Brief Report

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The *in vitro* and *in vivo* anti-virulence activities of *Cinnamomum bejolghota* by inhibiting type three secretion system effector proteins of Salmonella

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SUMMARY The bark of *Cinnamomum bejolghota* (Buch.-Ham.) Sweet (*C. bejolghota*) is widely used as medicine to treat bacterial diarrhea in Myanmar. We previously reported that the bark extract of *C. bejolghota* significantly inhibited secretion effector proteins of the type three secretion system (T3SS) in Salmonella. This study is designed to investigate the anti-virulence potential of the *C. bejolghota* bark extract against *Salmonella* Typhimuriumin *in vivo* and *in vitro* experiments. The results suggested that the polar fraction Fr.M_1 inhibited the secretion of effector proteins SipA, SipB, SipC and SipD without affecting bacteria growth and the translocation of SipC into MDA-MB-231 cells. In addition, Fr.M_1 alleviated inflammatory symptoms of mice in Salmonella-infected mouse model. Overall, the results provide evidence for medicinal usage of *C. bejolghota* bark to treat diarrhea in Myanmar.

Keywords *C. bejolghota*, T3SS effector protein, anti-virulence, *Salmonella* Typhimurium

1. Introduction

With the increasing of antibiotic resistance, many new targets for the development of non-traditional antibacterial drugs have been revealed, and the "anti-virulence strategy" is a promising avenue as an alternative (*1-3*). Most of the antibiotics target the key functions for bacterial survival and resistance to them is widespread. The "anti-virulence strategy" targets the virulence that not required for the survival of pathogens. Therefore, it is regarded as a promising target for the development of anti-virulence compounds (*4*). Among them, T3SS is a typically virulence mechanism employed by several gram-negative pathogens. Many medicinal plants have been reported with antibacterial and anti-infective activity, but the molecules from them showed weak antimicrobial activity, which means they may target virulence factors rather than bactericide (*5-7*).

C. bejolghota (Buch.-Ham.) Sweet, previously known as *C. obtusifolium* (Roxb.) Nees (*8*), is a large robust tree, distributed throughout the central and outer parts of eastern Himalayas and Burma. The stem barks were used locally as a substitute of cinnamon spice. The bark and its infusions have variety of local medicinal use for the treatment of headache, fever, urinary stone trouble, stomach disorder and diarrhea (*8,9*). More and more studies have confirmed that *C. bejolghota* has a wide range of pharmacological effects, including antihyperglycemic and anti-oxidative activity (*10*). Our recent results also indicated that *C. bejolghota* is one of the promising candidates as anti-virulence drug resource for treatment of Salmonella infection by inhibiting the effector proteins (*11*), which maybe the reason for traditional usage of *Cinnamomum* on the stomach disorder, gastroprotective activity and diarrhea (*12-15*). Therefore, to fulfill the speculation, we try to disclose the possible anti-virulence effectiveness of *C. bejolghota in vitro* and *in vivo* and the possible mechanism.

2. Materials and methods

2.1. Plant material and preparation of extracts and fractions

The *C. bejolghota* barks were collected from Kalaw Reserve Forest of Tanggyi Shan State (20°35'57.98N, 96°31'50.29E) in May 2017. It was authenticated by Yu Zhang and a voucher specimen of *C. bejolghota* bark (MB201705KLW009) was deposited in Kunming Institute of Botany, Chinese Academy of Sciences.

The barks (1.0 kg) were cut into pieces, powdered and extracted with 95% ethanol for three times. The ethanol extract was concentrated using a rotary evaporator *in vacuum* at 40°C to yield a residue. Then, the residue was dispersed in water and extracted with petroleum ether (PE), ethyl acetate (EA), and *n*-Butanol (Bu) to obtain PE, EA, Bu and water (W) extracts, respectively. Further, the Bu extract was dissolved in MeOH to obtain MeOH soluble fraction (Fr.M). Each extract was dissolved in DMSO to make 100 mg/mL stock solution. All the extracts (PE, EA, Fr.M and W) were screened for their inhibitory effects on the secretion of T3SS effector proteins of *S.* Typhimurium. The results suggested that Fr.M showed excellent anti-T3SS activity. Therefore, Fr.M was subjected to MPLC (RP-18 silica gel, 140 g) and eluted with gradient MeOH in water (H_2O, 30%, 50%, 70%, 90% and 100% MeOH) to obtain Fr.M_1-M_6. Then, Fr.M_1-M_6 was respectively dissolved in DMSO to make 100 mg/mL stock solutions for anti-T3SS screening.

2.2. Culture conditions of bacteria strain and cell line

Salmonella enterica serovar Typhimurium UK-1 χ8956 (*S.* Typhimurium) (*16*) was cultured in Luria-Bertani (LB) broth or on LB agar medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4) with 0.2% arabinose at 28°C or 37°C overnight. The quantity of bacteria was counted by measuring the OD_{600}. Bacteria were harvested by centrifugation at 12,000 rpm for 5 min, then suspended in phosphate-buffered saline (PBS) or LB media and used for the next experiments.

Human breast cancer cells MDA-MB-231 were cultured in RPMI-1640 medium (containing 10% fetal bovine serum) without antibiotics at 37°C and 5% CO_2.

2.3. Isolation and detection of T3SS effector proteins

The potential inhibitory effects of the different extracts and fractions against T3SS associated effector proteins were screened at the concentration of 100 μg/mL using sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie blue and western blotting analysis as described in the previously published literature (*16,17*).

2.4. Cytotoxicity assay

MDA-MB-231 cells were seeded in 96-cell plates (Corning, USA) with a density of 1.2×10^5 per well and the cells were incubated overnight at 37°C with 5% CO_2. Each well was filled with 100 μL of medium with different concentrations of Fr.M_1 ranging from 6.25 to 100 μg/mL, and the DMSO control group was filled with 200 μL medium, and there are triplicates for each experiment. After that, the 96-well plates were incubated in a constant temperature incubator at 37°C with 5% CO_2 for 48 h. Ten μL CCK-8 reagent (Hanbio) was added to each well, then incubate for 4 h. The OD_{480} value was measured with a microplate reader, and the survival rate of co-cultured cells with extracts was calculated according to the following formula. Survival rate (%) = (OD_{480} of experimental group – OD_{480} of blank group)/(OD_{480} of control group – OD_{480} of blank group) × 100%.

2.5. Gentamicin protection assay

In order to test whether Fr.M_1 inhibited *S.* Typhimurium invasion into host cells, the gentamicin protection assay was carried out (*16,18*). Overnight cultures of the *S.* Typhimurium in LB medium (0.2% L-arabinose) in 25°C incubator were diluted 1:10 with fresh LB broth. Fr.M_1 was added at the indicated concentration of 100 μg and cultures were placed in 37°C/220 rpm for 4 h. MDA-MB-231 cells were seeded in 96-cell plates with a density of 1.2×10^5 per well and the cells were incubated overnight at the atmosphere of 37°C with 5% CO_2. Wash cells three times with PBS, and 200 μL cell culture medium was added to each well, then the plate was incubated at 37°C for 30 minutes. *S.* Typhimurium was added to MDA-MB-231 cells at the multiplicity of infection (MOI) of 20. After incubating for 1 h at 37°C, cell culture medium containing 100 μg/mL gentamicin was replaced to each well to kill noninvasive *S.* Typhimurium cells for 1 h at 37°C. Then, MDA-MB-231 cells were washed three times with PBS and 100 μL 1% TritonX-100 with 1 mM PMSF was added to each well to lyse cells. The colony forming units (CFU) of bacteria in the cell lysis solutions were counted by the method of plating 1:10, 1:100, 1:1,000 dilution in LB agar plate with 0.2% L-arabinose.

2.6. Detection of SipC in the invasion assay

MDA-MB-231cells were seeded in 60 mm flat-bottom plates, incubated for 16 h at 37°C, and 5% CO_2 in RPMI-1640 supplemented with 10% FBS. Then MDA-MB-231 cells were cultured for 30 min in RPMI-164 medium without FBS, and infected with *S.* Typhimurium at the MOI of 50 and 1,000. After the addition of Fr.M_1 to the final concentrations of 100 μg/mL, the co-culture of Salmonella-MDA-MB-231 cells were incubated for 15, 30 and 60 min at 37°C, respectively. The co-culture was centrifuged for 5 min at 12,000 g at 4°C. The cells were washed with PBS, and lysed with 1% Triton X-100 solution with PMSF at the final concentration of 1 mM. Cell lysate was collected and per 50 μL of lysate was mixed with 150 μL loading buffer. After 95°C denaturation, proteins SipC and GADPH in different groups were analyzed by western blotting. All experiments were performed in triplicates.

2.7. Measurement of bacterial growth

S. Typhimurium was cultured in LB broth with 0.2% L-arabinose at 28°C and then diluted at 1:200 in fresh LB broth and incubated for 4 h at 37°C with $Fr.M_1$ or DMSO. OD_{570} of the culture was measured every hour using a microplate reader (Bio-Rad 680, USA) and three replicates for each experiment.

2.8. Animal experiments

Kunming mice were purchased from the Experimental Animal Center of Shandong University. The mouse experiment is overseen by the Animal Welfare and Ethical Committee of Shandong University (Approval No, 20026). Mouse model for Salmonella infection was induced as described before (*19-21*). Mice were provided with water containing streptomycin (5 mg/mL) for two days before treated with free water and food. Drinking water was offered before Salmonella infection, followed by infection or sterile PBS treatments. Bacteria were successively incubated in a shaker at 25°C and 37°C to induce the production of T3SS effector proteins. Bacteria strains were washed three times with sterilized PBS before infection. By measuring the OD_{600} value, the CFU of the bacterial solution was determined to be properly concentration. Sixty 6-8 weeks old Kunming mice were divided into six groups, and every group included five males and five females. To make the infection model, each group mouse (n = 10) was infected with appropriately 3×10^8 of bacterial cells. Each mouse in groups A, B, C was respectively treated with 5 mg, 10 mg and 20 mg $Fr.M_1$ for 5 days at 12-h intervals every day. In each case, control groups received sterile PBS, the positive group was treated with streptomycin (5 mg/mL). The weight of the mice was measured daily for 10 days.

After 10-day post-infection, mice were sacrificed by cervical dislocation. The spleen, liver and kidney index of the Kunming mice were weighed and calculated.

To evaluate bacteria loads in spleen, liver and kidney tissue, all the samples were homogenized in cold PBS and serial dilutions of the homogenates were plated on LB plates under Salmonella-selective plating medium, followed by overnight incubation in 37°C.

For histopathological analysis, segments of kidney, liver and spleen were fixed and embedded in paraffin according to standard procedures (*22-24*). Cryosections were mounted on glass slides and stained with hematoxylin and eosin (H&E). Pathological evaluation was performed by using microscope (ZEISS, Axio Observer A1m, Germany) in a proper manner.

2.9. Statistical analyses

Means and standard deviations were calculated and analyzed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA). The two-way analysis of variance (ANOVA) method was used in this software to evaluate the statistical significance between the different groups. p values of < 0.05 were considered statistically significance.

3. Results and Discussion

3.1. $Fr.M_1$ inhibit the secretion of effector proteins SipA, SipB, SipC and SipD of *S.* Typhimurium

C. bejolghota is a Myanmar medicinal plant, its bark soaking water or bark pasta made with water has profound potential for the treatment of severe diarrhea (*8*). Our recently screening results have suggested that the *C. bejolghota* bark extract showed inhibitory activity on the secretion of T3SS effectors of *S.* Typhimurium (*11*). In this study, the SDS-PAGE and western blotting results suggested that 100 μg/mL Fr.M showed significant anti-T3SS effects on the secretion of effector proteins SipA, SipB, SipC and SipD (Figure 1a). Further fractionation Fr.M by MPLC to obtain $Fr.M_1$ to M_6, and anti-T3SS results suggested that $Fr.M_1$ was the most active fraction (Figure 1b) inhibiting the secretion of effector proteins without an evident effect on the growth of bacteria (Figure 1c).

3.2. $Fr.M_1$ inhibited *S.* Typhimurium invasion into MDA-MB-231 cells

The cytotoxicity of $Fr.M_1$ was measured using the CCK-8 assay (*25*). 100 μg/mL $Fr.M_1$ showed no inhibitory effects on the growth of MDA-MB-231 cells (Figure 2a). Then we investigated the protective ability of $Fr.M_1$ by blocking *S.* Typhimurium into MDA-MB-231 cells. The gentamicin protection assay suggested that $Fr.M_1$ significantly reduced bacterial invasion into MDA-MB-231 cells compared to control group (Figure 2b). Meanwhile, the SipC level in MDA-MB-231 cells was detected by western blotting. The results suggested that $Fr.M_1$ reduced the SipC lever in MDA-MB-231 cells compared to control (Figure 2c), which suggested that $Fr.M_1$ can reduce *S.* Typhimurium invasion into MDA-MB-231 cells.

3.3. $Fr.M_1$ alleviated *S.* Typhimurium infection *in vivo*

In the infected experiment, 3 g/kg $Fr.M_1$ was orally administered to Kunming mice, but no death was observed after 10 days. To investigate the impact of $Fr.M_1$ treatment, the weight of Salmonella infected mice was measured daily for 10 days and the organ indexes were finally measured. As shown in Figure 3a, the weight loss of infected mice was significant after three days, whereas mice treated with $Fr.M_1$ and Streptomycin could reduce the degree of weight loss. The organ index is a sensitive indicator representing the effects of drugs on animal organs. An increased organ index indicates congestion, oedema or hypertrophy of the organs, *etc.*,

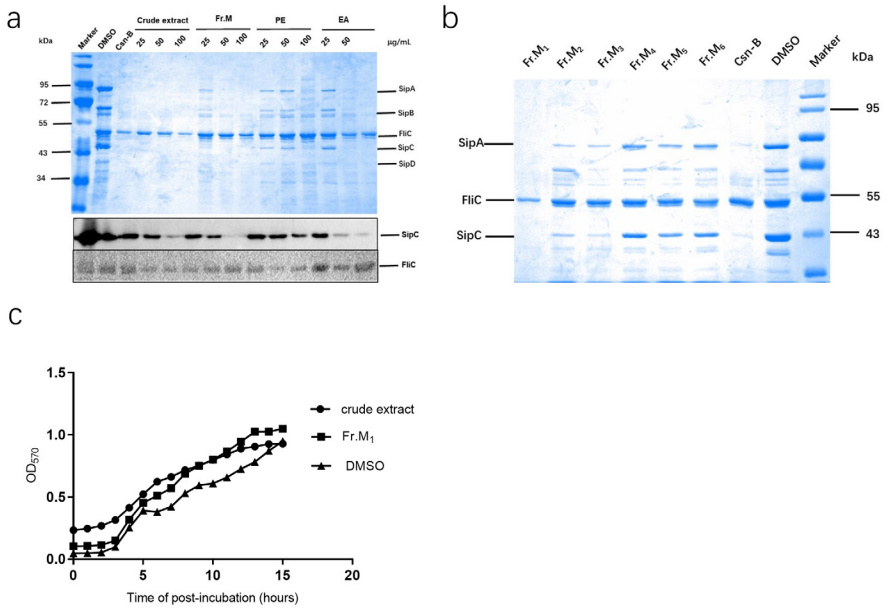

Figure 1. a) The screening results of different extract of *C. bejolghota* on the secretion of T3SS effector proteins SipA, SipB, SipC and SipD of *S.* Typhimurium (M, marker; cytosporone B, (Csn-B), positive control; DMSO, control); **b)** Fr.M_1 inhibited the secretion of effector proteins SipA, SipB, SipC and SipD of *S.* Typhimurium; **c)** *C. bejolghota* bark extract and Fr.M_1 did not significantly inhibit the bacterial growth at a concentration of 100 μg/mL.

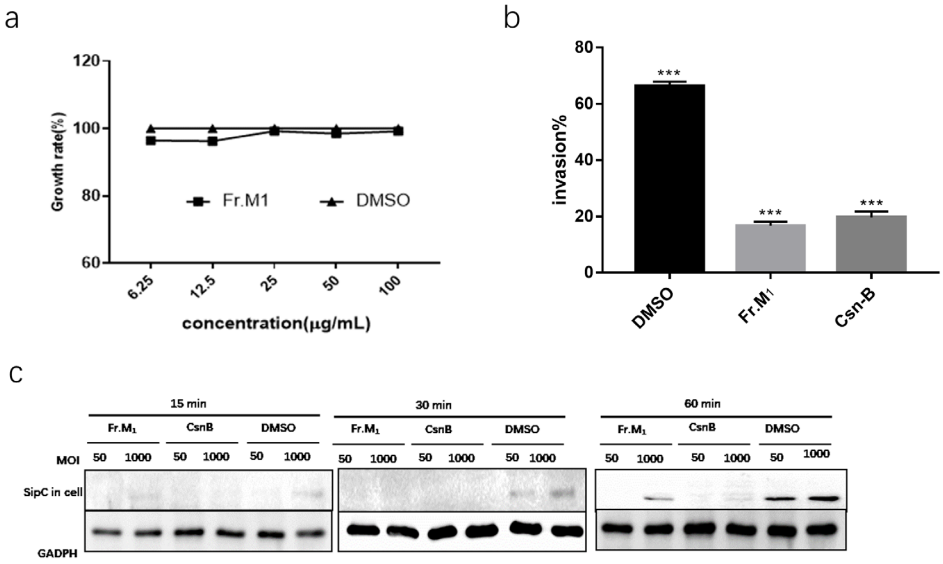

Figure 2. a) Fr.M_1 has no cytotoxic activity to MDA-MB-231 cells; **b)** Fr.M_1 inhibited S. Typhimurium invasion into MDA-MB-231 cells (The values represent percentages of invasion bacteria as related to the initial input, and error bars indicate standard deviations from the means. The levels of statistical significance is indicated as $^{***}p \leq 0.001$. The *p* value was calculated by comparing the value for the DMSO negative control.); **c)** Fr.M_1 significantly reduced the translocation of effector protein SipC into MDA-MB-231 cells compared to DMSO control.

while a decreased organ index indicates organ atrophy and other degenerative changes. The liver and kidney indexes of the infected mice were significantly higher than that of the Fr.M_1 treated mice, which indicated that Fr.M_1 treatment could reduce inflammation and edema of the organs (Figure 3b).

By observing the tissue sections of liver, spleen and kidney, we found that Salmonella infection generally caused organ congestion and edema, with a marked inflammatory cell infiltration around the vessels. Meantime, the pathological conditions of mice have improved after treatment with 20 mg/mouse Fr.M_1 (Figure 3c).

In recent years, many molecules from medicinal plants have been reported as T3SS inhibitors, such as cinnamaldehyde (*26*), paeonol (*27*), licoflavonol (*28*), thymol (*29*), epigallocatechin-3-gallate (*30*). The *in vitro* or *in vivo* bioassays suggested that Fr.M_1 was responsible for the anti-virulence activity of *C. bejolghota*. As a widely used medicinal and edible plant, *C. bejolghota*

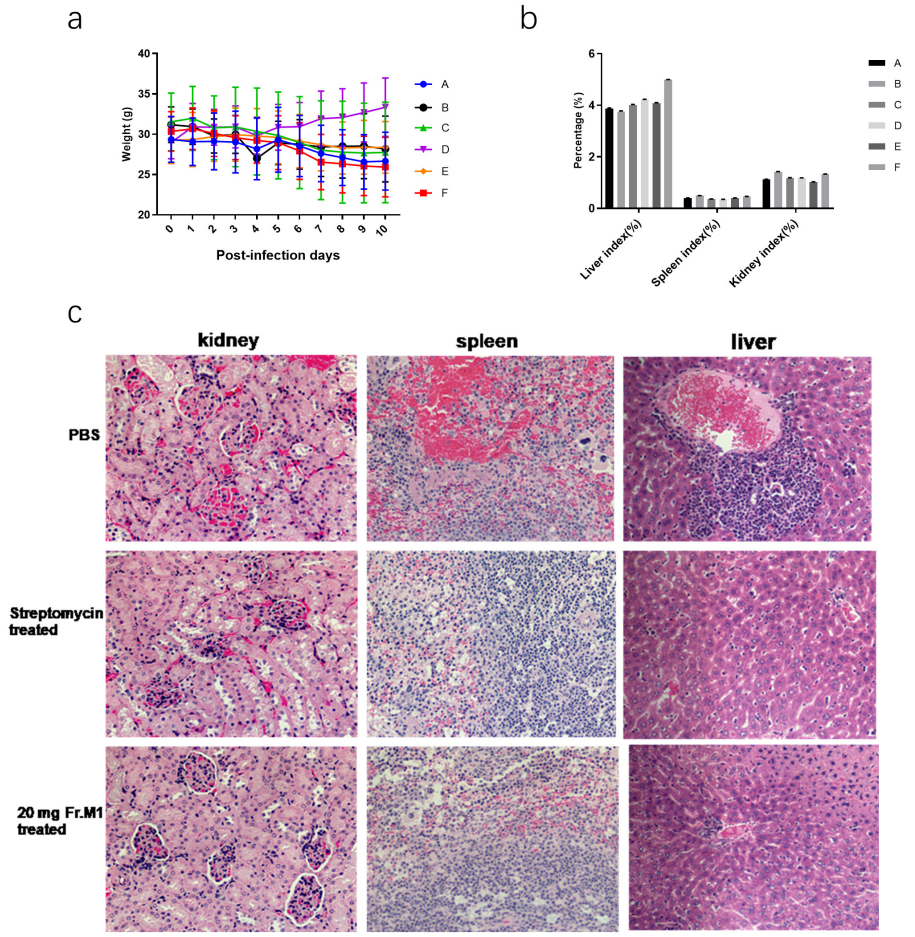

Figure 3. Protective effects of Fr.M$_1$ on *S.* Typhimurium-infected Kunming mice *in vivo*. a) the weight loss caused by Salmonella infection was alleviated; **b)** the organ indexes of liver, kidney and spleen are improved by Fr.M$_1$; **c)** Histopathology of the kidney, spleen and liver tissues were stained with hematoxylin and eosin (H&E) and observed by optical microscope (20 × 10).

also reported with diverse bioactivities such as anti-inflammation, antipyretic and analgesic activity (*31*) and antihyperglycemic property (*10*). The main constituents of *Cinnamomum* have been reported (*9,31,32*), and as one of the main constituents, cinnamaldehyde was reported to be an inhibitor of Salmonella T3SS by affecting the expression of key effector proteins, and reducing the translation of multiple virulence genes of the bacteria (*26*). Unlike cinnamaldehyde, Fr.M$_1$ was water soluble. Therefore, there must be undiscovered active ingredients in Fr.M$_1$ and need to be further explored.

Our study disclosed the polar fraction of *C. bejolghota* bark extract performed an excellent anti-virulent and anti-infectious property in *in vitro* and *in vivo* experiments. Therefore, we conclude that *C. bejolghota* bark could be used as an ideal material for the prevention and treatment of Salmonella caused infection or as supplementary food to be taken up by human or animals, and absorbed by the body together with a normal diet, which may become a new anti-virulence therapy.

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