bacteria per unit area were distinct at the surfaces of bioflims treated by various concentration extracts, with the significant alterations of bacterial density (Figs. 1-3). It might mean that the aqueous extracts could prompt the bacteria to die off or live in the planktonic mode, which will help the body's immune system to remove the biofilm survive remained cell[15, 16, 27].

Taken together, it is likely that the aqueous extracts from *Portulaca oleracea* L. have good inhibitory activities against bacteria bioflims. The extracts may have a potential application in treatment of bioflim-bacteria. Therefore, further related studies should be based on this point of view, and a certain effort are urgently needed to be devoted on its active ingredients in the fields of fine extraction and identification.

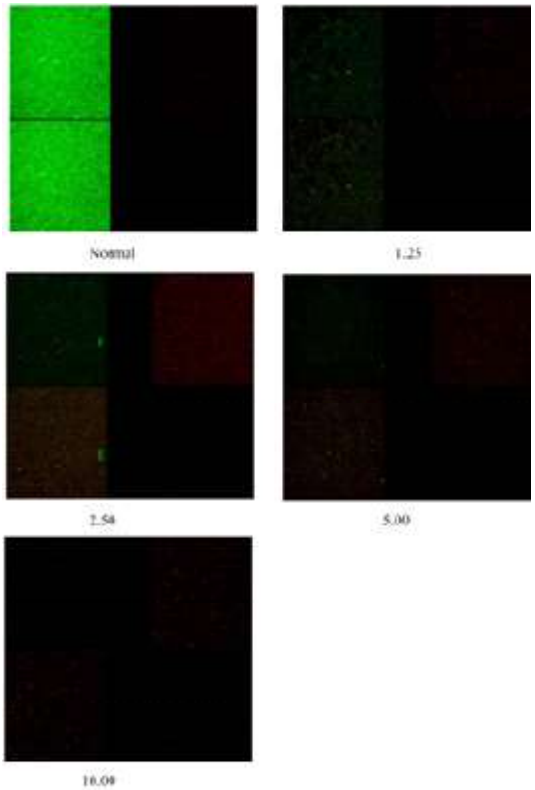

Fig. 3. *Escherichia coli* biofilms after the treatment, The 5.00 % (v/v) C_2H_5OH was treated as the positive control group. The confocal laser scanning micrographs show the section in the xz plane. Live cells appear green because of fluorescein diacetate; dead cells in red, staining with propidium iodide

CONCLUSIONS

Due to the virulence of *Portulaca oleracea* L. aqueous extracts, it can work as natural antimicrobial agent in pharmaceutical industry or food supplement. But there have been few studies on the activities. In this study, we evaluated its inhibiting activity against several common bacteria by the MIC and MBC determinations, and then estimated its antimicrobial effectiveness on artificial bacteria bioflims, via the confocal laser scanning.

Through the studies on the antimicrobial activity, it was found that the aqueous extracts have good inhibitory activities against bacteria (*Staphylococcus aureus, Staphylococcus epidermidis* and *Escherichia coli*). Further CLSM image analysis revealed that the aqueous extracts could inhibit the bacteria in the biofilm rather well. While, there are concentration- and time-dependent relationships between bacteria biofilm inhibition activities and extracts. It was indicated that the aqueous extracts can contribute to the biofilm surface bacteria died off or lived as planktonic, thereby reducing the density of bacteria within the bacterial biofilm surface per unit area, which will help the body's immune system to remove the biofilm survive remained cell. We hope that our results open the possibility of using the aqueous extracts from *Portulaca oleracea* L. for the agent against the bacteria biofilm.

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