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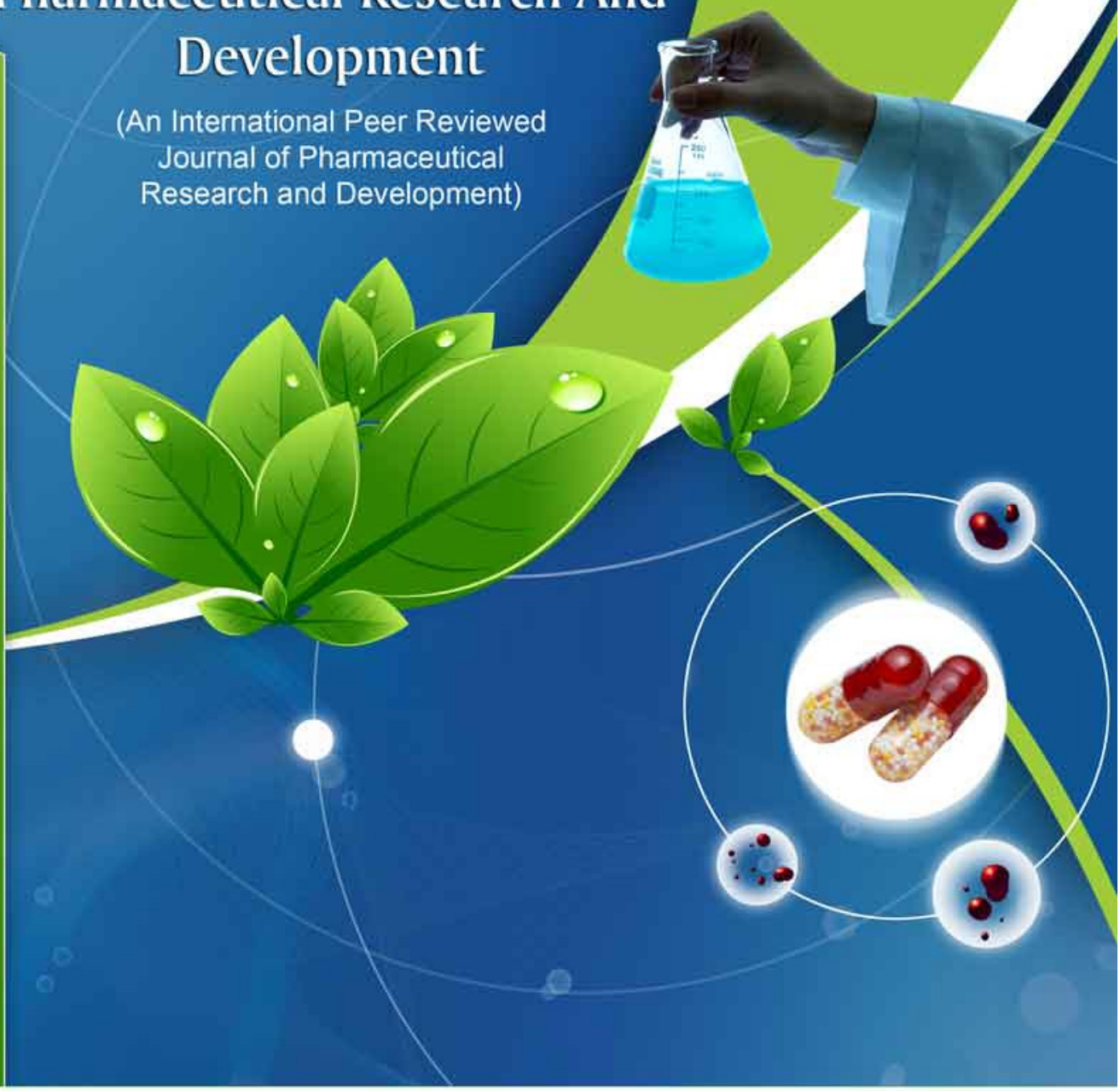

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Short Notes

THE *IN VITRO* ANTI-BIOFILM ACTIVITY OF THE ETOAC EXTRACT OF *PORIA COCOS* AGAINST *ESCHERICHIA COLI*

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ABSTRACT

The aim of this study was to evaluate the effects of the EtOAc extract of Poria cocos and pachymic acid on Escherichia coli biofilm formation. At concentrations ranging from 32 µg/ml to 256 µg/ml, the EtOAc extract of Poria cocos and pachymic acid showed activity against Escherichia coli biofilms in a concentration-dependent manner. Scanning electron microscopy (SEM) analysis showed that the Escherichia coli biofilm formation was completely inhibited by the EtOAc extract of Poria cocos. Moreover, the EtOAc extract of Poria cocos was more effective in inhibiting the biofilm of Escherichia coli compared with pachymic acid. So it is difficult to attribute the anti-biofilm activity of Poria cocos to one single component. Future research should therefore concentrate on the investigation of the additional compounds in the EtOAc extract of Poria cocos that involved in anti-biofilm activity. In conclusion, this study suggests that Poria cocos may have a therapeutic potential for urinary tract infections caused by Escherichia coli.

Key Words: *Poria cocos, Anti-biofilm, Escherichia coli*

INTRODUCTION

Urinary tract infections (UTI) may be caused by a variety of different organisms, most commonly bacteria. It is understood that a primary cause of chronic urinary tract infection is the formation of colonies of multiple species of pathogenic and non-pathogenic microorganisms called biofilm. Biofilm is an effective microbial defense mechanism against host defenses and antimicrobial agents [1]. Disassembly of the biofilm could be exploited to treat infections [2].

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Natural products are supposed as an important source of chemicals with anti-biofilm properties [3-5]. *Poria cocos* (Schw.) Wolf (Polyporaceae) is frequently appearance as a constituent of many preparations in Chinese medicine. *Poria cocos* contains two principal groups of chemicals, the triterpene fraction and the polysaccharide fraction, and has wide pharmacological properties, which were reviewed extensively by Ríos [6]. In traditional Chinese medicine, *Poria cocos* has been used as a diuretic for thousands years. Moreover, almost all formulas of Chinese medicine used to treat urinary tract infection (UTI) contain *Poria cocos*. The purpose of this study is to investigate the anti-biofilm activity of *Poria cocos* using *in vitro* methods. The *Poria cocos* was chosen in this study on the basis of its beneficial effects on UTI. In this paper we report the effect of the EtOAc extract of *Poria cocos* on *Escherichia coli* biofilm formation

MATERIALS AND METHODS

Materials

The *Poria cocos* was collected on July 27, 2011 from the Liping County, Guizhou province, China, and identified by authors. A voucher specimen was maintained in the herbarium of the Pharmacy Laboratory of Guizhou University, China. Pachymic acid and ursolic acid were purchased from Chengdu Biopurify Phytochemicals Ltd.(Chengdu, China). *Escherichia coli* U 07, a clinical strain, was obtained from the Department of Urinary Surgery of Guiyang Medicinal College Hospital, Guiyang, China.

Extraction and TLC analysis of EtOAc extract

The air-dried chips (200 g) of middle layer or *bai-fu-ling* (in Chinese) of *Poria cocos* were refluxed with 95% EtOH (300ml × 3). After filtration and evaporation of the EtOH at reduced pressure to afford a bright yellow syrup (12 g), the EtOH extract was mixed thoroughly with EtOAc. Filtration and evaporation of the EtOAc then gave a EtOAc-soluble fraction (1.5 g). The extract was stored in brown bottles at room temperature until use. The EtOAc extract of *Poria cocos* was checked by Thin Layer Chromatography (TLC) on analytical plate over silical gel 60 GF_{254} (Merck) and pachymic acid used as reference compound. The solvent system was dichloromethane / methanol = 100 : 2. The eluted spots were visualized by Phosphomolybdic Acid (PMA) Stain. As shown in Fig. 1, four fractions were good separated from the EtOAc extract of *Poria cocos* with R_f values of 0.36, 0.31, 0.26, and 0.15 respectively. The R_f value for pachymic acid was 0.26.

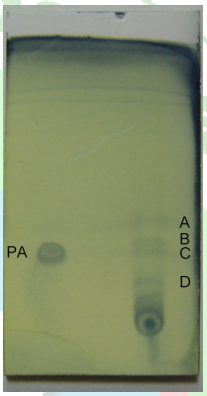


Figure 1: TLC analysis of EtOAc extract of *Poria cocos*. Pachymic acid (PA) is reference compound (R_f, 0.26). Dot A (R_f, 0.36), B (R_f, 0.31), C (R_f, 0.26), and D (R_f, 0.15) are four main fractions of EtOAc extract. The plate was stained by phosphomolybdic acid.

Bacterial biofilm inhibition

The EtOAc extract of *Poria cocos* and pachymic acid were tested for their in vitro anti-biofilm activity against *Escherichia coli* U 07 in commercially available presterilized, polystyrene, flat bottom 96-well microtitre plates by the method described previously [6,7] with modification. Each well was filled to a final volume of 200 µl. Initially, a 150 µl sample of sterile Mueller-Hinton broth was added to each well and then followed by 50 µl of bacterial inoculum at a density of 1×10^7 cfu/ml. The concentration of test samples in each well was ranging from 256-32 µg/ml. The plates were covered and then placed on a shaker for 24 h at 35℃ After incubation, the plates were removed from the shaker and immediately analyzed with a microtiter plate reader at 630 nm and were then rinsed and stained. The absorbance reading taken at 630 nm prior to rinsing the wells was compared to negative controls consisting of medium and inoculum. The results demonstrated that the EtOAc extract of *Poria cocos* and pachymic acid could not inhibited the growth of *Escherichia coli* U 07 even at the highest concentration of 256 µg/ml. The aim of rinse is to remove all nonadherent bacteria. Briefly, the biofilm-coated wells of microtitre plates were vigorously shaken for 2 min. The remaining attached bacteria were washed twice with 200 µl of PBS and air-dried for 45 min. The biofilm was then stained with 100 µl of 0.4% aqueous crystal violet solution for 10 min. Each well was rinsed again four times to remove any excess stain from the system and then eluted with 250 µl of ethanol. The plate was then immediately analyzed with a microtiter plate reader at 540 nm. The samples

and controls were analyzed in triplicate. Negative and positive controls are run for every plate. The positive control substance was ursolic acid, previously documented to moderately inhibit the formation of biofilms [8]. Negative controls included wells with only an appropriate volume of Mueller–Hinton broth and inoculum of *Escherichia coli* U 07. Comparing the average of OD of the negative control wells with that of the sample wells, we calculated the inhibition percentages for each concentration of the test samples by the following formula:

$$[(OD_{negative\ control} - OD_{sample})/ OD_{negative\ control}]\times 100$$

Scanning electron microscopy (SEM)

Biofilms grown on the surfaces of the wells of plate for 24 h were rinsed once with distilled water and fixed using 2.5% buffered glutaraldehyde at room temperature for 1 h. Fixed samples were then dehydrated through a graded series of ethanol concentrations, air-dried, mounted and sputter-coated with gold. Samples were analyzed by scanning electron microscopy (SEM) (Hitachi VP-SEM S-3400N).

RESULTS

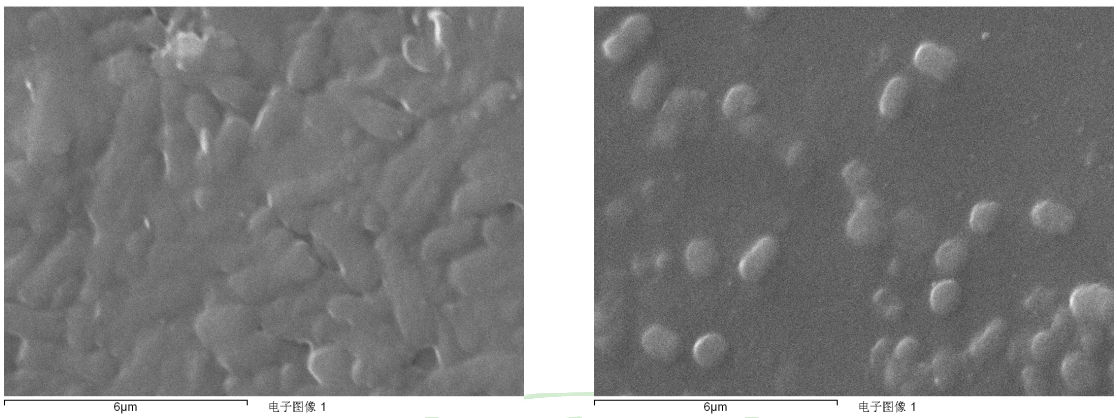
TLC analysis (Fig.1) showed the presence of pachymic acid and other compounds in the EtOAc extract of *Poria cocos*. As shown in Table 1, the EtOAc extract of *Poria cocos* and pachymic acid could inhibit the biofilm formation of *Escherichia coli* U 07 in a concentration-dependent manner. However, the EtOAc extract of *Poria cocos* was more potent than pachymic acid on biofilm formation inhibition. Ursolic acid was used as positive control. It was found to be the best inhibitor against the bacterial biofilm *Escherichia coli* U 07 in this experiment. The ursolic acid could completely inhibit the growth of *Escherichia coli* U 07 at concentration of 256 μg/ml.

Table 1: Antibiofilm activity of the EtOAc extract of *Poria cocos* and pachymic acid against *Escherichia coli* U 07 biofilm

		Inhibition rate (%)*		
Samples	***32 μg/ml***	***64 μg/ml***	***128 μg/ml***	***256 μg/ml***
EtOAc extract	48±7	62±5	74±4	87±6
Pachymic acid	29±3	44±6	53±5	72±8
Positive control (ursolic acid)	58±6	74±4	82±5	100
Negative control	0	-	-	-

* Data were expressed as the means ± S.D.

During the bacterial incubation period, *Escherichia coli* U 07 formed well developed biofilms inside the wells of plate (Fig. 2a). After treatment with the EtOAc extract of *Poria cocos*, the biofilm formation was obviously inhibited (Fig. 2b).



a **b**

Figure 2 SEM image of biofilm in negative control (a) and the EtOAc extract of *Poria cocos* (b) groups.

DISCUSSION

Biofilm protects the microbial cells from attacks by the immunity system as well as from the effect of antibiotics. Therefore, the biofilms are considered to be important virulence factor. Currently no therapies which target microbial biofilms exist, therefore new anti-biofilm agents, treatments and strategies are needed.

The EtOAc extract of *Poria cocos* was tested against the biofilm of *Escherichia coli* U 07. It exhibited anti-biofilm activity at low concentration of 32 μg/ml. Scanning electron microscopy (SEM) has proved to be a precious and invaluable tool for analyzing the structure and growth of biofilm. The anti-biofilm activity of the EtOAc extract of *Poria cocos* was also confirmed by SEM analysis of *Escherichia coli* U 07 biofilm grown on the surfaces of well of the plate. The results suggest that the *Poria cocos* could be a useful source for the development of promising anti-biofilm agents. Also the anti-biofilm effect of *Poria cocos* may explain its use in treatment of urinary tract infection in traditional Chinese medicine. Further research should be done to investigate the anti-biofilm activity of *Poria cocos* in animal model.

Pachymic acid is a component of the EtOAc extract of *Poria cocos*. As far as we know, the pachymic acid has not previously been tested as anti-biofilm agents. The results presented in this article indicate that the pachymic acid is active against *Escherichia coli* U 07 biofilms. It is very interesting that the EtOAc extract of *Poria cocos* was more effective in inhibiting the biofilm of *Escherichia coli* U 07 compared with pachymic acid. It can therefore be assumed that the other compounds in the EtOAc extract of *Poria cocos*, besides the pachymic acid, have anti-biofilm properties also. The pharmacological polyvalence of many constituents comes into action [9]. Future research should therefore concentrate on the investigation of the additional compounds in the EtOAc extract of *Poria cocos* that may involve in anti-biofilm activity.

ACKNOWLEDGEMENT

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