Inhibition of quorum-sensing-controlled virulence factors of *Pseudomonas aeruginosa* by *Murraya koenigii* essential oil: a study in a *Caenorhabditis elegans* infectious model

P. Sankar Ganesh and Ravishankar Vittal Rai

Department of Studies in Microbiology, University of Mysore, Manasagangotri, Mysore 570006, Karnataka, India

The global emergence of antibiotic-resistant strains of *Pseudomonas aeruginosa* poses a major threat in both hospital environments and the community. *P. aeruginosa* is an opportunistic human pathogen, and it also infects a wide range of model organisms including the nematode *Caenorhabditis elegans*. Quorum sensing (QS) mediates cell-to-cell communication in bacteria and has an important role in regulating virulence genes, antibiotic resistance and biofilm formation, which are crucial for establishment of the infection. Expression of many virulence factors such as pyocyanin and proteases in *P. aeruginosa* is under the control of the QS system, and are mediated by small molecules such as acyl homoserine lactones. Thus, interfering with the QS system would provide alternative ways of controlling the pathogenicity. *Murraya koenigii* is a medicinal plant widely used in India. The present study investigated the *in vivo* inhibitory activity of *M. koenigii* essential oil (EO) on QS-controlled virulence factors of *P. aeruginosa* PAO1 using *C. elegans*. *M. koenigii* EO significantly inhibited the pyocyanin production and staphyloytic LasA activity of *P. aeruginosa* PAO1. As compared to the control group with 100% killing of *C. elegans*, *M. koenigii* EO was able to rescue an average of 60% of *C. elegans* from death due to the toxic effect of *P. aeruginosa*. Thus, the present study suggests the anti-QS potential of *M. koenigii* EO which therefore can be considered as a future therapeutic agent for management of *P. aeruginosa*-mediated infections.

**INTRODUCTION**

*Pseudomonas aeruginosa* is a Gram-negative opportunistic human pathogen that mainly infects patients suffering from cystic fibrosis, pulmonary diseases, AIDS and severe burns, and is one of the leading causes of nosocomial infections (Bjarnsholt *et al.*, 2005; Mendelson *et al.*, 1994; Van Delden & Iglewski, 1998). It also causes urinary tract infection (Shigemura *et al.*, 2006), meningitis (Pai *et al.*, 2016), dermatitis (Hopkins *et al.*, 1981), wound sepsis (Steinstraesser *et al.*, 2002) and ventilator-associated pneumonia (Crouch Brewer *et al.*, 1996). *Pseudomonas aeruginosa* is known to establish persistent biofilms on indwelling medical devices (Gogoi *et al.*, 2015). It is reported to be the second most frequently isolated pathogen from intensive care unit patients and is mainly transmitted through hospital co-workers and colonized patients (Spencer, 1996). The persistence of *Pseudomonas aeruginosa* infections can be attributed to its ability to secrete many extracellular virulence factors such as pyocyanin (phenazine) (Liang *et al.*, 2011), rhamnolipid, alginate, exotoxin A, elastase, LasA and alkaline protease, the expression of which is controlled by the quorum sensing (QS) system (Nicas & Iglewski, 1985).

In Gram-negative bacteria, QS is controlled by a group of small signalling molecules such as N-acyl homoserine lactones (AHLs) (Fuqua & Greenberg, 1998). *Pseudomonas aeruginosa* possesses two AHL-based QS systems, namely, *las* and *rhl* (Gambello & Iglewski, 1991; Ochsner & Reiser, 1995). The *las* system comprises the transcriptional regulator, LasR, and its cognate AHL signal, N-(3-oxododecanoyl)-l-homoserine lactone, synthesized by AHL synthase LasI (Pearson *et al.*, 1994). Similarly, the *rhl* system is composed of RhlR together with its cognate AHL, N-butyryl-l-homoserine lactone, synthesized by RhlII AHL synthase (Pearson *et al.*, 1995). These QS molecules play

**Abbreviations:** AHL, acyl homoserine lactone; BHI, brain–heart infusion; EO, essential oil; LB, Luria–Bertani; OD, optical density; QS, quorum sensing.

One supplementary figure is available with the online Supplementary Material.
significant roles in the establishment of biofilm and production of virulence factors (De Kievit et al., 2001).

*Pseudomonas aeruginosa* is intrinsically resistant to many antibiotics, due to low outer membrane permeability and adaptive resistance mechanisms (Hancock & Speert, 2000). Moreover, the emergence of clinical strains with modified virulence factors makes treatment difficult (Gooderham & Hancock, 2009). The current therapeutic options, such as β-lactam and aminoglycoside drugs, usually fail because of widespread antibiotic resistance (Lister et al., 2009). To circumvent this challenge, increasing attention has been paid in recent years to anti-QS compounds as potential therapeutic agents. Anti-QS compounds, when administered along with antibiotics, have the potential to increase the pathogen’s susceptibility to antibiotics (Brackman et al., 2011). The first anti-QS compound, a furanone derivative, was characterized from the seaweed *Delisea pulchra* (Manefield et al., 1999). Some anti-QS compounds have been identified from various plant sources, namely, garlic extract (Bjarnsholt et al., 2005), *Pisum sativum* seedlings (Teplitski et al., 2000), *Melicope lunu-ankenda* leaves (Tan et al., 2012), *Vaccinium macrocarpon* (Harjai et al., 2014), *Conocarpus erectus*, *Buedia bueras* and *Callistemon viminalis* (Adonizio et al., 2008), *Chamaesyce hypericifolia*, *Callistemon viminalis*, *Tetrazygia bicolor* and *Quercus virginiana* (Adonizio et al., 2006). Similarly, there are reports of anti-QS compounds derived from fruits and essential oils (EOs) being used as alternative therapeutic agents for inhibiting biofilm formation by *Pseudomonas aeruginosa* (Vattem et al., 2007; Khan et al., 2009; Jaramillo-Colorado et al., 2012; Ganesh & Rai, 2015a; Husain et al., 2015).

EOs have been widely used in traditional systems of medicine on account of their antibacterial, antifungal, antiviral, insecticidal (Bassole & Juliani, 2012) and immunomodulatory properties (Orhan et al., 2016). Some EOs confer antimicrobial activity by damaging the cell membrane or cell wall or by bringing about cell lysis and leakage of cell content of bacteria (Burt, 2004). Plant EOs are known for their bioactive potential, but studies on QS inhibitory activities of EO on virulence factors are scarce (Khan et al., 2009). *Murraya koenigii* is a small aromatic tree belonging to the family Rutaceae. The plant is known for its medicinal values, which are exploited in the preparation of tonics and medicines to treat dysentery (Schmelzer & Gurb-Fakim, 2013). The plant also possesses anti diarrhoeal (Mandal et al., 2010), anti diabetic (Yada et al., 2002), antioxidant (Sasidharan & Menon, 2011), anti cancer (Noolu et al., 2013), antiviral (Shah et al., 2015), antimicrobial (Vats et al., 2011), antifungal (Vats et al., 2011) and wound healing properties (Nagappan et al., 2012).

In this study, we used *Caenorhabditis elegans* N2 as a model organism to study the in vivo effects of *Murraya koenigii* EO on *Pseudomonas aeruginosa* infections. *Caenorhabditis elegans* is one of the simplest invertebrate worms normally found in soil, and is a model organism used to study host–pathogen interaction (Mahajan-Miklos et al., 1999) and pharmaceutical drug delivery (Zheng et al., 2013) because of its simple growth requirements and short generation time (Brenner, 1974). *Caenorhabditis elegans* is usually propagated in a Petri dish containing *Escherichia coli* OP50 as a food source (Stiernagle, 1999). *Pseudomonas aeruginosa* is able to kill *Caenorhabditis elegans* in two different ways. In rich, high-salt medium, *Pseudomonas aeruginosa* can kill the nematodes within a few hours (fast killing), is mediated by the production of virulence factors such as phenazines (pyocyanin pigment), which may act through the generation of active oxygen species (Mahajan-Miklos et al., 1999). In paralytic killing, *Caenorhabditis elegans* grown on brain–heart infusion (BHI) medium with *Pseudomonas aeruginosa* becomes paralysed (lethal paralysis) and subsequently dies (Darby et al., 1999). Paralytic killing is mediated by a diffusible factor (such as hydrogen cyanide) that is under the control of both LasR and RhlR QS regulators and results in rapid neuromuscular paralysis (Darby et al., 1999; Gallagher & Manoil, 2001). The *Pseudomonas aeruginosa*-mediated killing of *Caenorhabditis elegans* mainly depends on QS-controlled virulence factors. Plant extracts (Adonizio et al., 2008b) and EO (Husain et al., 2015) are reported to attenuate QS-controlled virulence factors of *Pseudomonas aeruginosa* in infectious models of *Caenorhabditis elegans*.

We previously reported the antimicrobial and antibiofilm activities of *Murraya koenigii* EO against the clinical strain of *Pseudomonas aeruginosa* PAO1 and other food-spoiling *Pseudomonas* spp. (Bai & Rai, 2014; Ganesh & Rai, 2015b). The antibiofilm property of *Murraya koenigii* EO has been attributed to the presence of compounds such as cinnamaldehyde and spathulenol (Ganesh & Rai, 2015b). These compounds have previously been shown to possess anti-QS and antibiofilm properties (Gilabert et al., 2011; Kim et al., 2015). Moreover, cinnamaldehyde and spathulenol were reported to be potent anti-tumour agents when tested on various cancer cell lines (Chuang et al., 2012; Haiyan et al., 2016). Cinnamaldehyde inhibits the Rhl QS system by interfering with substrate binding and, in turn, attenuates pyocyanin production in *Pseudomonas aeruginosa* (Chang et al., 2014). Similarly, the leaf extract of *Murraya koenigii* has been reported to be a potent source of proteasome inhibitors, having anticancerous properties (Noolu et al., 2013). The present investigation was carried out to assess the efficacy of *Murraya koenigii* EO in inhibiting the effect of QS-controlled virulence factors in the model organism *Caenorhabditis elegans*. This is perhaps the first study to evaluate the anti-QS activity of *Murraya koenigii* EO against *Pseudomonas aeruginosa* PAO1 in a *Caenorhabditis elegans* infection model.

**METHODS**

*Murraya koenigii* EO. The *Murraya koenigii* EO, extracted with supercritical fluid CO₂, was purchased from South East Agro Industries, Mysore, India, and was stored in a sterile, airtight container at 4 °C for further analysis.
Bacterial strains and growth conditions. In this study, wild-type *Pseudomonas aeruginosa* PAO1 (Stover et al., 2000), mutant strain *Pseudomonas aeruginosa* PAO1-JP2 (both las and rhlI mutant) (Pearson et al., 1997) and *Staphylococcus aureus* ATCC 25923 (Treonang et al., 2014) were grown in Luria–Bertani (LB) medium containing tryptone–water 1 g, yeast extract 0.5 g, agar 1.5 g and 100 ml distilled H2O (pH 7.4). A fresh single colony was inoculated into 25 ml LB broth and incubated at 37°C for 24 h at 250 r.p.m. *E. coli* OP50 (Brenner, 1974) was grown in LB broth and incubated at 30°C for 24 h at 250 r.p.m.

**Caenorhabditis elegans** (N2) stock and culture maintenance. The *Caenorhabditis elegans* wild-type Bristol strain N2 was used for parasitic and fast-killing assays, and maintained under standard culture conditions with *E. coli* OP50 as a food source (Brenner, 1974).

**Caenorhabditis elegans** N2 hermaphroditic worms were synchronized using bleaching solution (5 M KOH+5% sodium hypochlorite 1:1 concentration) (Stiernagle, 1999). The hatched eggs were poured onto the lawn of *E. coli* OP50 on nematode growth medium (Brenner, 1974), and plates were incubated at 25°C. The synchronized L1-stage worms were used in subsequent assay (Stiernagle, 1999).

**Bacterial growth curve analysis.** The growth curve for *Pseudomonas aeruginosa* PAO1 cultured in the presence and absence of *Murraya koenigii* EO was analysed. Briefly, an overnight culture of *Pseudomonas aeruginosa* PAO1 was inoculated separately into 100 ml LB broth at different concentrations (0.1–0.5%, v/v) of *Murraya koenigii* EO and without *Murraya koenigii* EO (control). The culture set-up was incubated at 37°C and the optical density (OD) at 600 nm was read every 1 h for up to 24 h.

**Quantification of pyocyanin pigment.** Pyocyanin was extracted from the cell-free supernatant of *Pseudomonas aeruginosa* PAO1 culture as described by Essar et al. (1990). Briefly, a single colony of *Pseudomonas aeruginosa* PAO1 culture was inoculated in 1 ml nutrient broth [peptone 1 g, beef extract 1 g, NaCl 0.5 g (pH 7.3±0.1), agar 100 ml distilled H2O] containing concentrations of *Murraya koenigii* EO varying from 0.1% to 0.5%, v/v, and control tubes without *Murraya koenigii* EO (control). The plate set-up was incubated at 37°C and, the optical density (OD) at 600 nm was read every 1 h for up to 24 h.

**Fast-killing assay.** The fast-killing assay plates were prepared according to Mahajan-Miklos et al. (1999) with slight modifications. Briefly, a fresh peptone/glucose medium was prepared, and 10 ml of peptone/glucose medium (1% Bacto-Peptone, 1% NaCl, 1% glucose, 1% Bacto-Agar with 0.15 M sorbitol) was overlaid with 0.3% (v/v) of agar with 0.15 M sorbitol. The plates were kept for 24 h at 37°C for the bacteria to form bacterial lawns. A plate seeded with *E. coli* OP50 strain (without *Murraya koenigii* EO treatment) was used as a control for estimating the natural death of the nematode.

The synchronized L1-stage worms were collected in a sterile 1.5 ml Eppendorf tube from the stock plate by adding M9 buffer [3 g KH2PO4, 6 g Na2HPO4, 5 g NaCl (pH 6.5), 1 ml 1 M MgSO4 and 1000 ml H2O] for transferring the worms (to Eppendorf tube) and also to remove surface-bound bacterial cells. The tube was centrifuged at 425 g for 2 min and again washed twice with M9 buffer to remove bacterial cells (*E. coli* OP50) adherent to the worms. A total of 10 worms were seeded onto the BHI plate (with or without *Murraya koenigii* EO) containing test bacterial lawns (*E. coli* OP50, *Pseudomonas aeruginosa* PAO1-JP2 and *Pseudomonas aeruginosa* PAO1), and the plates were incubated at 25°C for 12 h. Each assay was carried out in triplicate. After 12 h, the survival and death of the worms were observed every 1 h up to 4 h. Dead worms were confirmed by their non-responsiveness to touch with a platinum wire pick and the absence of pharyngeal movement by stereo zoom microscopic analysis.

**Statistical analysis.** All experiments were performed in triplicate. Statistical significance for the quantification of pyocyanin was determined by paired t-test (two tailed). Paralysis and fast killing were analysed by one-way ANOVA (variance with P-value of 0.01) using GraphPad Prism 5.03.
RESULTS AND DISCUSSION

Inhibition of pyocyanin pigment

The biosynthesis of phenazine pigment in *Pseudomonas aeruginosa* depends on the media used and is regulated by the QS system. The inhibition of pyocyanin production in *Pseudomonas aeruginosa* is important from the perspective of disease progression. In the present study, we analysed the inhibition of pyocyanin production by *Murraya koenigii* EO at the sub-MIC level of 0.3 % (v/v) (Fig. 1). A maximum of 64.2 % inhibition of pyocyanin production was observed at a concentration of 0.3 % (v/v) *Murraya koenigii* EO [Figs 1 and S1 (available in the online Supplementary Material)]. The presence of *Murraya koenigii* EO (0.3 %, v/v) did not interfere with bacterial growth, as evidenced by the growth curve assay (Fig. 2). Our results are in accordance with the findings of a previous study wherein the anti-QS activity of peppermint oil was also shown to inhibit the production of pyocyanin in *Pseudomonas aeruginosa* PAO1 (Husain et al., 2015). EOs from *Ferula asafoetida* and *Dorema aucheri* have recently been reported to possess inhibitory action on the production of pyocyanin (Sepahi et al., 2015). Furthermore, EOs of *Syzygium aromaticum* (0.097 %, v/v) and *Cinnamomum verum* (0.1 %, v/v) were also found to reduce pyocyanin production in a clinical isolate of *Pseudomonas aeruginosa* (MM-1) (Ganesh & Rai, 2015a).

Inhibition of LasA staphyloytic activity

*Pseudomonas aeruginosa* is known to produce a variety of extracellular protease enzymes, such as protease IV, alkaline protease, elastase A and elastase B, that are associated with the virulence of this opportunistic pathogen (Liu, 1974; Caballero et al., 2001; Le Berre et al., 2008). Elastase is one of the prototype virulence factors for *Pseudomonas aeruginosa* regulated by the QS cascade and it degrades infected tissue, promoting bacterial invasion (Hoge et al., 2010). LasA protease is an endopeptidase that can cleave the pentaglycine bridge present in the peptidoglycan of *Staphylococcus aureus* cell walls (Kessler et al., 1993). In the present study, a significant reduction in LasA staphyloytic activity was observed in the treated culture as compared to that of the control. At the end of 60 min, the level of LasA staphyloytic activity was found to be reduced by 63.1±1.2 % (Table 1). Our results are similar to the findings of Adonizio et al. (2008a), which showed that plant extracts such as *Bucida buceras* (96 %), *Conocarpus erectus* (94 %), *Tetracygia bicolor* (89 %), *Callistemon viminalis* (71 %) and *Chamaesyce hypericifolia* (49 %) resulted in drastic reduction of LasA protease in *Pseudomonas aeruginosa*. Furthermore, Singh et al. (2012) observed that *Lagerstroemia speciosa* fruit extract induced a substantial decrease in LasA protease activity (5.02–71.53 %) when compared with the control.

Paralytic assay

*Caenorhabditis elegans* is commonly used as an *in vivo* animal model for studying bacterial pathogenesis. The efficacy and host toxicity of anti-infective agents are readily studied in this 'nematode' model (Adonizio et al., 2008b). The present study revealed that 70 % of the infected worms survived at the end of 3 h of incubation in the presence of *Murraya koenigii* EO (0.3 %, v/v) (Fig. 3a), and the survival rate was 55 % to 60 % as observed after 4 h. There was 100 % mortality of non-treated worms at the end of 4 h. No deaths were observed when *Caenorhabditis elegans* was fed with *E. coli* OP50 (Fig. 3a). Similarly, the nematodes infected with the QS mutant strain of *Pseudomonas aeruginosa* PAO1-JP2 showed 90 % survival after 4 h (Fig. 3a). There was no marked toxic effect of *Murraya koenigii* EO on the survival and lifespan of *Caenorhabditis elegans* fed with *E. coli* OP50, suggesting the absence of toxic compounds in the former (data not shown). The results of the paralytic assay suggest that the addition of 0.3 % (v/v) *Murraya koenigii* EO decreased the expression of virulence factors by interfering with the QS system. Many plant extracts, such as garlic, *Conocarpus erectus*, *Callistemon viminalis*, *Bucida buceras* and *Mentha piperita* EO have been reported to be inhibitors of QS-dependent pathogenicity in infection models of *Caenorhabditis elegans* (Rasmussen et al., 2005; Adonizio et al., 2008b; Husain et al., 2015). Hydrogen cyanide is a primary toxin released by *Pseudomonas aeruginosa* and is responsible for rapid killing of nematodes (Gallagher & Manoil, 2001). Production of hydrogen cyanide by *Pseudomonas aeruginosa* PAO1 paralysed the nematodes through the *hcn* operon, which is controlled by the QS regulators LasR and RhlR at high cell density (Pessi & Haas, 2000). Based on the results of paralytic assay, it is tempting to speculate that *Murraya koenigii* EO...
koenigii EO has a role in reducing cyanide production by attenuating the hcn operon via the QS system.

Fast-killing assay

Pseudomonas aeruginosa can kill Caenorhabditis elegans due to the production of the virulence factor phenazine (pyocyanin pigment), which is under the control of the QS system (Mahajan-Miklos et al., 1999). In the fast-killing method, 40% of infected nematodes died within 2 h and the remaining 60% were completely dead after 4 h (Fig. 3b). Following treatment with Murraya koenigii EO (0.3%, v/v), 60% of the nematodes remained alive at the end of 4 h (Fig. 3b). Nematode death was not observed on E. coli OP50 plates at the end of the incubation time (Fig. 3b). Similarly, infection with the QS mutant strain of Pseudomonas aeruginosa PAO1-JP2 (both lasI and rhII mutants) resulted in only 10% nematode death within 4 h (Fig. 3b). Our results suggest that Caenorhabditis elegans infected with the lasI and rhII mutant strains of Pseudomonas aeruginosa PAO1-JP2 demonstrates reduced mortality because of lack of production of phenazine (pyocyanin pigment). Similar results were observed by Mahajan-Miklos et al. (1999), where a mutant strain of Pseudomonas aeruginosa PA14 (transposon Tn phoA) failed to kill the nematodes. Treatment with Murraya koenigii EO significantly reduced the death rate in Caenorhabditis elegans infected with Pseudomonas aeruginosa PA01 when compared to absence of Murraya koenigii EO treatment (Fig. 3b). The expression of virulence factors such as phenazine by Pseudomonas aeruginosa PA01 mediated rapid killing of Caenorhabditis elegans (Mahajan-Miklos et al., 1999).

In conclusion, targeting of the QS system is gaining much attention as an infection control strategy, especially in the wake of widespread drug resistance among bacterial pathogens. Our study documents the attenuation of QS-controlled virulence factors of Pseudomonas aeruginosa by Murraya koenigii EO in an infection model of Caenorhabditis elegans. Murraya koenigii EO significantly reduced the

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*The percentage of LasA inhibition was calculated with respect to control OD at 600 nm.
death rate of Caenorhabditis elegans by inhibiting the toxicity of Pseudomonas aeruginosa. Our results suggest that Murraya koenigii EO is a source of anti-QS agents for controlling Pseudomonas aeruginosa infections.

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Attenuation of virulence factors of P. aeruginosa by M. koenigii essential oil


