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Journal Name:	**Microbiology Research Journal International**
Manuscript Number:	**Ms_MRJI_51659**
Title of the Manuscript:	**Anti-virulence Activity of Three Medicinal Plants: Cassia occidentalis L., Crossopteryx febrifuga (Afzel ex G. Don) Benth. and Zanthoxylum zanthoxyloides (Lam) Zep. and Timl**
Type of the Article	**Original Research Article**

General guideline for Peer Review process:

This journal's peer review policy states that **NO** manuscript should be rejected only on the basis of '**lack of Novelty'**, provided the manuscript is scientifically robust and technically sound.
To know the complete guideline for Peer Review process, reviewers are requested to visit this link:

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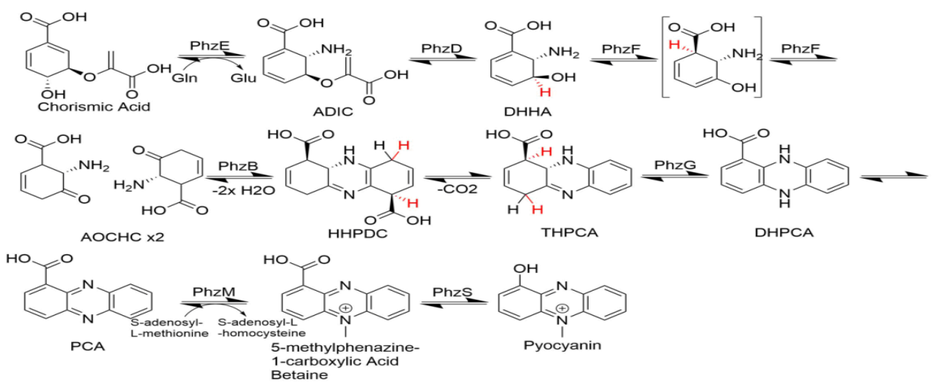
PART 1: Review Comments

	Reviewer's comment	**Author's comment** *(if agreed with reviewer, correct the manuscript and highlight that part in the manuscript. It is mandatory that authors should write his/her feedback here)*
Compulsory REVISION comments	The paper Ms_MRJI_51659 described the effects of plant extracts to reduce procyanin and elastase in vitro as virulence marker. However, toxin genes or Tra and Tbr genes could be studied? However, have no growth inhibitory activities? Without purification , the study has no value because high concentration was used. PCR technology could be used to detect the mRNA. The paper could be published only after correction of the following statement and needs more work. Recently, phyto-medicine has given priority but you have to work substantial to give idea of chemicals by HPLC, NMR, FTIR and Mass. Literature Search performed:  shikimic acid → chorismic acid → phenazine-1-carboxylic acid → 5-methylphenazine-1-carboxylic acid betaine → pyocyanin[6] Mavrodi, D. V.; Bonsall, R. F.; Delaney, S. M.; Soule, M. J.; Phillips, G.; Thomashow, L. S. (2001). "Functional Analysis of Genes for Biosynthesis of Pyocyanin and Phenazine-1-Carboxamide from Pseudomonas aeruginosa PAO1". *Journal of Bacteriology*. **183** (21): 6454–6465. doi:10.1128/JB.183.21.6454-6465.2001. In the cystic fibrosis lung, intracellular pyocyanin converts molecular oxygen to the superoxide free radical by oxidizing NADPH to $NADP^+$. Pyocyanin inactivates catalase by reducing its gene's transcription as well as directly targeting the enzyme itself. Another target of pyocyanin is caspase 3-like proteases which can then go on to initiate apoptosis and necrosis. Quorum sensing (QS) via acyl-homoserine lactone (HSL), controls the expression of an array of virulence genes in *P. aeruginosa.* The autoinducer synthase, LasI, synthesises N-(3-oxododecanoyl) homoserine lactone (3OC12-HSL), which regulates the production of elastase, exotoxin A and alkaline protease, while RhlI synthesizes the autoinducer N-butyryl homoserine lactone (C4-HSL), which regulates the production of rhamnolipid, alkaline protease, elastase, cyanide and pyocyanin [4,5]. Winson MK, Camara M, Latifi A, Foglino M, Chhabra SR, Daykin M, Bally M, Chapon V, Salmond GP, Bycroft BW. Multiple N-acyl-L-homoserine lactone signal molecules regulate	

production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa* . Proc Natl Acad Sci U S A. 1995;92:9427–9431

Virulence genes in this study were selected on the basis of their importance. Isolates were screened for QS systems, alkaline protease (*aprA*), rhamnolipid AB (*rhlAB*), phospholipase (*plcH*) and elastase (*lasB*). They were also screened for exotoxin A (*toxA*) which is highly conserved in *P. aeruginosa* and not in other species of this genus. Highly conserved flagellin'b' and heterologous flagellin 'a' were also screened in the uroisolates.

Nikbin VS, Aslani MM, Sharafi Z, Hashemipour M, Shahcheraghi F, Ebrahimipour GH. Molecular identification and detection of virulence genes among *Pseudomonas aeruginosa* isolated from different infectious origins. Iran J Microbiol. 2012;4:118–23.

Amplifications were carried out in 25 µl volumes containing template DNA (50 ng), Taq buffer (1X), DMSO (Dimethyl sulfoxide), Magnesium chloride (2 mM), each primer (10 pM/µl), nucleotides (dATP, dCTP, dGTP, dTTP) (200 µM, Thermo Scientific) and Taq polymerase (1 U/µl, FIREpol). Amplifications were carried out in a Biorad Thermal Cycler for 30 cycles consisting of pre-denaturation, denaturation, annealing, extension and post elongation. The parameters for the amplification cycles used in each PCR experiment are represented in Table 1. A set of oligonucleotide primers (Eurofins Genomics) that allowed to amplify whole QS genes (*lasI, lasR, rhlI and rhlR*) [13] were selected. Also, PCR assays were used to detect the extracellular virulence genes encoding alkaline protease (*aprA*) [14], exotoxin A (*toxA*) [15] and rhamnolipid AB (*rhlAB*), phospholipase H (*plcH*), elastase (*lasB*) [7]. A set of conserved oligonucleotide primers CW45, CW46 [16] was also used to analyse flagellin subtypes in the clinical strains. The sequences of specific primers used in PCR reactions and the molecular weight of the obtained amplicons are presented in Table 2. After amplification, 10 µl sample was subjected to electrophoresis on a standard 1% agarose gel for 1 h at 100 V, stained with ethidium bromide (Sigma) and detected by UV transillumination

Vasil ML, Chamberlain C, Grant CCR. Molecular studies of Pseudomonas exotoxin A gene. Infect Immun. 1986;52:538–548.

P. aeruginosa strains produce two distinct types of O antigen (O-Ag): a common polysaccharide antigen (A-band) composed of a homopolymer of d-rhamnose, and an O-specific antigen (B-band) composed of a heteropolymer of three to five distinct sugars in its repeat units. So far, *P. aeruginosa* isolates have been classified into 20 serotypes by the International Antigenic Typing Scheme (IATS) [2]. The lipopolysaccharide (LPS) of *P. aeruginosa* is less toxic than that of other Gram-negative rods, facilitating its establishment of chronic infections by eliciting a low inflammatory response [3] *P. aeruginosa* also possesses two other LuxR-type regulators, both of which affect the expression of the *rhlI* and *lasI* genes. QscR (*q*uorum *s*ensing *c*ontrol *r*epressor) negatively affects the production of RhlI- and LasI-made AHLs in an AHL-independent manner, despite being able to bind AHLs (Chugani *et al.*, 2001).

Major points are:

The ability of plant extracts to inhibit the production of pyocyanin was assessed according to previously described procedures [10]. Overnight culture of *P. aeruginosa* PAO1 was diluted and supplemented with plant extract dissolved in DMSO. After 18 h of incubation at 37 °C, 175 rpm, tubes were sampled to assess bacterial growth through turbidity (OD_{600nm}). Supernatant was used for pyocyanin determination (A_{380nm}). Pyocyanin was extracted successively with chloroform and 0.2 M HCl.

	Elastase production contained in the supernatant was assessed according to [14]. Briefly, 750 µL cell free supernatant was added to 250 µL elastin congo red solution (5 mg/mL in 0.1 M Tris-HCl pH 8; 1 mM $CaCl_2$) and the mixture was incubated at 37 °C for 16 h at 200 rpm. The mixture was centrifuged at 3000 g for 10 min and absorbance was read at 495 nm to estimate elastase activity. Question 1. Why the incubation is 16-18 hrs? Make a time course and show the optimum temperature. Your reaction mixture will be dried at 37^{O}C for 18 hrs?????? Question 2. 100µg/ml concentration is too high??? So, make Thin Layer Chromatography (20x10cm), scratch the bands and show which chemical is active and then you can lower the concentration. C. febrifuga has 24mg/100mg extract which is exceptionally high. Check the data. If so then TLC will give you good result if it is a candidate inhibitor??? Chieda Y, Iiyama K, Lee JM et al. Inactivation of pyocyanin synthesis genes has no effect on the virulence of *Pseudomonasaeruginosa* PAO1 toward the silkworm, *Bombyx mori*. *FEMS Microbiol Lett* 2008, 278: 101–107. Clatworthy AE, Pierson E, Hung DT. Targeting virulence: a new paradigm for antimicrobial therapy. Nat Chem Biol. 2007;3:541–548. . Question 3. Cassia extract has antibacterial activity and you growth inhibition- confusing. Give the correct data???? Unless you have inhibitory activity what could the implication of the study????? Question 4. You should inject Pseudomonas bacteria into rats and show the rat survived longer or no symptoms of disease when plant extracts were injected. Question 5. Give the reaction kinetics of procyanin and elastin congo red????? Why you used 0.2M HCl or 1mM CaCl2. Justify Question 6. Add few more references as described.	
Minor REVISION comments		
Optional/General comments		

PART 2:

	Reviewer's comment	**Author's comment** *(if agreed with reviewer, correct the manuscript and highlight that part in the manuscript. It is mandatory that authors should write his/her feedback here)*
Are there ethical issues in this manuscript?	*(If yes, Kindly please write down the ethical issues here in details)*	

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