Anti-infective potential of castor oil and ricinoleic acid against selected human-pathogenic bacteria

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Castor oil (CO) and Ricinoleic acid (RA) were evaluated for their anti-infective activity against four different human-pathogenic bacteria, employing the nematode worm *Caenorhabditis elegans* as a model host. *In vitro* assays to study interaction of these two items with test bacteria with respect to bacterial growth, quorum-regulated pigment production, biofilm formation, and few other clinically relevant traits were also performed.

CO and RA were procured from CDH and Sigma-Aldrich respectively. Methodology of the *in vitro* and *in vivo* assays was adopted from our previous publications (Joshi et al., 2019; Patel et al., 2019), which also contains details of the organisms used.

Results explaining effect of CO and RA are presented through Figures 1-15, which indicate CO and RA both to be capable of attenuating virulence of pathogenic bacteria. Latter exerted activity at concentrations much lesser than that of CO. While major observations are mentioned below, full details of this study can be seen in Biswas et al. (2018).

CO (0.5-2% v/v) could attenuate virulence of three of the test bacteria by 25-56%. It could enhance susceptibility of all the four pathogens to lysis in presence of human serum upto 20-39%. CO could eradicate pre-formed *P. aeruginosa* biofilm by 30%. Neither *P. aeruginosa* nor *S. aureus* could develop complete resistance to CO even after repeated exposure to this plant oil. CO was able to modulate quorum-regulated pigment production in all the three multidrug-resistant gram-negative bacteria used in this study. Antibiotic susceptibility of all the test pathogens was modulated upon CO-treatment.

RA (1-20 µg/mL) could attenuate virulence of all the three gram-negative pathogens by 4-63%. Even repeated exposure of *P. aeruginosa* or *S. marcescens* to RA did not induce complete resistance in these pathogens. Susceptibility of three of the test pathogens to lysis in presence of human serum was enhanced upon RA-treatment by 21-77%. Haemolytic potential of *P. aeruginosa* and *C. violaceum* was curbed upto 23-41% as a result of RA-pretreatment. Production of quorum-modulated pigment in all four test pathogens was modulated under influence of RA. Antibiotic susceptibility of all the four pathogens was also modulated as a result of RA-treatment.

As RA seemed to exert anti-pathogenic potential at concentrations lower than that of CO, and since it has been known to be a constituent of CO (Patel et al., 2016); RA can be said to be largely (not necessarily solely) responsible for the anti-infective potential of CO.

Figure 1. Effect of castor oil on *Chromobacterium violaceum*

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; AS: Antibiotic susceptibility; QS: Quorum sensing; CO: Castor Oil

(A) Effect of CO on growth and QS-regulated violacein production in *C. violaceum*: Bacterial cell density was quantified as OD₇₅₀; OD of violacein was measured at 570 nm, and Violacein Unit was calculated as the ratio OD₅₇₀/OD₇₅₀ (an indication of violacein production per unit of growth); **(B) CO acts as a signal-response inhibitor against *C. violaceum*.** **(C) CO-pre-treatment enhances susceptibility of *C. violaceum* to different antibiotics.** **(D) CO had no effect on catalase activity and haemolytic activity of *C. violaceum*, but it modulates susceptibility of *C. violaceum* to lysis by human serum:** Catalase activity was assessed by monitoring disappearance of H₂O₂ at 240 nm. Hemoglobin concentration was measured as OD₄₉₀. 1% triton served as the positive control (OD₄₉₀ = 1.6 ± 0.004), and PBS served as the negative control (OD₄₉₀ = 0.02 ± 0.004) for this assay. 'Control' in serum-dependent lysis assay was CO-unexposed cells of *C. violaceum* incubated with human serum. **(E) CO-treatment attenuates virulence of *C. violaceum* towards *C. elegans*:** Catechin (50 µg/mL) and ampicillin (500 µg/mL) employed as positive controls conferred 100% protection on worm population, and CO at 0.05% v/v and 0.25% v/v conferred 4% ***±0.28 and 7.33% ***±0.15 survival benefit, respectively. Survival benefit refers to the difference between number of worms surviving in experimental and control wells. CO at tested concentrations showed no toxicity towards the worm.

Figure 2. Effect of castor oil on *Serratia marcescens*

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; AS: Antibiotic susceptibility; QS: Quorum sensing; CO: Castor Oil

(A) Effect of CO on growth and QS-regulated prodigiosin production in *S. marcescens*: Bacterial cell density was quantified as OD₇₅₀; OD of prodigiosin was measured at 490 nm, and Prodigiosin Unit was calculated as the ratio OD₄₉₀/OD₇₅₀ (an indication of prodigiosin production per unit of growth). **(B) CO-pre-treatment modulates susceptibility of *S. marcescens* to different antibiotics.** (C) CO enhances catalase activity of *S. marcescens*: Catalase activity was assessed by monitoring disappearance of H₂O₂ at 240 nm. (D) CO had no effect on haemolytic activity of *S. marcescens*: Hemoglobin concentration was measured as OD₄₉₀. 1% triton served as the positive control (OD₄₉₀ = 1.6 ± 0.004), and PBS served as the negative control (OD₄₉₀ = 0.02 ± 0.004) for this assay. **(E) CO modulates susceptibility of *S. marcescens* to lysis by human serum:** 'Control' in serum-dependent lysis assay was CO-unexposed cells of *S. marcescens* incubated with human serum. **(F) CO-treatment reduces virulence of *S. marcescens* towards *C. elegans*:** Catechin (50 µg/mL) and ofloxacin (0.1 µg/mL) employed as positive controls conferred 100% and 80% protection on worm population, respectively. Pre-treatment of bacteria with CO at 0.05, 0.1, 0.125, 0.25, 0.5, 1, 1.25, 1.5 and 2% v/v conferred 28.34%*** ± 2.28, 29.94%*** ± 3.33, 31.64%*** ± 2.84, 41.64%*** ± 3.28, 38.34%*** ± 4.28, 34.94%*** ± 2.28, 44.94%*** ± 3.34, 46.64%*** ± 4.46 and 56.64%*** ± 4.48 survival benefit, respectively; Survival benefit refers to the difference between number of worms surviving in experimental and control wells. CO at tested concentrations showed no toxicity towards the worm.

Figure 3. Effect of castor oil on various traits of *S. marcescens*, in vitro

* $p < 0.05$, ** $p < 0.01$; CO: Castor Oil

(A) Demonstration of post-extract effect of CO on *S. marcescens* in vitro. (B) Effect of CO on *S. marcescens* growth, and pigment production altered after repeated exposure to CO.

Bacterial cell density was quantified as OD₇₅₀; OD of prodigiosin was measured at 490 nm, and Prodigiosin Unit was calculated as the ratio OD₄₉₀/OD₇₅₀ (an indication of prodigiosin production per unit of growth).

Figure 4. Effect of castor oil on *Pseudomonas aeruginosa*

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; AS: Antibiotic susceptibility; QS: Quorum sensing; CO: Castor Oil; PBS: Phosphate buffer saline

(A) Effect of CO on growth and QS-regulated pigment production in *P. aeruginosa*: Bacterial cell density was quantified as OD₇₅₀; OD of pyoverdine was measured at 405 nm. Pyocyanin was measured at 490 nm. Pyoverdine Unit was calculated as the ratio OD₄₀₅/OD₇₅₀ (an indication of pyoverdine production per unit of growth); Pyocyanin Unit was calculated as the ratio OD₄₉₀/OD₇₅₀ (an indication of pyocyanin production per unit of growth). **(B) CO acts as a signal-response inhibitor against *P. aeruginosa*.** **(C) CO-treatment made *P. aeruginosa* more susceptible to cephalixin** **(D) CO had no effect on catalase activity, enhances haemolytic activity, and increases susceptibility of *P. aeruginosa* to lysis by human serum:** Catalase activity was assessed by monitoring disappearance of H₂O₂ at 240 nm. Hemoglobin concentration was measured as OD₄₉₀. 1% triton served as the positive control (OD₄₉₀ = 1.6 ± 0.004), and PBS served as the negative control (OD₄₉₀ = 0.01 ± 0.00) for this assay. 'Control' in serum-dependent lysis assay was CO-unexposed cells of *P. aeruginosa* incubated with human serum. **(E) CO reduced *P. aeruginosa* biofilm formation, eradicated pre-formed biofilm, but it enhanced biofilm viability:** Crystal violet assay was performed to measure biofilm formation, and biofilm eradication, followed by the measurement of OD at 570 nm; Cell viability in biofilm was estimated through MTT assay, wherein OD was measured at 570 nm.

Figure 5. Effect of CO on various traits of *P. aeruginosa*, *in vitro* and *in vivo*

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; CO: Castor Oil

(A) Effect of CO on *P. aeruginosa* growth and pyoverdine production remained unaltered, while pyocyanin production was altered after repeated exposure to CO. **(B) No Post Extract Effect of CO on *P. aeruginosa* was observed *in vitro*:** Bacterial cell density was quantified as OD₇₅₀; OD of pyoverdine was measured at 405 nm, Pyocyanin was measured at 490 nm. Pyoverdine Unit was calculated as the ratio OD₄₀₅/OD₇₅₀ (an indication of pyoverdine production per unit of growth); Pyocyanin Unit was calculated as the ratio OD₄₉₀/OD₇₅₀ (an indication of pyocyanin production per unit of growth) **(C) CO-treatment reduces the virulence of *P. aeruginosa* towards *C. elegans*:** Catechin (50 µg/mL) and gentamicin (0.1 µg/mL) employed as positive controls conferred 100% protection on worm population. CO at 1, 1.25, 1.5 and 2% v/v conferred, 5%*** ± 0.82, 25%*** ± 2.28, 40%*** ± 3.28 and 45%*** ± 4.45 survival benefit respectively. Survival benefit refers to the difference between number of worms surviving in experimental and control wells. CO at tested concentrations showed no toxicity towards *C. elegans*. **(D) Post-extract effect of CO on *P. aeruginosa* *in vivo***

Figure 6. Effect of castor oil on *Staphylococcus aureus*

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; AS: Antibiotic susceptibility; QS: Quorum sensing; CO: Castor oil, PBS: Phosphate buffer saline

(A) Effect of CO on growth and QS-regulated pigment production in *S. aureus*: Bacterial cell density was quantified as

OD₇₅₀; OD of staphyloxanthin was measured at 450 nm, and Staphyloxanthin Unit was calculated as the ratio OD₄₅₀/OD₇₅₀ (an indication of staphyloxanthin production per unit of growth). **(B) CO-treatment made *S. aureus* more susceptible to gentamicin.** **(C) CO had no effect on catalase activity, enhances haemolytic activity, and modulates susceptibility of *S. aureus* to lysis by human serum:**

Catalase activity was assessed by monitoring disappearance of H₂O₂ at 240 nm; Hemoglobin concentration was measured as OD₄₉₀. 1% triton served as the positive control (OD₄₉₀ = 1.6 ± 0.004), and PBS served as the negative control (OD₄₉₀ = 0.02 ± 0.004) for this assay; 'Control' in serum-dependent lysis assay was CO-unexposed cells of *S. aureus* incubated with human serum. **(D) CO reduced *P. aeruginosa* biofilm formation, had no effect on eradicated pre-formed biofilm, but enhanced biofilm viability:** Crystal violet assay was performed to measure biofilm formation, and biofilm eradication, followed by the measurement of OD at 570 nm; Cell viability in biofilm was estimated through MTT assay, wherein OD was measured at 570 nm. **(E) CO-treatment reduces the virulence of *S. aureus* towards *C. elegans*:** Catechin (50 μg/mL) and gentamicin (0.1 μg/mL) employed as a positive controls conferred 100% protection on worm population. 0.05, 0.1, 0.125, 0.25, 0.5, 1, 1.25, 1.5 and 2% v/v conferred 0%, 10% ± 1.01, 27.5% ± 2.28, 30% ± 3.23, 45% ± 4.42, 52.5% ± 3.23, 52.5% ± 2.28, 37.5% ± 4.22 and 40% ± 3.52 survival benefit, respectively. Survival benefit refers to the difference between number of worms surviving in experimental and control wells. CO at tested concentrations showed no toxicity towards *C. elegans*. **(F) Post-extract effect of CO on *S. aureus* in vivo**

Figure 7. Effect of CO on mix-species culture of *P. aeruginosa* and *S. aureus*

(A) Biofilm formation **(B)** Anti-virulence effect of CO on mix-species culture of *P. aeruginosa* and *S. aureus*

Figure 8. Effect of ricinoleic acid on *Chromobacterium violaceum*

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; AS: Antibiotic susceptibility; QS: Quorum sensing; RA: Ricinoleic acid

(A) Effect of RA on growth and QS-regulated violacein production in *C. violaceum*: Bacterial cell density was quantified as OD₇₅₀; OD of violacein was measured at 570 nm, and Violacein Unit was calculated as the ratio OD₅₇₀/OD₇₅₀ (an indication of violacein production per unit of growth). **(B) RA acts as a signal-response inhibitor against *C. violaceum*.** **(C) RA-pre-treatment modulates susceptibility of *C. violaceum* to different antibiotics.** **(D) RA enhances catalase activity and inhibits haemolytic of *C. violaceum* but RA modulates susceptibility of *C. violaceum* to lysis by human serum:** Catalase activity was assessed by monitoring disappearance of H₂O₂ at 240 nm. Hemoglobin concentration was measured as OD₄₉₀. 1% triton served as the positive control (OD₄₉₀ = 1.5 ± 0.005), and PBS served as the negative control (OD₄₉₀ = 0.03 ± 0.004) for this assay. 'Control' in serum-dependent lysis assay was RA-unexposed cells of *C. violaceum* incubated with human serum. **(E) RA-treatment attenuates virulence of *C. violaceum* towards *C. elegans*:** Catechin (50 μg/mL) and ampicillin (500 μg/mL) employed as positive controls conferred 100% protection on worm population, and RA at 1 μg/mL, 5 μg/mL, 10 μg/mL, 20 μg/mL conferred 13.33% ± 1.28, 23.33% ± 1.25, 40% ± 2.28, 63.33% ± 3.23 survival benefit, respectively. Survival benefit refers to the difference between number of worms surviving in experimental and control wells. DMSO present in the 'vehicle control' at 0.5% v/v did not affect virulence of the bacterium towards *C. elegans*; DMSO (0.5% v/v) and RA at tested concentrations showed no toxicity towards the worm.

Figure 9. Effect of ricinoleic acid on *Serratia marcescens*

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; AS: Antibiotic susceptibility; QS: Quorum sensing; RA: Ricinoleic acid

(A) Effect of RA on growth and QS-regulated prodigiosin production in *S. marcescens*: Bacterial cell density was quantified as OD₇₅₀; OD of prodigiosin was measured at 490 nm, and Prodigiosin Unit was calculated as the ratio OD₄₉₀/OD₇₅₀ (an indication of prodigiosin production per unit of growth). **(B) RA acts as a signal-response inhibitor against *S. marcescens*.** **(C) RA-pre-treatment modulates susceptibility of *S. marcescens* to different antibiotics.** **(D) CO inhibits catalase activity but had no effect on haemolytic activity, and it enhances susceptibility of *S. marcescens* to lysis by human serum.** Catalase activity was assessed by monitoring disappearance of H₂O₂ at 240 nm. Hemoglobin concentration was measured as OD₄₉₀. 1% triton served as the positive control (OD₄₉₀ = 1.6 ± 0.004), and PBS served as the negative control (OD₄₉₀ = 0.03 ± 0.005) for this assay. 'Control' in serum-dependent lysis assay was RA-unexposed cells of *S. marcescens* incubated with human serum.

Figure 10. Effect of ricinoleic acid on various traits of *S. marcescens*, in vitro and in vivo

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; RA: Ricinoleic acid

(A) Effect of RA on *S. marcescens* growth and Prodigiosin Unit altered after repeated exposure to RA: Bacterial cell density was quantified as OD₇₅₀; OD of prodigiosin was measured at 490 nm. Prodigiosin Unit was calculated as the ratio OD₄₉₀/OD₇₅₀ (an indication of prodigiosin production per unit of growth). **(B) RA-treatment reduces the virulence of *S. marcescens* towards *C. elegans*:** Catechin (50 μg/mL) and gentamicin (0.1 μg/mL) employed as positive controls conferred 100% protection on worm population. RA at 10 μg/mL, 20 μg/mL conferred 28.33 and 46.66 survival respectively. Survival benefit refers to the difference between number of worms surviving in experimental and control wells. DMSO present in the 'vehicle control' at 0.5% v/v did not affect virulence of the bacterium towards *C. elegans*; DMSO (0.5% v/v) and RA at tested concentrations showed no toxicity towards *C. elegans*. **(C) No Post Extract Effect of RA on *S. marcescens* was observed in vitro** **(D) Post-extract effect of RA on *S. marcescens* in vivo**

Figure 11. Effect of ricinoleic acid on *Pseudomonas aeruginosa*

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; AS: Antibiotic susceptibility; QS: Quorum sensing; RA: Ricinoleic acid; PBS: Phosphate buffer saline

(A) Effect of RA on growth and QS-regulated pigment production in *P. aeruginosa*: Bacterial cell density was quantified as OD₇₅₀; OD of pyoverdine was measured at 405 nm, Pyocyanin was measured at 490 nm. Pyoverdine Unit was calculated as the ratio OD₄₀₅/OD₇₅₀ (an indication of pyoverdine production per unit of growth); Pyocyanin Unit was calculated as the ratio OD₄₉₀/OD₇₅₀ (an indication of pyocyanin production per unit of growth). **(B) RA-pre-treatment modulates susceptibility of *P. aeruginosa* to different antibiotics.**

(C) RA enhances catalase activity and inhibits haemolytic activity, but had no effect on susceptibility of *P.*

***aeruginosa* to lysis by human serum:** Catalase activity was assessed by monitoring disappearance of H₂O₂ at 240 nm; Hemoglobin concentration was measured as OD₄₉₀. 1% triton served as the positive control (OD₄₉₀ = 1.5), and PBS served as the negative control (OD₄₉₀ = 0.01) for this assay; 'Control' in serum-dependent lysis assay was RA-unexposed cells of *P. aeruginosa* incubated with human serum. **(D) RA had no effect on *P. aeruginosa* biofilm formation, eradication, and biofilm viability:** Crystal violet assay was performed to measure biofilm formation, and biofilm eradication, followed by the measurement of OD at 570 nm; Cell viability in biofilm was estimated through MTT assay,

wherein OD was measured at 570 nm.

Figure 12. Effect of ricinoleic acid on various traits of *P. aeruginosa*, in vitro and in vivo

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; RA: Ricinoleic acid

(A) Effect of RA on *P. aeruginosa* growth and pigment productions altered after repeated exposure to RA:

Bacterial cell density was quantified as OD₇₅₀; OD of pyoverdine was measured at 405 nm, Pyocyanin was measured at 490 nm. Pyoverdine Unit was calculated as the ratio OD₄₀₅/OD₇₅₀ (an indication of pyoverdine production per unit of growth); Pyocyanin Unit was calculated as the ratio OD₄₉₀/OD₇₅₀ (an indication of pyocyanin production per unit of growth) **(B) RA-treatment reduces the virulence of *S. marcescens* towards *C. elegans*:** Catechin (50 µg/mL) and gentamicin (0.1 µg/mL) employed as positive controls conferred 100% protection on worm population RA at 10 µg/mL, 50 µg/mL, 100 µg/mL, and repeated exposure 10 times on RA (100 µg/mL) containing media is 0%, 16.67%***± 2.35, 23.34%***± 1.18 survival benefit respectively. Survival benefit refers to the difference between number of worms surviving in experimental and control wells. DMSO present in the 'vehicle control' at 0.5% v/v did not affect virulence of the bacterium towards *C. elegans*; DMSO (0.5% v/v) and

RA at tested concentrations showed no toxicity towards *C. elegans*. **(C) No Post Extract Effect of RA on *P. aeruginosa* was observed in vitro** **(D) Post-extract effect of RA on *P. aeruginosa* in vivo**

Figure 13. Effect of ricinoleic acid on *Staphylococcus aureus*

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; AS: Antibiotic susceptibility; QS: Quorum sensing; RA: Ricinoleic acid, PBS: Phosphate buffer saline

(A) Effect of RA on growth and QS-regulated pigment production in *S. aureus*: Bacterial cell density was quantified as OD₇₅₀; OD of staphyloxanthin was measured at 450 nm, and Staphyloxanthin Unit was calculated as the ratio OD₄₅₀/OD₇₅₀ (an indication of staphyloxanthin production per unit of growth). **(B) RA pre-treatment modulates susceptibility of *S. aureus* to different antibiotics.** **(C) RA had no effect on catalase activity, enhances haemolytic activity, and modulates susceptibility of *S. aureus* to lysis by human serum:** Catalase activity was assessed by monitoring disappearance of H₂O₂ at 240 nm. Hemoglobin concentration was measured as OD₄₉₀. 1% triton served as the positive control (OD₄₉₀ = 1.5), and PBS served as the negative control (OD₄₉₀ = 0.03) for this assay. 'Control' in serum-dependent lysis assay was RA-unexposed cells of *S. aureus* incubated with human serum. **(D) RA could not affect the biofilm formation, eradication, and biofilm viability:** Crystal violet assay was performed to measure biofilm formation, and biofilm eradication, followed by the measurement of OD at 570 nm; Cell viability in biofilm was estimated through MTT assay, wherein OD was measured at 570 nm.

Figure 14. No Post Extract Effect of ricinoleic acid on *S. aureus* was observed (A) *in vitro* (B) *in vivo*

$p < 0.05$, $**p < 0.01$; RA: Ricinoleic acid

Bacterial cell density was quantified as OD₇₅₀; OD of staphyloxanthin was measured at 450 nm, and Staphyloxanthin Unit was calculated as the ratio OD₄₅₀/OD₇₅₀ (an indication of staphyloxanthin production per unit of growth).

Figure 15. Effect of RA on mix-species culture of test pathogens

RA: Ricinoleic acid

(A) Anti-virulence effect of RA on mix-species culture of *P. aeruginosa* and *S. aureus* (B) Anti-virulence effect of RA on mix-species culture of *S. marcescens* and *C. violaceum*

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