Tropical Journal of Pharmaceutical Research January 2019; 18 (1): 43-48
**ISSN:** 1596-5996 (print); 1596-9827 (electronic)


Available online at http://www.tjpr.org
**http://dx.doi.org/10.4314/tjpr.v18i1.7**

**Original Research Article**

# Root tuber extract of *Polygooum cillinerve* (Nakai) Ohwi exerts antimicrobial and immunomodulatory activities

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*Sent for review: 24 October 2018* *Revised accepted: 27 December 2018*

## Abstract

***Purpose:*** *To investigate the antibacterial and immunomodulatory activities of the root tuber extract of Polygonum ciliinerve (Nakai) ohwi, a traditional Chinese medicine.*
***Methods:*** *Minimum inhibitory concentrations (MICs) of root tuber extract of Polygonum ciliinerve (Nakai) ohwi (rPC) and the synergistic effects between rPC and antibiotics were evaluated by broth dilution. In vitro adhesion and invasion assays were used to determine the effect of rPC on bacterial adhesion and invasion. Furthermore, the immunomodulatory effects of rPC were assessed by western blot.*
***Results:*** *rPC treatment inhibited the growth of Staphylococcus aureus (MIC = 12 mg/ml) and Escherichia coli (MIC = 64 mg/mL). rPC also showed synergistic effects with penicillin (fractional inhibitory concentration, FIC = 0.45), vancomycin (FIC = 0.333), moxifloxacin (FIC = 0.25), and levofloxacin (FIC = 0.356). The adhesion and invasion of bacteria were also suppressed by rPC treatment. Moreover, rPC exhibited an immunomodulatory effect during bacterial infection.*
***Conclusion:*** *rPC shows antibacterial and immunomodulatory activites when assessed by multiple methods, and therefore is a potential therapeutic alternative agent for the treatment of bacterial infections.*

***Keywords:*** *Polygonum ciliinerve root tuber, Synergistic effect, Immunomodulatory, Antibacterial*



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## INTRODUCTION

The widespread clinical use of antibiotics combined with a shortage of new antimicrobials, has resulted to bacterial pathogens with drug resistance [1]. Consequently, infectious diseases caused by bacterial pathogens have resulted in increased morbidity and mortality worldwide and are a serious threat to public health [2].

The pharmaceutical industry is devoted to developing new antibiotics to overcome antimicrobial resistance. Screening bioactive compounds from microbes and improving the chemical structures of these bioactive compounds are the most common strategy. In addition, natural products are an important treasury for new antibacterial drugs and have historically provided people with a source of organic molecules to treat infections [3]. Modern

pharmacology has shown that natural products have antimicrobial activity and show a synergistic effect with antibiotics [3,4].

*Polygonum ciliinerve (Nakai) ohwi*, belonging to Polygonum of *Polygonaceae*, is popularly used as an antimicrobial in China. As an herbal medicine, rPC is the effectual component of *Polygonum ciliinerve (Nakai) ohwi* used for disease treatment by recordation. Modern pharmacological analysis showed polysaccharides and anthraquinones were regarded as the active ingredients of rPC [5-7]. Although early studies showed that rPC possesses anti-oxidant and antifungal activities, the antimicrobial activities of rPC remain to be explored [6,8,9].

This study elucidated the antibacterial activities of rPC using several assays, including traditional bacteriostasis assessments, drug combination assays, bacterial adhesion and invasion assays, and assessments to determine immunomodulatory effects. Based on these results, rPC may be expected to be a therapeutic alternative for infectious diseases and may also provide a lead for the development of herbal antimicrobial medicines.

## EXPERIMENTAL

### rPC preparation

rPC was purchased from Hu Qing Yu Chinese Pharmacy. Pulverized rPC was extracted by twice refluxing in 30 % ethanol for 3 h at 100 $^{o}$C. The extract was passed through a 50-μm filter and isolated as a lyophilized powder.

### Bacterial strains, cells, and chemicals

*S. aureus* ATCC6538 and *E. coli* ATCC25922 were incubated in Luria Broth (LB) medium at 37 ℃. Fetal human colon (FHC) cells were purchased from the Chinese Academy of Sciences. Gentamicin, penicillin, vancomycin, moxifloxacin, and levofloxacin were purchased from Sigma-Aldrich. A cell counting kit-8 (CCK-8) was acquired from Dojindo.

### Evaluation of MIC and time-kill assay

MICs were measured by broth dilution in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI). Serial dilutions of rPC/antibiotics were prepared in 96-well plates, and bacteria ($10^5$ CFU/mL) were inoculated into each well. The 96-well plates were incubated for 24 h at 37 ℃, and absorbance at a wavelength of 600 nm, was measured. The minimum concentrations of rPC/antibiotics that inhibited the growth of bacteria were the MICs. Absorbance was measured with a SpectraMax M3 multi-mode microplate reader (Molecular Devices) to monitor the number of bacteria.

*S. aureus* and *E. coli* were cultured in LB medium with different concentrations of rPC (0, 0.125, 0.25, 1 × MIC) at 37 ℃ for 24 h. The samples were removed, diluted (1:10), and the absorbance measured at 600 nm wavelength hourly. Standard growth curves constructed by plotting absorbance against colony forming units per milliliter (CFU/mL) in order to calculate CFU/mL of *S. aureus* and *E. coli*.

### Synergism test

Different concentrations of rPC and antibiotics were put together in 96-well plates (each concentration was lower than the determined MIC). *S. aureus* ($10^5$ CFU/mL) was inoculated into each well. After incubation for 24 h at 37 ℃, the $OD_{600}$ of the 96-well plates was measured. The combinations that yielded minimal concentrations of rPC and antibiotics that inhibited the growth of bacteria indicated the combined MIC.

For component A, the FIC of A was calculated as the combined MIC of A divided by the MIC of A alone. The FIC index was the sum of the FICs for each combined component. The interactions are considered antagonistic if FIC index > 2, indifference if 1 < FIC index < 2, additive if FIC index = 1, and synergistic if FIC index < 1.

### Adhesion and invasion assay

FHC cells ($10^5$ cells per well) were seeded into 24-well plates 1 day before the assay, and the medium was changed to serum-free medium 1 h before infection. *S. aureus* ($10^6$ CFU) was added to each well in addition to different concentrations of rPC (0, 0.25, 0.5, 1 × MIC), and the plates were centrifuged (2000 rpm, 10 min) to allow infection. For the adhesion assay, FHC cells were lysed (0.1 % Triton) after washing, and the $OD_{600}$ was measured. For the invasion assay, the 24-well plates were incubated for 40 min and washed three times with PBS. Cells were incubated with gentamicin-containing medium (25 μg/mL) for another 1 h and lysed to determine CFUs.

### Western blot

FHC cells ($10^6$ cells per well) were seeded into 6-well plates and treated with *S. aureus* ($10^7$ CFU)

and rPC (0, 0.25, 0.5, 1 × MIC) for 24 h. Cells were lysed in RIPA lysis and extraction buffer (Thermo Fisher Scientific) with protease inhibitors (Sigma-Aldrich). A BCA Protein Assay Kit (Thermo Fisher Scientific) was used to determine protein concentrations. Ten-microliter protein samples were electrophoresed with SDS polyacrylamide gels and transferred to PVDF membranes (Millipore). The membranes were blocked by TBST (tris-buffered saline with Tween 20) containing 5% skim milk for 3 h and washed three times with TBST. The primary antibodies against GAPDH (Abcam), p38 (Abcam), phosphorylated-p38 (Abcam), ERK (Abcam), phosphorylated-ERK (Abcam), IKBα (Abcam) and phosphorylated-IKBα (Abcam) were incubated with the membranes overnight at 4℃. After washing with TBST, horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam) were incubated with the membranes at room temperature for 1 h. The chemiluminescence induced by SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific) was detected with ImageQuant LAS 4000mini (GE Healthcare Life Sciences).

### Statistical analysis

Data were collected from three independent experiments and are expressed as mean ± standard error of mean (SEM). The statistical significance of differences between two groups was evaluated with the Mann-Whitney test. SPSS and Prism software was used to perform the statistical analysis, with $p < 0.05$ indicating significance.

## RESULTS

### Antimicrobial activity of rPC

The antibacterial activities of rPC against *S. aureus* and *E. coli* were determined through by broth dilution. The MICs of rPC against *S. aureus* and *E. coli* were 12 and 64mg/ml, respectively (Figure 1A). These results suggest that rPC exhibits antimicrobial activities against both gram-negative and gram-positive bacteria.

Based on the MIC of rPC, time-kill curves of *S. aureus* and *E. coli* were also obtained (Figure 1B). After exposure to rPC (0, 0.125, 0.25, 0.5, 1.0 × MIC), the viable count was tested every hour through measurement of $OD_{600}$. When compared with the control, rPC successfully restricted viable cell count. Moreover, rPC showed effective and prolonged bactericidal ability at the MIC concentration within 24h. Taken together, rPC has broad and strong antibacterial activities.

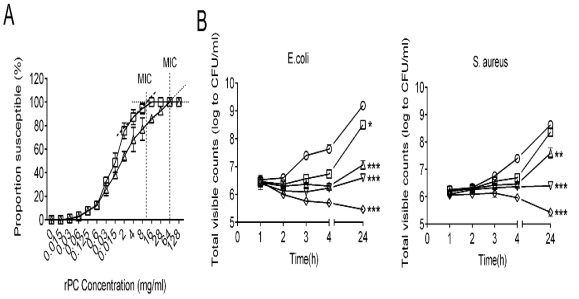


**Figure 1:** Antimicrobial activity of rPC.(A) Inhibition of the growth of *S. aureus* and *E. coli* by various concentrations of rPC*;* □:*E.coli*, △:*S.aureus*. (B) Time-kill curves for *S. aureus* and *E. coli* treated with rPC (0, 0.125, 0.25, 0.5, 1 × MIC); ***p*< 0.01, ****p*< 0.001, compared with control group; ○: Control; □:0.125 × MIC rPC; △:0.25 × MIC rPC; ▽:0.5 × MIC rPC; ◇:1.0 × MIC rPC

### Synergistic effect of rPC and antibiotics

To further evaluate potential applications of rPC, the synergistic activity of rPC and antibiotics was tested. FIC index was used as indicators of the synergistic effect against *S. aureus*. The FIC index of rPC and antibiotics ranged from 0.2 to 0.5, indicative of synergistic effects (FIC ≤ 0.5) (Table 1). The combined use of rPC and Moxifloxacin showed the strongest synergistic effect (FIC = 0.25). The results indicate that rPC could be used in clinical treatment to improve the utilization efficiency of antibiotics and decrease the overuse of antibiotics.

**Table 1:** Effect of combined antibacterial agents on the growth of *S. aureus* in LB medium at 37 $^{o}$C. FIC index < 0.5 considered as synergistic

| **Component** | | **Single MIC** | | **Combined MIC** | | **FIC** |
|---|---|---|---|---|---|---|
| *A* | *B* | *A (mg/mL)* | *B (μg/mL)* | *A (mg/mL)* | *B (μg/mL)* | |
| rPC | Penicillin | 12 | 0.12 | 3 | 0.024 | 0.45 |
| rPC | Vancomycin | 12 | 8 | 1 | 2 | 0.333 |
| rPC | Moxifloxacin | 12 | 60 | 1 | 10 | 0.25 |
| rPC | Levofloxacin | 12 | 0.53 | 2 | 0.1 | 0.356 |

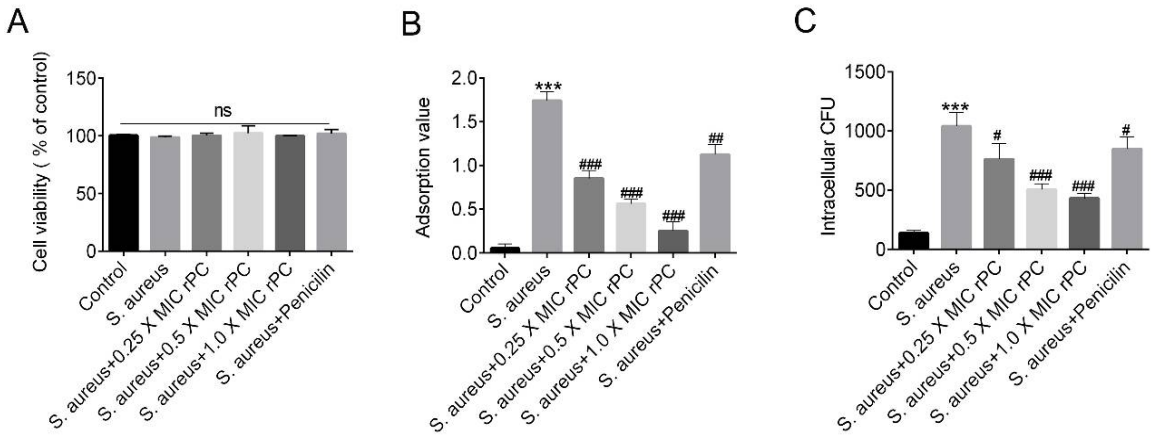


**Figure 2:** Effect of rPC on *S. aureus* infection of enterocytes *in vitro*. (A) FHC cells were exposed to *S. aureus* and rPC (0, 0.25, 0.5, 1 × MIC) for 2 h. Cell viability was determined with a CCK-8 assay. (B and C) The effects of rPC (0, 0.25, 0.5, 1 × MIC) on *S. aureus* adhesion to (B) and invasion of (C) FHC cells during bacterial infection. ***$P$ < 0.001, compared with control group; #$p$ < 0.05, ##$p$ < 0.01 ###$p$ < 0.001 compared with *S. aureus* infection group

### rPC inhibited *S. aureus* infection of enterocytes *in vitro*

To identify whether the antimicrobial activity of rPC correlated with the inhibition of bacterial infection, the adhesion and invasion of enterocytes by *S. aureus* was quantified with conventional infection assays. First, the cytotoxic effect of rPC (0, 0.25, 0.5, 1 × MIC) and *S. aureus* on FHC cells was determined, and no obvious cytotoxic effects was found (Figure 2A). In the assay, Penicillin was used as a positive control. rPC reduced *S. aureus* adhesion and invasion to FHC cells in a dose-dependent manner (Figures 2 B and C). Furthermore, rPC had a stronger effect on inhibition of bacterial adhesion and invasion than penicillin which only inhibits bacterial growth, indicating that rPC has a direct effect on bacterial adhesion and invasion (Figure 2 B and C).

### rPC ameliorated the inflammatory response of infected cells

*S. aureus* may induce cellular immune responses after invasion by activating the MAPK/ERK and NF-κB signaling pathways[10,11]. Therefore, FHC cells were infected with *S. aureus*, and the expression of typical proteins was analyzed after rPC treatment (0, 0.25, 0.5, 1 × MIC). Phosphorylation of p38 (MAPK14), ERK, and IKBα were observed after *S. aureus* infection, suggesting that the MAPK/ERK and NF-□B pathways were activated (Figure 3). However, rPC treatment significantly decreased the activation of p38, ERK, and IKBα, indicating a reduced inflammatory response in enterocytes (Figure 3). These results demonstrate that rPC could act to maintain cellular immune homeostasis during bacterial infection.

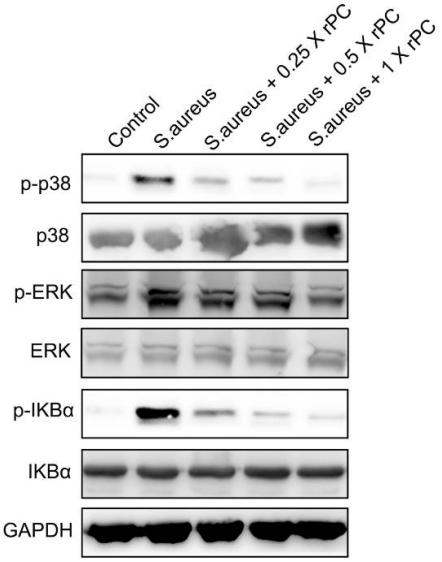


**Figure 3:** rPC ameliorated the inflammatory response of infected cells.The expression of phosphorylated-p38, p38, phosphorylated-ERK, ERK, phosphorylated-IKBα, and IKBα in FHC cells with different treatment

## DISCUSSION

In order to decrease the morbidity and mortality of infectious diseases, it is important to discover new drugs with effective antibacterial activities. Traditional herbal medicines were considered to possess effective antimicrobial activities and hard to acquire drug resistance [12]. Therefore, rPC, an anti-infective drug in traditional Chinese medicine, has the potential to become a novel therapy for bacterial infections. In this study, the antibacterial activity of rPC and its synergistic

effect with antibiotics were evaluated. In addition, other antibacterial activities of rPC were also assessed, particularly inhibition of bacterial adhesion and invasion, which in-turn modulates the immune responses of infected cells.

In the present study, rPC showed antimicrobial activity against *S. aureus* and *E. coli* (MIC = 12and 64 mg/mL, respectively). Consistent with the records of Bencao Tujing (published in 1061, China), rPC served as an anti-infection agent. Emodin and physcion, the effective of rPC, have strong bactericidal effects [5,13,14]. Therefore, rPC possessed natural antibacterial activity. Moreover, the time-kill assays illustrated that rPC can inhibit bacteria growth at concentrations $\geq 0.5 \times$ MIC.

Synergistic effects were observed between rPC and antibiotics. The combined concentrations of rPC and antibiotics inhibiting bacterial growth were much lower than single use. Therefore, rPC probably can be used as an adjuvant to effectively prevent drug resistance to antibiotics. However, the experimental conditions in this study were limited (bacteria was incubated in culture medium within a 24 h period); also, the pathophysiological condition of human body is more complicated and hence further studies need to be done.

Pathogenic bacteria, such as *S. aureus* and *E. coli*, have sophisticated adhesion systems, like flagella and fimbriae, conducting efficient cellular adhesion and invasion [15,16]. In addition, microorganisms can utilize extracellular matrix such as fibronectin, to promote adhesion and invasion [17]. The findings of this study suggest that rPC suppresses bacterial adhesion and invasion.

NF-κB and p38/MAPK signaling pathways link extracellular stimuli with inflammatory responses [18,19]. In the current study, rPC treatment reduced the activation of p38, ERK and IKBα compared with the *S. aureus* group, which indicated that rPC can attenuate inflammation of infected cells. Therefore, the immunomodulatory effects of rPC should be further explored.

## CONCLUSION

This study demonstrates that rPC possesses strong antibacterial activities and has a synergistic effect with antibiotics. Moreover, the results suggest that rPC inhibits bacterial adhesion, suppresses bacterial invasion, and plays an immunomodulatory role in host cells. Based on these results, rPC is a promising medicinal agent for the treatment of infectious diseases, and may potentially help to tackle antibiotic resistance.

## DECLARATIONS

### *Acknowledgement*

This work was funded as part of projects titled ‘A study on the effects of Polygonum cillinerve on Staphylococcus aureus and TLR/NOD pathway on the basis of mutant selection window’ (grant no. 2017JM8021) and ‘A Study on the effects of total flavonoid in ligustrum lucidum ait on lipidmetabolite and the related mechanism’ (grant no. 2010Jk476), respectively.

### *Conflict of interest*

The authors declare that there is no conflict of interest associated with this work.

### *Contribution of authors*

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Lanxiu Cao and Juan Lv designed all the experiments and revised the paper. Xian Zhao and Jiang Wang performed the experiments while Li Ma wrote the paper.

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