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Modulation of quorum sensing-controlled virulence factors by *Nymphaea tetragona* (water lily) extract

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ABSTRACT

Ethnopharmacological relevance: *Nymphaea tetragona* is a widely distributed ornamental species with ethnomedicinal uses in the treatment of diarrhea, dysentery, eruptive fevers, and infections. The anti-infectious activities of this herb have already been assessed to clarify its traditional use as a medicine.
Aim of study: In this study, we aimed to verify the inhibitory effects of *N. tetragona* 50% methanol extract (NTME) on quorum sensing (QS)-controlled virulence factors of bacteria since QS and its virulence factors are novel targets for antimicrobial therapy.
Materials and methods: The antibacterial activity of this extract was evaluated against *Chromobacterium violaceum* and *Pseudomonas aeruginosa*. The inhibition of the violacein pigment of *C. violaceum* by NTME was determined qualitative and quantitative using standard methods. The effects of NTME on swarming motility, biofilm viability, pyocyanin production, and LasA protease activity were evaluated using *P. aeruginosa*. Finally, the *in vitro* and *in vivo* cytotoxicity of NTME were verified by MTT assay and oral administration to rats, respectively.
Results: The extract had concentration-dependent antibacterial activity against gram-negative bacteria. NTME at 1/2× minimum inhibitory concentration (MIC), 1× MIC and 2× MIC significantly lowered the levels of violacein of *C. violaceum* compared to that of the control. The swarming motility of *P. aeruginosa* was inhibited by ≥70% by treatment with 1/2× MIC of NTME. There were remarkable reductions in pyocyanin production and LasA protease activity in the overnight culture supernatant of *P. aeruginosa* supplemented with NTME when compared with that of the untreated control. The confocal micrographs of 24 h biofilms of *P. aeruginosa* exposed to NTME exhibited a lower number of live cells than the control. No toxic effect was observed in *in vitro* and *in vivo* cytotoxicity assays of NTME.
Conclusions: NTME was demonstrated to have significant concentration-dependent inhibitory effects on quorum sensing-mediated virulence factors of bacteria with non-toxic properties, and could thus be a prospective quorum sensing inhibitor.

1. Introduction

Nymphaea tetragona belongs to the family Nymphaceae and, is the Asia-tropical representative of the diminutive water lilies. It is widely distributed, and can be found in Asia-temperate, Asia-tropical, Europe, and northern America (Nguyen, 2013; Tandon et al., 2010). *N. tetragona* has ethno-medical uses since the rhizome is used by tribal herbal practitioners in the Indian region to cure

Abbreviations: NTME, *N. tetragona* 50% methanol Extract; QS, quorum sensing; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MIC, minimum inhibitory concentration; LB, Luria-Bertani; MHB, Mueller Hinton Broth; MHA, Mueller Hinton Agar; TSA, Tryptic Soy Agar; rpm, rotations per minute; MBC, minimum bactericidal concentration; CLSI, Clinical and Laboratory Standards Institute; CFU, colony-forming unit; OD, optical density; PBS, phosphate-buffered saline; FBS, fetal bovine serum; LPS, lipopolysaccharide; DMSO, dimethyl sulfoxide, IC_{50}, inhibitory concentration fifty percent; OECD, Organization for Economic Co-operation and Development

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acute diarrhea and dysentery (Tandon et al., 2010). In folk medicine, different parts of the plant are used for the treatment of diarrhea with fever, enteritis, dysentery, eruptive fevers, painful discharge of urine, and infections of the urinary passages (Bown, 1995; Dash et al., 2013; El-Ghazali et al., 1994; Raja et al., 2010). In addition, practitioners of herbal medicine have also used water lily to treat kidney pain and congestion of the bronchi (Herbs, 2000). Thus, this plant has traditional use for anti-infective properties. Recently, phytotherapists and scientists from different fields have investigated the possible pharmacological effects of this plant.

In one study, geraniin was isolated from *N. tetragona*, an opportunistic water lily species, and was proven to inhibit the growth of fish pathogenic bacteria (*Aeromonas salmonicida* and *Pseudomonas fluorescens*) (Kurihara et al., 1993). It was also reported that *N. tetragona* effectively inhibited the growth of *Microcystis aeruginosa* in a liquor co-culture (Li and Hou, 2007). Nine biologically-active higher fatty acids were isolated from *N. tetragona* flowers and identified (Durgapal and Kothari, 2011). In our recent study, *N. tetragona* was demonstrated to have synergistic effects with antibiotics, and many bioactive compounds were identified from this plant (Hossain et al., 2014). Non-quantitative screening of the quorum sensing inhibition of this plant was also reported in that article (Hossain et al., 2014).

Quorum sensing is a bacterial signaling system that operates through diffusible chemical signal molecules and is dependent on their cell density (DeAngelis et al., 2008; Defoirdt et al., 2008). Bacterial virulence factor production and enzyme secretion are potentially controlled by this signaling system (Babić et al., 2010; Niu et al., 2006). In addition to virulence, QS regulates swarming motility, biofilm development, and drug resistance in bacteria (Wagner and Iglewski, 2008; Williams and Camara, 2009). Thus, QS inhibition has become a novel strategy for combating infections, pathogenesis, and bacterial drug resistance (Hong et al., 2012). *Pseudomonas aeruginosa* is a representative bacteria with widespread occurrence and severe virulence, and is regulated by the QS signaling system (Wagner and Iglewski, 2008).

The quorum sensing inhibition activity of *N. tetragona* was assessed in our previous study but was not quantitatively measured (Hossain et al., 2014). The plant extract was studied to determine the interactions when used in combination with commercial antibiotics in bacterial inhibition, and to determine the plant's compound profile (Hossain et al., 2014). However, in order to prove the potential of *N. tetragona*, it is important to determine the plant's QS inhibition in a quantifiable manner and to evaluate the effect of this plant on other virulence factors, neither of which has been reported to date. Thus, in the current study, we have expanded our previous work to quantitatively evaluate the effects of this plant extract on QS inhibition and the repression of other virulence factors regulated by the QS system. Moreover, we conducted *in vitro* and *in vivo* cytotoxicity tests to determine the safety profile of this plant extract for further commercial use. These data support the use of *N. tetragona* extract as a potential candidate for combatting bacterial virulence.

2. Materials and methods

2.1. Bacterial strains and culture media

Chromobacterium violaceum ATCC12472, *Pseudomonas aeruginosa* PAO1, and *Staphylococcus aureus* ATCC25923 were used in this study to determine the effects of the NTME. This study utilized Luria-Bertani (LB, Difco, USA) broth, Mueller Hinton Broth, Mueller Hinton Agar (MHB, MHA, Difco, USA) and Tryptic Soy Agar (TSA, Bacto, USA).

2.2. Extraction of plant material

N. tetragona whole plant powder was collected from Chamsamgol Lotus Farm (Chungju, South Korea). Previously reported extraction methods (Sikder et al., 2012; Yisa, 2009) were slightly modified and used to extract the plant. The plant powder (100 g) was placed into a 2000 mL round-bottom boiling flask (Schott Duran, NY, USA) with 1000 mL of 50% methanol and boiled on a non-asbestos surface at a temperature of 100 °C. The extraction was validated by checking the percent Brix, pH, absorbance at various wavelengths, and QS inhibition of *C. violaceum* (ATCC12472) by standard disk diffusion assay every 30 min for 6 h (data not shown). The percent Brix, absorbance, and QS inhibition of the extract were the highest from 3 h of extraction onwards. Thus, the supernatant of NTME after 3 h extraction was collected after passing through a filter paper of 70 mm porosity (Advantec®, Toyo Roshi Kaisha Ltd, Tokyo, Japan), and the filtrate solvent was then separated using a Buchi Rotavapor R-114 (BÜCHI Labortechnik AG, Flawil, Switzerland) at 10 rpm and an Eyela CCA-1111 (Tokyo Rikakikai Co. Ltd., China). A vacuum fridge dryer (Operon Co. Lid, South Korea) was maintained at −70 °C to solidify the dense supernatant. The total yield of dried material was 10.71 g.

2.3. Antibacterial activity

2.3.1. Determination of minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of NTME against *C. violaceum* (ATCC12472) and *P. aeruginosa* (PAO1) were evaluated according to Clinical and Laboratory Standards Institute (CLSI) guideline CLSI (Clinical and Laboratory Standards Institute) (2007). Briefly, NTME was successively diluted two fold in 96-well microplates to give final concentrations of 0.31, 0.63, 1.25, 2.50, 5.00, 10.00 and 20.00 mg/mL in different wells. Tetracycline was used as control in a concentration range of 0.50–256.00 μg/mL. Diluted bacterial culture (100 μL) was dispensed into each well of the 96-well plates so that the inoculum would be about 5×10^5 CFU/mL after inoculation. After incubation for 24 h, MICs were recognized as the lowest concentrations of the extract or drug that inhibited the increment of bacterial number compared to the initial number of bacteria. For MBC determination, 20 μL aliquots from the MIC wells and those above the MIC were plated onto MHA or TSA plates and incubated for 24 h at their respective incubation temperature. MBCs were considered as the lowest concentrations that completely inhibited the growth of bacteria on the agar plates.

2.3.2. Bacterial inhibition rate

The rates of inhibition of *C. violaceum* (ATCC12472) and *P. aeruginosa* (PAO1) were assessed by slightly modifying a previously described method (Dubuisson et al., 2010). Sterile MHB (10 mL) was supplemented beforehand with 1/2 × MIC (2.50 mg/mL), 1 × MIC (5.00 mg/mL), and 2 × MIC (10.00 mg/mL) of NTME in screw cap test tubes for the determination of the rates of inhibition of *C. violaceum* (ATCC12472). In the case of *P. aeruginosa* (PAO1), 1/2 × MIC (5.00 mg/mL), 1 × MIC (10.00 mg/mL), and 2 × MIC (20.00 mg/mL) of NTME were added to 10 mL of MHB in different test tubes. The final concentrations of bacteria in the MHB were approximately 5×10^5 CFU/mL. *C. violaceum* (ATCC12472) and *P. aeruginosa* (PAO1) were incubated at 30 °C and 37 °C, respectively, in a shaking incubator at 150 rpm for 24 h. Aliquots (100 μL) were collected at 0, 1, 2, 4, 8, 12 and 24 h of incubation and serially diluted tenfold. Colony-forming units (CFU) were evaluated by culturing 20 μL aliquots of even number of dilutions from the serial dilutions on TSA plates for 24 h at their respective temperatures.