

Linoleic acid acts as a potential anti-virulence agent in *Klebsiella pneumoniae*

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Abstract

Introduction The rise in antimicrobial resistance among bacterial pathogens is a global concern, and anti-virulence therapy may be an alternative strategy to address the issue. Multidrug resistant (MDR) hypervirulent *Klebsiella pneumoniae* (HvKp) is known to be associated with healthcare associated infections. These are often challenging to treat and here anti-virulence therapy may be a treatment option. The study of anti-virulence compounds against HvKp by in-silico prediction, in-vitro experiments and in-vivo assay enables to determine which anti-virulence compounds are suitable for an alternative approach MDR HvKp.

Methods Modeling of the proteins, ligand binding and molecular docking were performed targeting different hypervirulence genes viz., *rmpA*, *rmpA2* and, *iroC* by in-silico analysis using different bioinformatics tool and software. Minimum inhibitory concentration (MIC) was determined for six anti-virulence compounds; curcumin, eugenol, reserpine, linoleic acid, ϵ -anethole, and α -thujone by standard protocol. Quantitative real-time PCR was performed selecting two isolates harboring *rmpA*, *rmpA2* and *iroC* genes. *Galleria mellonella* larva killing assay was used for in-vivo experiment.

Results In-silico analysis observed that linoleic acid could be the best fit in comparison with the other compounds. None of the anti-virulence compounds showed any inhibitory activity and upon transcriptional expression analysis of the hypervirulence genes; *rmpA* was marginally increased for both the isolates when linoleic acid exposure was given.

Conclusions In-vivo study revealed that linoleic acid and reserpine showed anti-virulence activity.

Keywords Anti-virulence, antimicrobial resistance, hypervirulent *Klebsiella pneumoniae*, *rmpA*, *rmpA2*, *iroC*.

Introduction

Antimicrobial resistance is a global threat and the situation has become increasingly critical with no new group of antimicrobials on the pipeline.¹ Anti-virulence seems to be the most promising alternate strategy to tackle this global threat. This also aims to block the virulence factors within a bacterium thereby limiting its pathogenic behavior.² This strategy is found to

be advantageous as emergence of resistance against anti-virulence compounds is very low.³ Many compounds are investigated for potential anti-virulence properties and appear to be successful.⁴ Hypervirulent *Klebsiella pneumoniae* is known to be linked with healthcare associated infections and the investigators have already reported multidrug resistant (MDR) hypervirulent *Klebsiella pneumoniae* associated

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with fatal bloodstream infections within neonatal intensive care unit of a tertiary referral hospital, where it was evident that strategy regarding management of such infections is lacking.⁵ The World Health Organization has included MDR *K. pneumoniae* as global priority¹ and recently the department of Biotechnology, Government of India, published the Indian priority pathogen list⁶ where MDR *K. pneumoniae* has been included in the critical priority list of pathogens.⁶

With reference to hypervirulence in *K. pneumoniae*, several virulence genes have been implicated. In this regard, the *iroC* gene encodes an ABC (ATP-binding cassette) transporter required for transport of salmochelins⁷ and *rmpA* and *rmpA2* is a regulator of mucoid phenotype A, which contributes to the hypervirulent phenotype.⁸ At present there is no consensus on management of hypervirulent *K. pneumoniae* and an associated high mortality has been reported in earlier studies.^{9,10} With this background and with the endeavor to look for a solution for these hypervirulent strains causing infections, in the present study in-silico prediction, in-vitro expression and in-vivo assessment of anti-virulence compounds were done against multidrug resistant hypervirulent *Klebsiella pneumoniae*.

Methods

Modeling of the proteins

For this study, hypervirulence and hypermucoviscous genes *rmpA*, *rmpA2* and iron acquisition gene, *iroC* were considered. In silico studies were performed to determine the best fit for these hypervirulence genes. Structural profiles of these genes were studied to reveal their structural stability. However, owing to the unavailability of crystal structure of *rmpA*, *rmpA2* and *iroC* proteins in NCBI-BLASTp search in Protein Data Bank, computational modeling was employed for prediction of the protein structures. Complete structure of *rmpA*, *rmpA2* and *iroC* proteins were modeled using I-TASSER (<https://zhanglab.ccmb.med.umich.edu/ITASSE/>). This 3D structure prediction server uses available amino acid sequences to deduce protein structures following their default algorithm. The

best template having highest significance generated following the default algorithm was selected.¹¹⁻¹³

Ligands

Six natural compounds possessing anti-virulence properties were selected as ligands for docking analysis based on literature survey (Supplementary Table S1). Imipenem was considered as the known inhibitor of bacterial growth. The SMILES of ligands were extracted from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) and converted to sdf format using Open Babel toolbox.¹⁴

Molecular docking studies

For docking studies, Molegro Virtual Docker (MVD) was employed in order to find the best fit for the proteins under consideration. Individual receptors were imported into the software, followed by import of the ligands. The ligands were docked against the active site/cavities of the receptors. Docking radius of 20 Å and axis 50x50x50 was constructed around the cavities to focus on the specific area during docking. Overall, 10 independent docking runs, and 1500 iterations were set for each ligand which returned 1 pose in MVD. The details of the interacting residues and the chemical compounds have been provided as Supplementary Tables S2, S3 and Supplementary Figures S1-S3. MolDock score and Hydrogen Bond score generated after the run showed the efficacy of the target and ligand and were considered for further analysis. This score was derived from piecewise linear potential (PLP) based on cavity prediction and differential evolution.¹⁵⁻¹⁸

Bacterial strains and identification

The study was conducted at the Institute of Medical Sciences, Banaras Hindu University, in Varanasi, and Assam University, Silchar, Assam, India. Previously characterized and published isolates of MDR *K. pneumoniae* from cases of neonatal sepsis were considered. A total of 9 isolates were revived by subculture on MacConkey agar and were re-identified by a VITEK 2 compact system (bioMérieux, USA).

Assessment of inhibitory activity of selected anti-virulence compounds

Minimum inhibitory concentration (MIC) of isolates against anti-virulence compounds namely: curcumin, eugenol, reserpine, linoleic acid, ϵ -anethole and α -thujone (Sigma Aldrich, USA) were determined. Imipenem with a concentration range of 4-16 μ g/mL was used as control. The MIC of the anti-virulence compounds was evaluated by Resazurin microtiter assay against all the bacterial isolates.¹⁹ MICs were determined by the microbroth dilution method using a concentration range from 0.5 to 1024 μ L/mL. Suspension of the bacteria at log growth phase was made in cation adjusted Mueller Hinton broth (CAMHB) and matched with 0.5 MacFarland turbidity standard. Bacterial suspensions were incubated overnight with serial dilution of all the six compounds in 96 well microplate at 37°C. Following this, the plates were observed for visible turbidity prior to addition of resazurin. Standard antibiotic solutions of imipenem (Sigma-Aldrich Laboratories, India) were used as control at the same concentration range. Wells containing only sterile media were used as media control to validate the experiment. All experiments were performed in duplicates.

Transcriptional analysis of hypervirulence genes with and without anti-virulence compounds

Two isolates based on their imipenem MIC profile and carriage of *ompA*, *ompA2* and *iroC* (BK1 and BK2) were selected for expressional analysis. Expression of *ompA*, *ompA2* and *iroC* genes was determined by quantitative real-time PCR assay (Applied Biosystem, USA). Oligonucleotides specific for the respective genes were used for the study (Table 1). The isolates were inoculated into a freshly prepared 5 mL Luria Bertani broth (Hi-Media Laboratories Pvt. Ltd., India) containing 2 μ L volume of each extract (curcumin 0.1 g/mL, eugenol 1.067 g/mL, reserpine 0.2 g/mL, linoleic acid 0.5 g/mL, ϵ -anethole 0.968 g/mL, α -thujone 0.914 g/mL) respectively. Isolates were grown without anti-virulence compounds and incubated in a shaker incubator (160 rpm) at 37°C for 12-

16 hours. Mid-log phase bacterial cultures at OD600 (OD value 0.4-0.5) were used for the experiment. Total mRNA was extracted by using RNeasy mini kit (Qiagen, Germany) following the manufacturer's instructions and reverse-transcribed into cDNA by using QuantiTect® Reverse Transcription kit (Qiagen, Germany). Quantification of *ompA*, *ompA2* and *iroC* transcripts was performed by Picodrop (Pico 200, UK) and real-time PCR was performed using Power Sybr Green Master Mix (Applied Biosystem, UK) using Step One Plus real-time detection system (Applied Biosystem, USA), and expression was calculated based on their respective CT value.

In-vivo analysis of anti-virulence in *Galleria mellonella* infection model

The *Galleria mellonella* (wax moth) and larva were obtained from UDeS Honey Farms, Varanasi, India and reared in the parasitology section of the laboratory at 30°C in dark with natural beeswax diet. The last instar larvae, weighing 250 mg - 350 mg was used for the experiment. *G. mellonella* experiment was performed as described previously¹⁷ with slight modification. Briefly, bacterial suspension of hypervirulent *K. pneumoniae* was prepared by mixing 2-3 pure isolated colonies into sterile PBS, from overnight growth on MacConkey agar plates. The bacterial cell count was adjusted to 1.5×10^8 CFU/mL according to 0.5 McFarland. A total of 5 μ L of the bacterial suspension were injected into the last proleg of the larva with a microliter™ syringe (10 μ L glass syringe FN (701N) P/n 80300, Hamilton® Reno, USA). Thirty minutes post-injection, 2 μ L volume of each extract (α -thujone 0.914 g/mL, curcumin 0.1 g/mL, eugenol; 1.067 g/mL, anethol; 0.968 g/mL, linoleic acid; 0.5 g/mL, reserpine; 0.2 g/mL) was injected into the second-to-last proleg of the larvae. A set of control group i.e., larva injected with only bacterial suspension, injected with sterile PBS and non-injected larva were included with each experiment. The larva was kept at 37°C in dark and observed every 24 hours for consecutive 6 days. During this period, the survival of larva was assessed by any response to

Table 1: Oligonucleotide primers used for Real time PCR

Genes	Primer pairs	Sequence	Reaction condition	Amplified product size (bp)
<i>rmpA</i>	rmpA forward	CAATGGATGTGGCTTGACGTTT	95°C-2 mins 35 cycles: 95°C-20 secs 50°C-40 secs 72°C-1:20 mins 72°C-7 mins	196bp
	rmpA reverse	ATTGCAGCACTGCTTGTTTCCT		
<i>rmpA2</i>	rmpA2 forward	TGAAGGCTCGATGGATAAACCC		350bp
	rmpA2 reverse	CCCTCCTGGAGAGTAAGCATT		
<i>iroC</i>	iroC forward	CGGGAAATAGACGGCAAGGT		978bp
	iroC reverse	AGCCGAATTGTGATGCTGGA		

physical stimuli. The experiments were repeated two times with 4 larvae in each experimental group.

Statistical analysis

The Log-rank (Mantel-Cox) Test was used to determine the activity of extract and duration of death. Statistical analysis was done GraphPad Prism version 5.0 for Windows, GraphPad Software, USA.

Results

The final predicted structures of *rmpA*, *rmpA2* and *iroC* are depicted in Figure 1 A, B, and C respectively (Figure 1). In case of all the 3 receptors, the computational model showed that except Reserpine and Curcumin all the ligands could effectively bind to the active sites, but Linoleic acid showed the best fit (Supplementary Table S4). For H-bonds, among the effectively bonded complexes, linoleic acid had the highest number of H-bonds for all the 3 receptors. The compounds did not show any inhibitory activity on the hypervirulent *K. pneumoniae* isolates, even at concentrations ranging from 512-1024 µg/mL, and isolates showed growth in all the concentrations of the said compounds whereas they were inhibited by imipenem within 4-8 µg/mL range. Transcriptional expression pattern of the isolates (BK1 and BK2) harboring *rmpA* was marginally increased when linoleic acid stress was applied. However, no significant change in expression of the above-mentioned gene was observed the with rest of the compounds (Figure 2A). Similarly, *rmpA2* and *iroC* too did not show any significant expressional pattern with either of

the anti-virulence compounds (Figure 2B and Figure 2C).

The in-vivo *G. mellonella* infection study showed that α -thujone, curcumin, eugenol and anethol had no activity against hypervirulent *K. pneumoniae* (BK1 and BK2) as 100% of larvae died on day 1 to day 2 post-infection. However, linoleic acid and reserpine exhibited anti-virulence activity as the larvae showed 50% and 25% survival rates respectively at day 6 post-infection (Table 2). All the larva injected only with sterile PBS showed 100% survival rate. By day 3, 25% and by day 4, 100% larva, injected only with hypervirulent *K. pneumoniae* strain died. The log-rank (Mantel-Cox) test defined that the time to death for hypervirulent *Klebsiella pneumoniae* infected larva was reduced compared to extracts treated larvae ($p=0.08$) – Figure 3.

Table 2: Comparison of Survival Curves

Log-rank (Mantel-Cox) Test	
Chi square	12.39
df	7
P value	0.0884
P value summary	ns
Are the survival curves sig different?	No
Log rank test for trend	
Chi square	9.339
df	1
P value	0.0022
P value summary	**
Sig. trend?	Yes

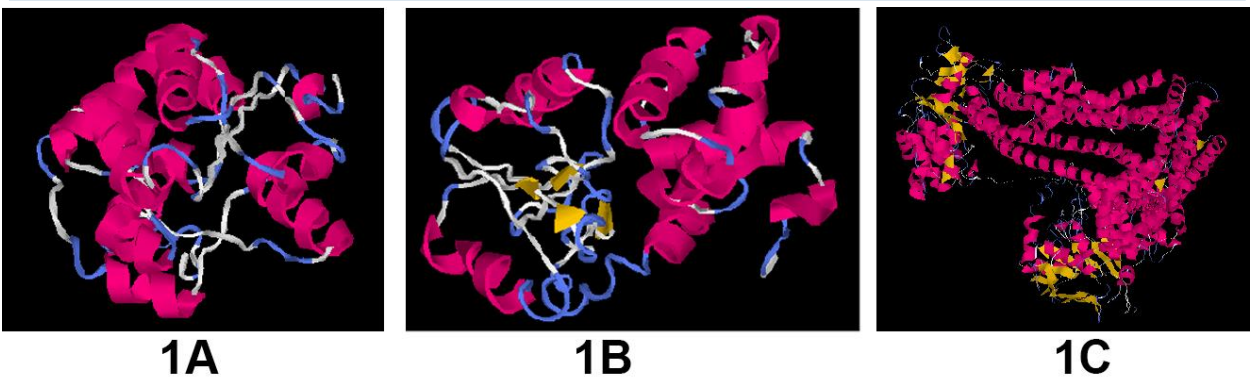


Figure 1. Figure 1A represents predicted structures of *rmpA*, 1B represents predicted structures of *rmpA2* and 1C showing predicted structures of *iroC*

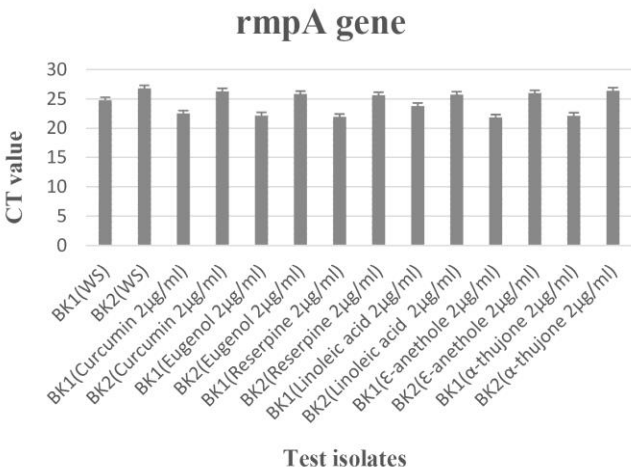


Figure 2A: Expressional analysis of *rmpA* gene harboring isolates against different anti-virulence compounds and without anti-virulence compound (Control).X-axis denotes test isolates whereas Yaxis shows CT value of the corresponding isolates

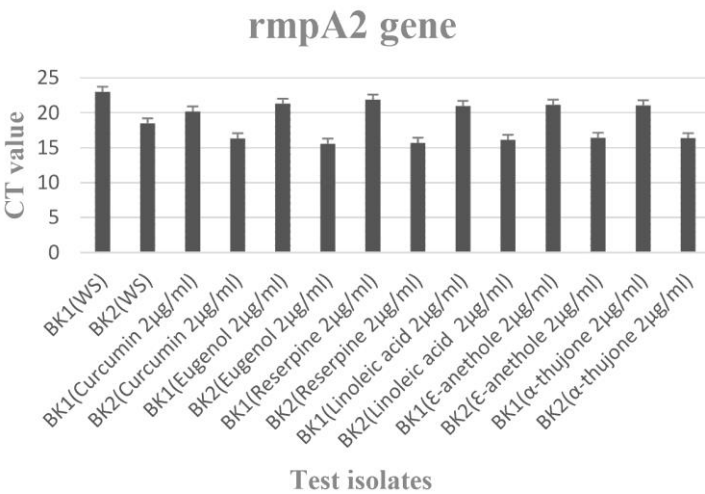


Figure 2B: Expressional analysis of *rmpA2* gene harboring isolates against different anti-virulence compounds and without anti-virulence compound (Control) X-axis denotes test isolates whereas Y-axis shows CT value of the corresponding isolates

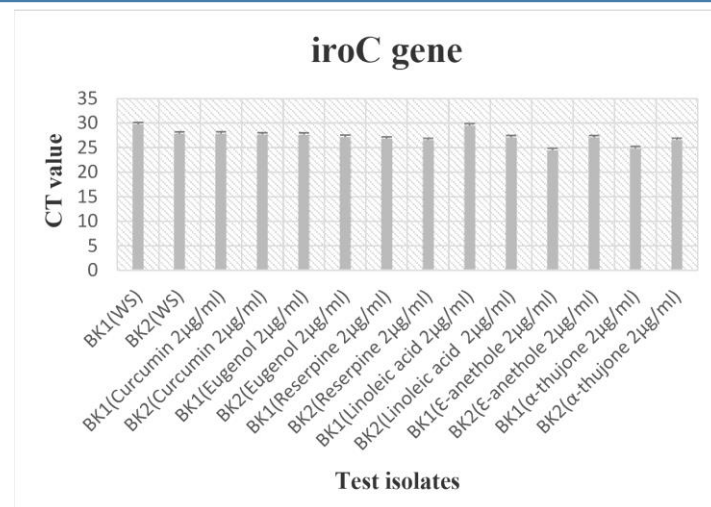


Figure 2C: Expressional analysis of *iroC* gene harboring isolates against different anti-virulence compounds and without anti-virulence compound (Control). X-axis denotes test isolates whereas Y-axis shows CT value of the corresponding isolates

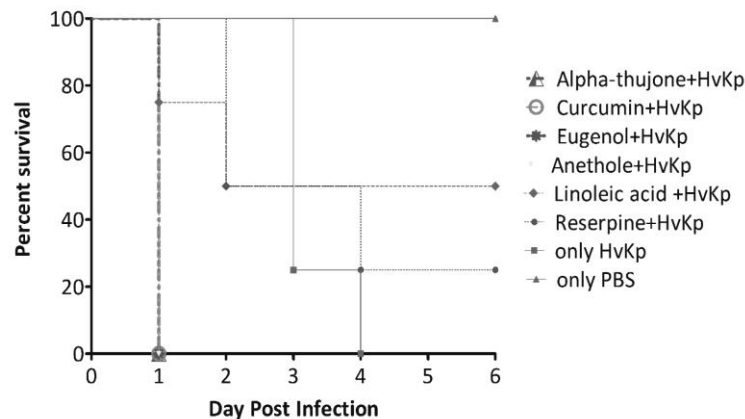


Figure 3: Kaplan-Meier survival curves showing survival of *Galleria mellonella* larvae infected with hypervirulent *Klebsiella pneumoniae* (HvKp)

Discussion

Anti-virulence compounds have an advantage of imposing little evolutionary and selection pressure in development of resistance compared to conventional antibiotics. As these compounds target virulence factors within a pathogen, it is less likely that they will have an impact on normal flora.^{19,20} This study tried to search for compounds to combat hypervirulence in *K. pneumoniae*, which is one of the emerging issues and there have been few studies in this respect.

Molecular docking is a powerful drug designing tool that predicts binding pattern/orientation of ligand with protein at lowest potential energy. This released energy is

the measure of stability of the ligand-protein complex i.e., the higher the docking score the higher is the ability of the ligand to inhibit the receptor's activity.²¹⁻²³ In earlier studies it has been observed that in *Staphylococcus mutans* and *K. pneumoniae*, linoleic acid could reduce biofilm accumulation without affecting their viability.^{9,11} Reports have suggested that anethole affects the virulence cascade in *Vibrio cholerae*.⁴ Eugenol affects exotoxin production in *Staphylococcus aureus*, thus depicting an anti-virulence activity besides affecting biofilm formation in *Escherichia coli*, *Pseudomonas aeruginosa*, *K. pneumoniae*, *S. aureus*,¹² while α -thujone interferes with quorum

sensing system of *P. aeruginosa*.^{9,12} In the expression analysis, transcriptional response of hypervirulence genes did not show any specific pattern in presence of the studied anti-virulence compounds. We may assume that this anti-virulence compound has no interference with the virulence genes and the binding of this compound takes place with translational form of these virulence genes.

Hypervirulence in *K. pneumoniae* represents a serious public health threat globally. In this study, we have investigated hypervirulent isolates with multiple anti-virulence compounds. In a very recent study by Zarandi et al., it has been reported that curcumin acquire antibiofilm, anti-efflux and anti-capsule activities, which may be used to boost anti-*Klebsiella* treatment.^{24,31} Several virulence factors of *K. pneumoniae* enable its spread and emergence as a global threat. There are several genetic markers of hypervirulence in *K. pneumoniae*; among these, as previously described, the most important ones include regulators of the mucoid phenotype, the genes *rmpA* and *rmpA2*, siderophores such as aerobactin, enterobactin, yersiniabactin, and salmochelin, and genes that regulate iron uptake and allantoin metabolism.^{8,25} Furthermore, *rmpA/rmpA2* genes along with siderophores appear to be associated with invasive infections.²⁶ All the MDR hypervirulent *K. pneumoniae* isolates in a study were phenotypically and genotypically hypermucoviscous with several types of siderophore-encoding genes; their high invasivity reflected in their ability to cause 100% mortality in the neonatal unit, as previously reported.⁵

The in-vivo experiment underscored anti-virulence activity of linoleic acid and reserpine in *G. mellonella* model, which is also in agreement with the in-silico finding. The present study has included a small number of bacterial isolates and a small number of insect larva and this warrants further investigation with a larger number of isolates. This study was successful with its approach for searching an effective anti-virulence compound by in-silico, in-vitro, and in-vivo analysis.

Conclusions

This study documented that linoleic acid and reserpine have anti-virulence property without any antimicrobial activity against the hypervirulent isolates. This study also laid the grounds for further studies on this aspect of curbing or tackling virulence, to combat morbidity and mortality thus caused by hypervirulent *K. pneumoniae* strains.

Author contributions statement: Data curation, TB; Investigation, JW, SB and SS; Methodology, TB, SB and AB; Software, SB and MAL; Supervision, AB; Validation, TB and MDC; Writing – original draft, JW and AB; Writing – review & editing, AB. All authors read and approved the final version of the manuscript.

Conflicts of interest: All authors – none to declare.

Funding: None to declare.

Ethics approval: The study was approved by the Ethics Committee of Assam University, Silchar, India.

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References

1. World Health Organization. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. Geneva: WHO Press; 2017.
2. Choby JE, Howard-Anderson J, Weiss DS. Hypervirulent *Klebsiella pneumoniae* clinical and molecular perspectives. *J Intern Med*. 2020;287:283-300. <https://doi.org/10.1111/joim.13007>
3. Wyres KL, Nguyen TNT, Lam MMC, et al. Genomic surveillance for hypervirulence and multi-drug resistance in invasive *Klebsiella pneumoniae* from South and Southeast Asia. *Genome Med*. 2020;12:11. <https://doi.org/10.1186/s13073-019-0706-y>
4. Buroni S, Chiarelli LR. Antivirulence compounds: a future direction to overcome antibiotic resistance? *Future Microbiol*. 2020;155:299-301. <https://doi.org/10.2217/fmb-2019-0294>
5. Banerjee T, Wangkheimayum J, Sharma S, Kumar A, Bhattacharjee A. Extensively drug resistant hypervirulent *Klebsiella pneumoniae* from a series of neonatal sepsis in a tertiary care hospital, India. *Front Med*. 2021;8:645955. <https://doi.org/10.3389/fmed.2021.645955>

6. WHO Country Office for India, Department of Biotechnology, Government of India. Indian Priority Pathogen List to Guide research, discovery, and development of new antibiotics in India. 2017.
7. Crouch ML, Castor M, Karlinsey JE, Kalthorn T, Fang FC. Biosynthesis and IroC-dependent export of the siderophore salmochelin are essential for virulence of *Salmonella enterica* serovar Typhimurium. *Mol Microbiol*. 2008;67:971-83. <https://doi.org/10.1111/j.1365-2958.2007.06089.x>
8. Lai YC, Peng HL, Chang HY. RmpA2, an activator of capsule biosynthesis in *Klebsiella pneumoniae* CG43, regulates K2 cps gene expression at the transcriptional level. *J Bacteriol*. 2003;185:788-800. <https://doi.org/10.1128/JB.185.3.788-800.2003>
9. Shankar C, Basu S, Lal B, et al. Aerobactin seems to be a promising marker compared with unstable RmpA2 for the identification of hypervirulent carbapenem-resistant *Klebsiella pneumoniae*: in silico and in vitro evidence. *Front Cell Infect Microbiol*. 2021;11:709681. <https://doi.org/10.3389/fcimb.2021.709681>
10. Yu VL, Hansen DS, Ko WC, et al. Virulence characteristics of *Klebsiella* and clinical manifestations of *K. pneumoniae* bloodstream infections. *Emerg Infect Dis*. 2007;13:986-93. <https://doi.org/10.3201/eid1307.070187>
11. O'Boyle NM, Banck M, James CA, Morley C, Vandermeersch T, Hutchison GR. Open Babel: an open chemical toolbox. *J Cheminform*. 2011;3:33. <https://doi.org/10.1186/1758-2946-3-33>
12. Lin JC, Chang FY, Fung CP, et al. High prevalence of phagocytic-resistant capsular serotypes of *Klebsiella pneumoniae* in liver abscess. *Microbes Infect*. 2004;6:1191-8. <https://doi.org/10.1016/j.micinf.2004.06.003>
13. Araújo BF, Ferreira ML, Campos PA, et al. Hypervirulence and biofilm production in KPC-2-producing *Klebsiella pneumoniae* CG258 isolated in Brazil. *J Med Microbiol*. 2018;67:523-8. <https://doi.org/10.1099/jmm.0.000711>
14. Wu SC, Liu F, Zhu K, Shen JZ. Natural products that target virulence factors in antibiotic-resistant *Staphylococcus aureus*. *J Agric Food Chem*. 2019;67:13195-211. <https://doi.org/10.1021/acs.jafc.9b05595>
15. Bitencourt-Ferreira G, de Azevedo WF Jr. Molegro Virtual Docker for docking. *Methods Mol Biol*. 2019;2053:149-67. https://doi.org/10.1007/978-1-4939-9752-7_10
16. Thomsen R, Christensen MS. MolDock: a new technique for high-accuracy molecular docking. *J Med Chem*. 2006;49:3315-21. <https://doi.org/10.1021/jm051197e>
17. Hong SS, Kim K, Huh JY, Jung B, Kang MS, Hong SG. Multiplex PCR for rapid detection of genes encoding class A carbapenemases. *Ann Lab Med*. 2012;32:359-61. <https://doi.org/10.3343/alm.2012.32.5.359>
18. Poirel L, Walsh TR, Cuvillier V, Nordmann P. Multiplex PCR for detection of acquired carbapenemase genes. *Diagn Microbiol Infect Dis*. 2011;70:119-23. <https://doi.org/10.1016/j.diagmicrobio.2010.12.002>
19. Sarker SD, Nahar L, Kumarasamy Y. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. *Methods*. 2007;42:321-4. <https://doi.org/10.1016/j.ymeth.2007.01.006>
20. Jacobs AC, Thompson MG, Black CC, et al. AB5075, a highly virulent isolate of *Acinetobacter baumannii*, as a model strain for the evaluation of pathogenesis and antimicrobial treatments. *mBio*. 2014;5:e01076-14. <https://doi.org/10.1128/mBio.01076-14>
21. Nie L, Lv Y, Yuan M, et al. Genetic basis of high-level aminoglycoside resistance in *Acinetobacter baumannii* from Beijing, China. *Acta Pharm Sin B*. 2014;4:295-300. <https://doi.org/10.1016/j.apsb.2014.06.004>
22. Kusumaningrum S, Budianto E, Kosela S, Sumaryono W, Juniarti F. The molecular docking of 1,4-naphthoquinone derivatives as inhibitors of Polo-like kinase 1 using Molegro Virtual Docker. *J Appl Pharm Sci*. 2014;4:47-53.
23. Morris GM, Lim-Wilby M. Molecular docking. *Methods Mol Biol*. 2008;443:365-82. https://doi.org/10.1007/978-1-59745-177-2_19
24. Karami-Zarandi M, Ghale HE, Ranjbar R. Characterization of virulence factors and antibacterial activity of curcumin in hypervirulent *Klebsiella pneumoniae*. *Future Microbiol*. 2022;17:529-40. <https://doi.org/10.2217/fmb-2021-0222>
25. Effah CY, Sun T, Liu S, Wu Y. *Klebsiella pneumoniae*: an increasing threat to public health. *Ann Clin Microbiol Antimicrob*. 2020;19:1. <https://doi.org/10.1186/s12941-019-0343-8>
26. Holt KE, Wertheim H, Zadoks RN, et al. Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in *Klebsiella pneumoniae*, an urgent threat to public health. *Proc Natl Acad Sci U S A*. 2015;112:E3574-81. <https://doi.org/10.1073/pnas.1501049112>
27. Silva LN, Zimmer KR, Macedo AJ, Trentin DS. Plant natural products targeting bacterial virulence factors. *Chem Rev*. 2016;116:9162-236. <https://doi.org/10.1021/acs.chemrev.6b00184>
28. Zahid MS, Awasthi SP, Asakura M, et al. Suppression of virulence of toxigenic *Vibrio cholerae* by anethole through the cyclic AMP (cAMP)-cAMP receptor protein signaling system. *PLoS One*. 2015;10:e0137529. <https://doi.org/10.1371/journal.pone.0137529>
29. Wu SC, Liu F, Zhu K, Shen JZ. Natural products that target virulence factors in antibiotic-resistant *Staphylococcus aureus*. *J Agric Food Chem*. 2019;67:13195-211. <https://doi.org/10.1021/acs.jafc.9b05595>
30. Choudhury D, Paul D, Ghosh AS, et al. Effect of single-dose carbapenem exposure on transcriptional expression of *bla_{NDM-1}* and *mexA* in *Pseudomonas aeruginosa*. *J Glob Antimicrob Resist*. 2016;7:72-7. <https://doi.org/10.1016/j.jgar.2016.07.015>

31. Karami-Zarandi M, Rahdar HA, Esmaeili H, Ranjbar R.
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mechanisms. Future Microbiol. 2023;18:65-81.
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Supplementary Table S1. Ligands with anti-virulence properties

Sl.no.	Ligand	SMILES	References
1.	Linoleic acid	<chem>CCCCC=CCC=CCCCCCCCC(=O) O</chem>	26
2.	Anethole	<chem>CC=CC1=CC=C(C=C1) OC</chem>	27
3.	Eugenol	<chem>COC1=C(C=CC(=C1) CC=C) O</chem>	26
4.	a-thujone	<chem>CC1C2CC2(CC1=O) C(C)C</chem>	26
5.	Curcumin	<chem>COC1=C(C=CC(=C1) C=CC(=O) CC(=O) C=CC2=CC(=C(C=C2) O) OC) O</chem>	28
6.	Reserpine	<chem>COC1C(CC2CN3CCC4=C(C3CC2C1C(=O) OC) NC5=C4C=CC(=C5) OC) OC(=O) C6=CC(=C(C(=C6) OC) OC) OC</chem>	26
7.	Imipenem	<chem>CC(C1C2CC(=C(N2C1=O) C(=O) O) SCCN=CN) O</chem>	29

Supplementary Table S2. Details of interacting residues

iroC					
Name	Ligand	MolDock Score	Rerank Score	HBond	Interacting Residue
[00]Anethole	Anethole	-70.3359	-57.5987	-1.37703	Arg 745
[00]Curcumin	Curcumin	900.791	-88.297	-4.7344	Trp 737
[00]Eugenol	Eugenol	-66.2379	-55.3592	-2.5	Lys 740
[00]Imipenem	Imipenem	961.792	-26.0014	0	None
[00]Linoleic acid	Linoleic acid	-104.833	-83.978	-2.21929	Gly 611, His 741
[00]Reserpine	Reserpine	1938.78	-45.7376	-1.29104	Leu596
[00]a-thujone	a-thujone	-40.1092	-33.1718	0	None
rmpA					
Name	Ligand	MolDock Score	Rerank Score	HBond	Interacting Residue
[01]Anethole	Anethole	-68.7182	-57.6121	-2.5	Tyr 187
[03]Curcumin	Curcumin	902.913	-81.6046	-5.79218	Tyr 187, Leu 76
[01]Eugenol	Eugenol	-51.5243	-45.2856	-3.50172	Arg 57, Asn 55
[04]Imipenem	Imipenem	934.956	-53.611	-4.64087	Arg 54, Ile 56
[01]Linoleic acid	Linoleic	-96.6963	-79.7245	-2.99186	Ala 49

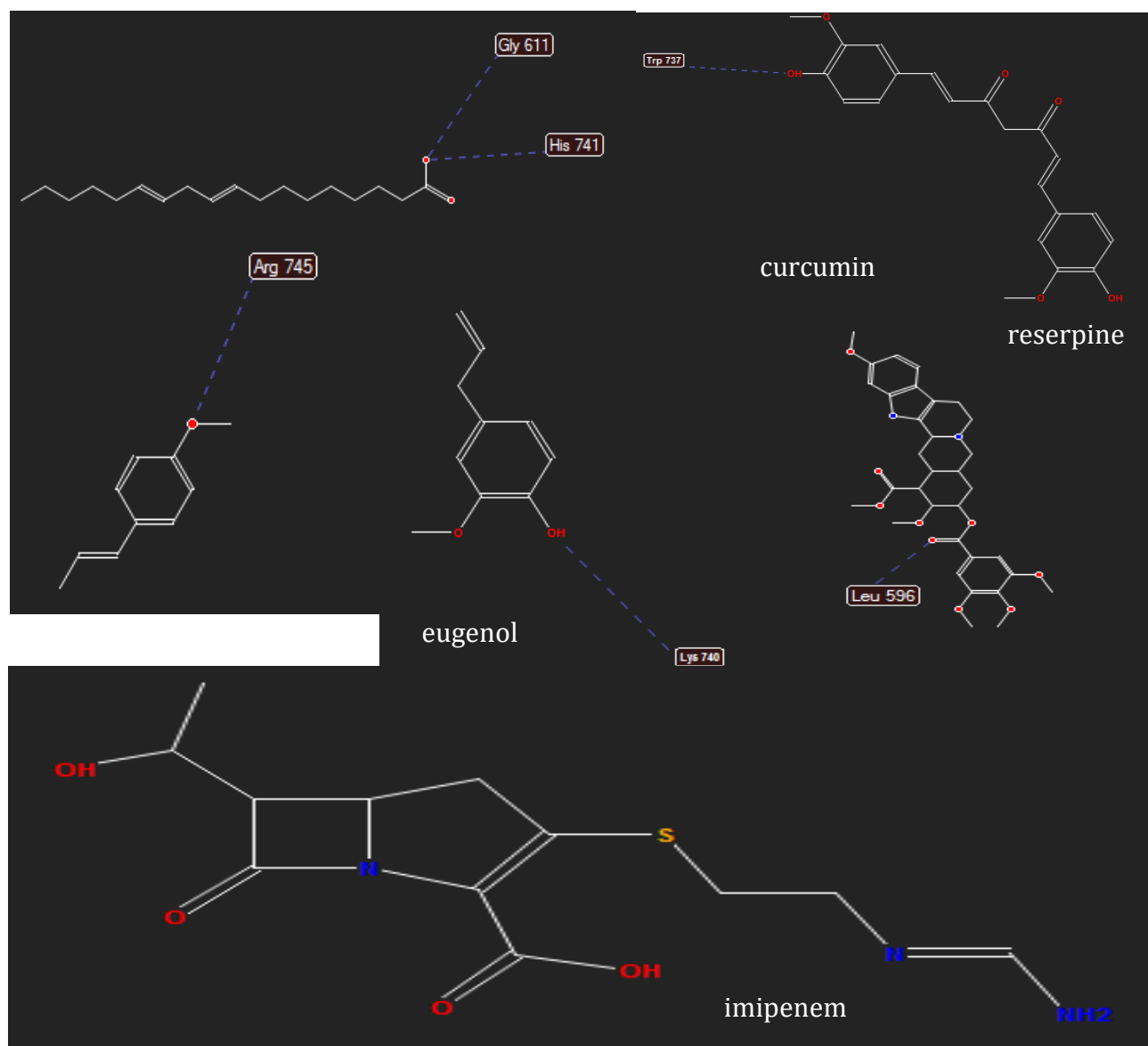
	acid				
[04]Reserpine	Reserpine	1949.13	211.053	-4.01985	Lys 53, Arg 175
[01]a-thujone	a-thujone	-38.7058	-32.5871	-0.50719	None
rmpA2					
Name	Ligand	MolDock Score	Rerank Score	HBond	Interacting Residue
[03]Anethole	Anethole	-66.5371	-27.623	-4.78696	Arg 54
[04]Curcumin	Curcumin	973.011	245.441	-5.92192	Arg 159, Glu 124, Tyr 6, 127,
[01]Eugenol	Eugenol	-68.4607	-57.0339	-4.09646	Arg 54
[03]Imipenem	Imipenem	922.948	-59.6065	-5.36771	Arg 159, Glu 161
[03]Linoleic acid	Linoleic acid	-121.81	-93.9299	-2.2632	Glu 174, Ser 178
[04]Reserpine	Reserpine	2101.83	1227.7	-7.28914	Ser 178, 84, Arg 54, Cys 179. 182
[00]a-thujone	a-thujone	-56.1563	-47.1471	-0.11931	

Supplementary Table S3. Details of chemical compounds

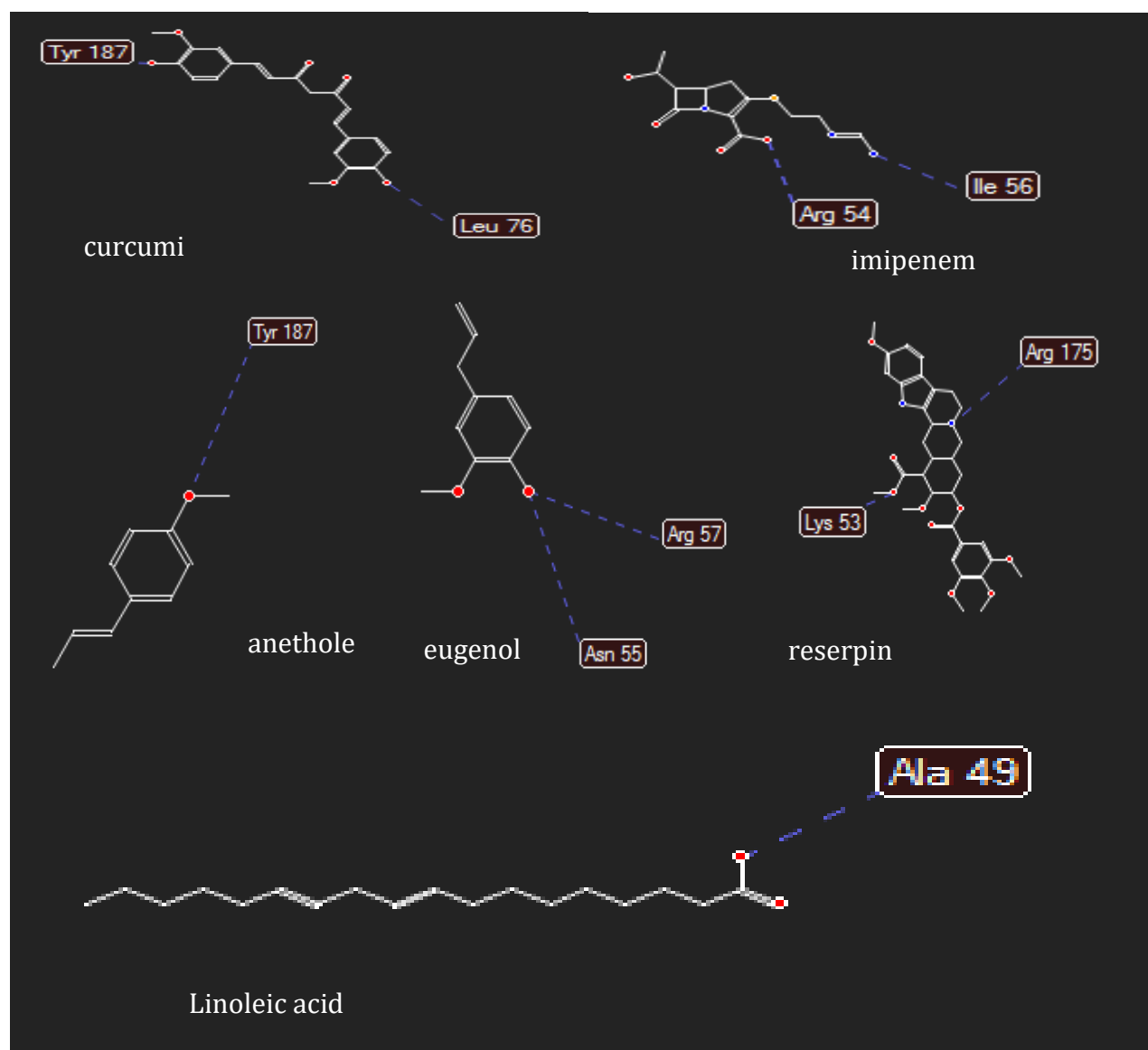
Compounds	Smiles
Curcumin	<chem>COC1=C(C=CC(=C1)C=CC(=O)CC(=O)C=CC2=CC(=C(C=C2)O)OC)O</chem>
Eugenol	<chem>COC1=C(C=CC(=C1)CC=C)O</chem>
Reserpine	<chem>COC1C(CC2CN3CCC4=C(C3CC2C1C(=O)OC)NC5=C4C=CC(=C5)OC)OC(=O)C6=CC(=C(C(=C6)OC)OC)OC</chem>
Linoleic acid	<chem>CCCCC=CCC=CCCCCCCCC(=O)O</chem>
Anethole	<chem>CC=CC1=CC=C(C=C1)OC</chem>
a-thujone	<chem>CC1C2CC2(CC1=O)C(C)C</chem>
Imipenem	<chem>CC(C1C2CC(=C(N2C1=O)C(=O)O)SCCN=CN)O</chem>

Supplementary Table S4. Docking Scores (MolDock Score and H bond) of different ligand against receptors

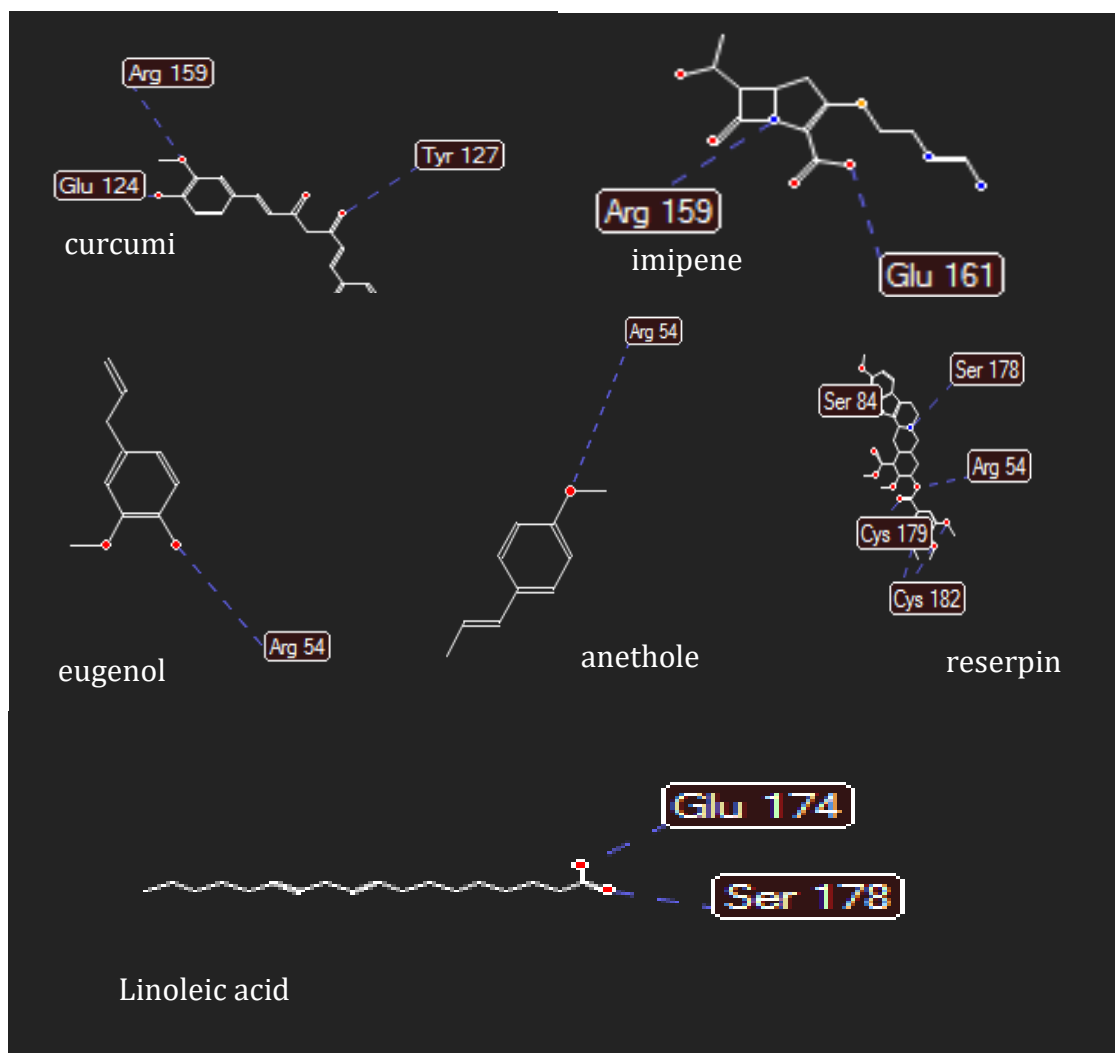
Ligand	<i>rmpA</i>		<i>rmpA2</i>		<i>iroC</i>	
	MolDock Score	HBond	MolDock Score	HBond	MolDock Score	HBond
Linoleic acid	-103.519	-3.59336	-95.6091	-5.35988	-99.8598	-2.5
Anethole	-83.1232	0	-73.3253	0	-70.1926	0
Eugenol	-76.9971	-2.5	-71.7414	-2.5	-68.1339	-0.08581
a-thujone	-68.3509	-1.77241	-57.2073	0.934993	-54.7913	0
Curcumin	900.132	-4.04844	902.903	-3.70036	912.212	-1.63852
Reserpine	1898.47	-2.78029	1960.73	-0.16373	1938.64	0
Imipenem	-33.703	-1.85883	-30.348	-8.10202	-29.485	-2.5199



Supplementary Figure S1. Images showing interacting residues of the anti-virulent compounds with iroC



Supplementary Figure S2. Images showing interacting residues of the anti-virulent compounds with rmpA



Supplementary Figure S3. Images showing interacting residues of the anti-virulent compounds with rmpA2