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Screening for Antibacterial Active Fractions from *Kummerowia striata*

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To screen the antibacterial effective fractions from the extract of *Kummerowia striata* and further mechanism of antibacterial activity. The effective fractions were screened with AB-8 macroporous resins and analyzed by high performance liquid chromatography, Minimal inhibitory concentrations (MICs) of the extract against *Staphylococcus aureus* were determined using serial dilution method. The ultrastructural morphology of cells of *S. aureus* was observed under electron microscopy. Three compounds including genistein, kaempferol, galuteolin from the powder were identifed. MIC of 70 % ethanol eluate, the most effective fraction, was 2.3 mg mL^{-1}. Through the observation of the ultrastructural morphology of cells of *S. aureus* under electron microscopy, the cell walls were broken and the edge of cell wall of *S. aureus* was irregular. The results show that the total flavones might be the main chemical components with antibacterial activity and the mechanism against *S. aureus* is breaking cell wall and disintegrating cytoplasm of bacteria.

Keywords: Genistein, Kaempferol, Galuteolin, Scanning electron microscope, Traditional Chinese medicine.

INTRODUCTION

Kummerowia striata (Thunb.) Schindl. is a herb belonging to the Leguminosae family. In the whole world, there exist 2 species plants belonging to the genus *Kummerowia*. Another species is *Kummerowia stipulacea* (Maxim.) Makino. The two plants are widely distributed in China. Herb of the genus plants are used as traditional Chinese drugs called Jiyancao. Based on the theories describing the medicinal properties of *K. striata* in the traditional Chinese medicine, it has functions of heat-clearing, detoxicating, promoting urination and checking diarrhea and has been used in the treatment of gastroenteritis, dysentery and urinary system infection. Nevertheless, there is little research on chemical composition, activities, pharmacology. *Luteolin 4'-O-glucopyranoside* has been identified as the IL-5 inhibitor from *Kummerowia striata* Thunb.[1]. *K. striata* (Thunb.) Schindl has a good antiinflammatory effect on LPS-stimulated RAW264.7 cell. On one hand, it can significantly inhibit the production of IL-1β, IL-6, NO, TNF-α, COX-2 in LPS-stimulated cell than that of single LPS stimulated cell ($p < 0.01$ or $p < 0.05$)[2]. Clinical researches are relatively more, and the main indications are infant chronic bacillary diarrhea[3-5], rotavirus enteritis[6]. epidemic keratoconjunctivitis[7] and anaphylactoid purpura[8], especially the first one.

K. striata is one of medicinal plants with richer flavones including galuteolin, kaempferide, genistein. The crude extract of *K. striata* has exhibited strong biological activity against MRSA. In this study, we expand the horizons of our researches. The effective fractions of the crude extract processed with macroporous resins. HPLC technique also made it clear to further characterize more chemical informations.

EXPERIMENTAL

Kummerowia striata were collected from Anguo City of Hebei province, China. The collection process was authenticated as *Kummerowia striata* (Thunb.) Schindl. by Prof. Chang Ji Yuan, School of Pharmaceutical Science, China Medical University. The voucher specimen has been deposited in the pharmacognosy laboratory under specimen number SY006.

Preparation of *Kummerowia striata* extract and HPLC analysis: *K. striata* was ground into powder and refluxed in 70 % ethanol (v/v) (100 g/1000 mL, 1.5 h × 2). The filtrate was concentrated under reduced pressure and processed with AB-8 macroporous resins (purchased from Cangzhou Baoen Chemical Industry Co.). It was successively eluted by water, 10 %, 30 %, 50 %,70 % and 95 % ethanol. The ethanol effluent was separately collected and ethanol in the effluent was recycled. The residue was dried in an oven. The resulting powder was used. A Waters 2695 series HPLC system, equipped with a UV-visible detector, was used for liquid chromatography analysis of the powders. A stainless steel column (4.6 × 150

mm) packed with C18, 5 µm particle diameter, at 35 ± 0.5 °C, was used. The mobile phase contained methanol-0.1 % phosphonic acid (63:37). The sample (10 µl) was injected into the column. The flow rate was maintained at 1 mL/min and UV absorbance detection was carried out at 370 nm.

Determination of minimal inhibitory concentration (MIC) against *Staphylococcus aureus*: Minimal inhibitory concentration (MIC) of the different concentration ethanol eluates of extract of *K. striata* against *Staphylococcus aureus* (ATCC 25923) was determined by the serial dilution method. The sample was diluted with LB medium and 100 CFU/mL bacteria was added. The bacteria was then incubated in an aerobic shaker for 24 h at 37 ºC. After incubation, the liquid was streaked in agar medium plate and then, incubated for 24 h at 37 ºC. MIC value was recorded. Through contrasting the MICs of different fractions, highly effective fraction was found.

Examination by scanning electron microscope (SEM): LB medium (50 mL) was added into four conical flasks. 70 % ethanol eluate was added three conical flasks and made final concentration into MIC, 1/2 MIC and 1/4 MIC and then, 100 CFU/mL bacteria (30 µL) was added. Another conical flask added only the same amount of bacteria. *Staphylococcus aureus* was examined by scanning electron microscope (SEM).

RESULTS AND DISCUSSION

HPLC analysis of *Kummerowia striata* eluates: HPLC-UV analysis was conducted to determine the components in the total extract, 10 %, 30 %, 50 %, 70 % and 95 % ethanol fractions of *K. striata*. Retention time of obtained peaks was compared with that of known standards under the same conditions. In total, three compounds, namely galuteolin, kaempferide and genistein, were determined. Results showed that there was larger variation in components. There were higher content of the three compounds in 70 % ethanol fraction than that in the others (Fig. 1).

Determination of MIC value: MIC of all the fractions were recorded. MIC of 10 % ethanol fraction was more than 20 mg mL^{-1}, that of 30 % ethanol fraction was 7.5 mg mL^{-1}, that of 95 % ethanol fraction was 6.5 mg mL^{-1}, that of 50 % ethanol fraction was 5.5 mg mL^{-1}, that of 70 % ethanol fraction was 2.3 mg mL^{-1} and was the lowest in the five fractions.

Scanning electron microscopy of *Staphylococcus aureus*: Under the scanning electron microscopy, the ultrastructural morphology of cells of *Staphylococcus aureus* in MIC, 1/2 MIC or 1/4 MIC solution of 70 % ethanol fraction and the cell walls were broken and the edge of cell wall was irregular. There are significant correlations between the activity and the concentration of the sample, while, the cell wall of *S. aureus* without drug was distinct and smooth. (Fig. 2).

The extract of *K. striata* was processed with AB-8 macroporous resins and was successively eluted by 10 %, 30 %, 50 %, 70 % and 95 % ethanol. The eluates were analyzed by HPLC methods. Results showed that chemical components in the eluates through AB-8 macroporous resins were different from each other and the separation action was obvious. Three kinds of compouds including genistein, kaempferol, galuteolin were identifed.

MIC refers to the lowest concentration of the antimicrobial agent which is required for the inhibition of visible growth of the tested isolate[9]. MICs of 30, 50, 70 and 95 % ethanol eluate were 2.3 to 7.5 mg mL^{-1}, that of 70 % ethanol eluate was the lowest and its activity against MRSA was the best in the several fractions. HPLC analyses shown that main chemical components were flavones. Based on the results, there were higher content of genistein, kaempferol and galuteolin in 70 % ethanol fraction. Genistein and kaempferol were flavonoid aglycones and belonged to low-polar compounds.

Scanning electron microscope is an important technique to produce high-resolution images of biological samples, Inner structure of cell samples can be observed in detail. Based on the observation of the ultrastructural morphology of cells of *Staphylococcus aureus* under electron microscopy, cell wall of normal *S. aureus* was distinct and smooth. When *S. aureus* was in MIC extract, all of cell wall of *S. aureus* was broken and unclear. When *S. aureus* is in 1/2 MIC extract, cell wall of *S. aureus* was usually disrupted and unclear and the individual was normal. When *S. aureus* is in 1/4 MIC extract, both of disrupted cell wall of *S. aureus* and normal cell wall could be found.

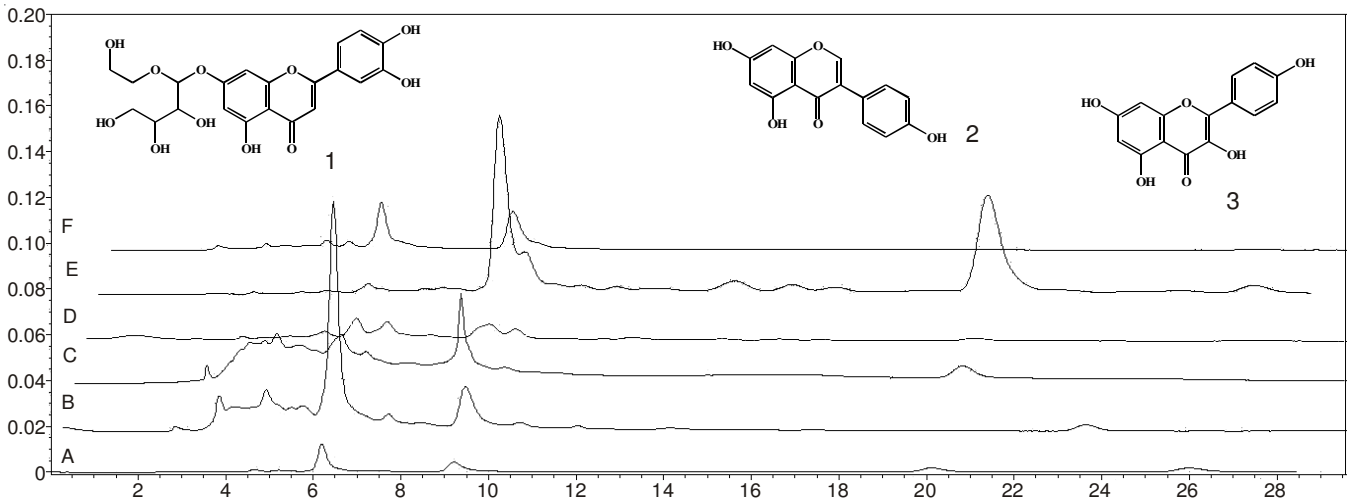

Fig. 1. HPLC chromatograms of *Kummerowia striata* eluates, HPLC chromatograms (A-total extract; B-10 % ethanol; C-30 % ethanol; D-50 % ethanol; E-70 % ethanol; F-95 % ethanol); Peaks (1- galuteolin; 2-genistein; 3-kaempferide)

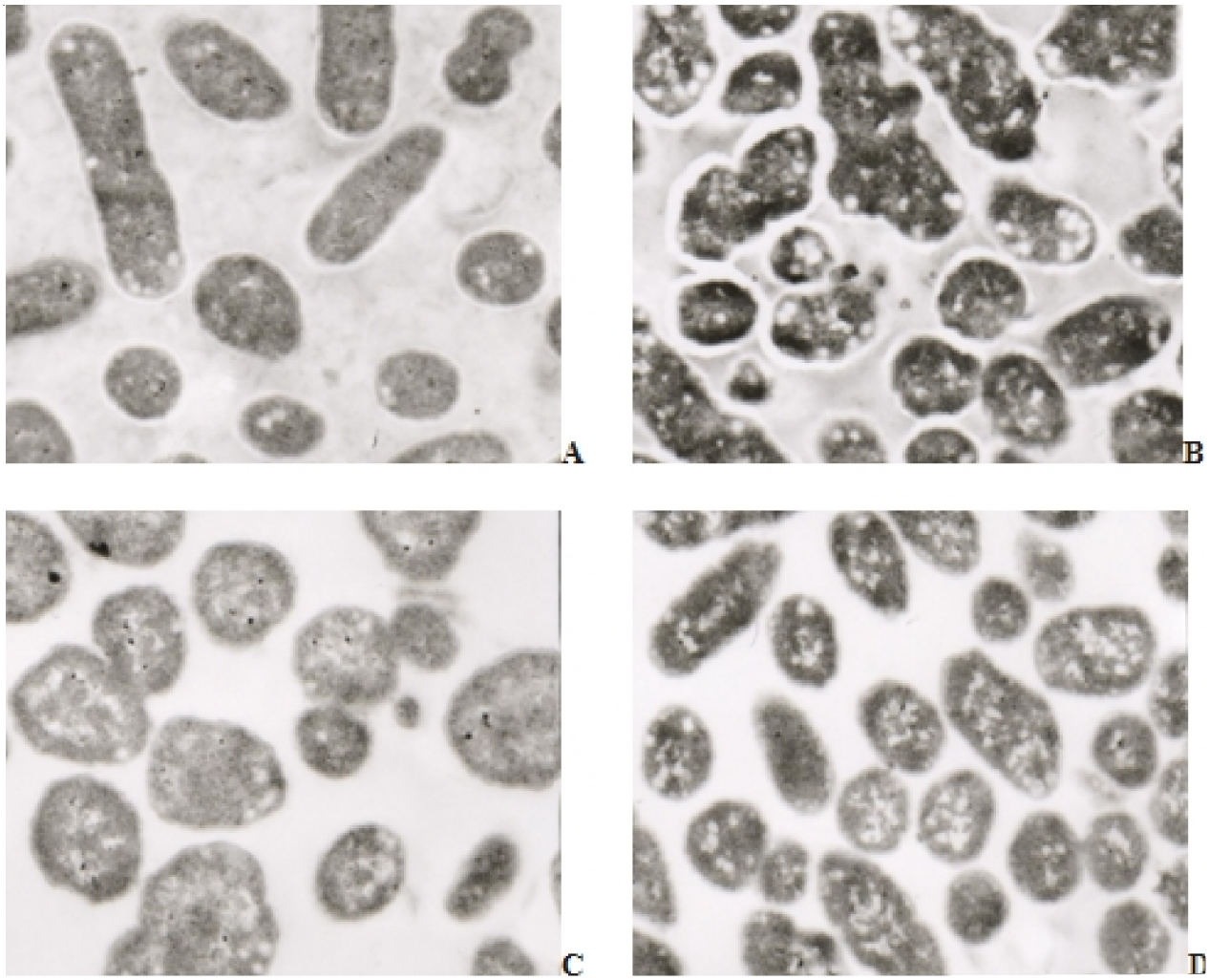

Fig. 2. The cell of *S. aureus* in *Kummerowia striata*, A: The cell wall of normal *S. aureus* was distinct and smooth; B: When the cell of *S. aureus* was in MIC extract, the cell wall was broken and unclear; C: When the cell of *S. aureus* was in 1/2MIC extract, the cell wall was usually disrupted and unclear, and the individual was normal. D: When the cell of *S. aureus* was in 1/4MIC extract, both of disrupted and normal cell wall could be found

Conclusion

Total flavones, especially, flavonoid aglycones, might be the main chemical components from *K. striata* with antibacterial activity. The mechanism against *S. aureus* is breaking cell wall and disintegrating cytoplasm of bacteria.

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