Proteomic and Systematic Functional Profiling Unveils Citral Targeting Antibiotic Resistance, Antioxidant Defense, and Biofilm-Associated Two-Component Systems of *Acinetobacter baumannii* To Encumber Biofilm and Virulence Traits

Anthonymuthu Selvaraj,a Alaguvel Valliammai,a Pandiyan Muthuramalingam,a,b Sivasamy Sethupathy,a,c Ganapathy Ashwinkumar Subramenium,a,d Manikandan Ramesh,a Shunmugiah Karutha Pandiana

aDepartment of Biotechnology, Alagappa University, Karaikudi, Tamil Nadu, India
bDepartment of Systems Biology, Science Research Centre, Yonsei University, Seoul, South Korea
cBiofuels Institute, School of the Environment and Safety Engineering, Jiangsu University, Zhenjiang, Jiangsu, China
dCollege of Medicine, Pennsylvania State University, Hershey, Pennsylvania, USA

**ABSTRACT** *Acinetobacter baumannii* has been reported as a multidrug-resistant bacterium due to biofilms and antimicrobial resistance mechanisms. Hence, novel therapeutic strategies are necessary to overcome *A. baumannii* infections. This study revealed that citral at 200 µg/ml attenuated *A. baumannii* biofilms by up to 90% without affecting viability. Furthermore, microscopic analyses and *in vitro* assays confirmed the antibiofilm efficacy of citral. The global effect of citral on *A. baumannii* was evaluated by proteomic, transcriptional, and *in silico* approaches. Two-dimensional (2D) gel electrophoresis and matrix-assisted laser desorption ionization–time of flight/time of flight (MALDI-TOF/TOF) analyses were used to assess the effect of citral on the *A. baumannii* cellular proteome. Quantitative real-time PCR (qPCR) analysis was done to validate the proteomic data and identify the differentially expressed *A. baumannii* genes. Protein-protein interactions, gene enrichment, and comparative gene network analyses were performed to explore the interactions and functional attributes of differentially expressed proteins of *A. baumannii*. Global omics-based analyses revealed that citral targeted various mechanisms such as biofilm formation, antibiotic resistance, antioxidant defense, iron acquisition, and type II and type IV secretion systems. The results of antioxidant analyses and antibiotic sensitivity, blood survival, lipase, and hemolysis assays validated the proteomic results. Cytotoxicity analysis showed a nontoxic effect of citral on peripheral blood mononuclear cells (PBMCs). Overall, the current study unveiled that citral has multitarget efficacy to inhibit the biofilm formation and virulence of *A. baumannii*.

**IMPORTANCE** *Acinetobacter baumannii* is a nosocomial-infection-causing bacterium and also possesses multidrug resistance to a wide range of conventional antibiotics. The biofilm-forming ability of *A. baumannii* plays a major role in its resistance and persistence. There is an alarming need for novel treatment strategies to control *A. baumannii* biofilm-associated issues. The present study demonstrated the strong antibiofilm and antivirulence efficacy of citral against *A. baumannii*. In addition, proteomic analysis revealed the multitarget potential of citral against *A. baumannii*. Furthermore, citral treatment enhances the susceptibility of *A. baumannii* to the host innate immune system and reactive oxygen species (ROS). Cytotoxicity analysis revealed the nonfatal effect of citral on human PBMCs. Therefore, citral could be the safest therapeutic compound and can be taken for further clinical evaluation for the treatment of biofilm-associated infections by *A. baumannii*.
Acinetobacter baumannii is a coccobacillus Gram-negative bacterium that causes nosocomial infections such as bacteremia, meningitis, wound infections, urinary tract infections, and pneumonia. It is mostly identified in hospital-associated infections worldwide. Initially, it was referred to as Iraqibacter due to untreatable infection spread by A. baumannii among injured U.S. military soldiers in a civilian hospital in Iraq (1). The Infectious Diseases Society of America categorized A. baumannii as one of the pathogens in the “ESKAPE” group of bacteria. This multidrug-resistant (MDR) bacterium has been noted as a high-priority pathogen by the World Health Organization (WHO) in a report about the list of MDR bacteria for the development of new drugs (2). Several antibiotic resistance mechanisms have been identified in A. baumannii, such as quorum sensing, biofilm formation, efflux pumps, and alteration of the outer membrane. Biofilm formation is one of the most important factors for the persistent survival on various surfaces and the antibiotic resistance of A. baumannii (3).

In a biofilm, bacterial communities are attached to biotic and abiotic surfaces and surrounded by a matrix of extracellular polymeric substances (EPSs), which include polysaccharides, proteins, extracellular DNA, and virulence factors. EPS acts as a barrier to antibiotics, and it provides more survival tolerance against environment stress and the human immune system. Therefore, bacteria in biofilms are much more resistant than planktonic cells. Moreover, antibiotic failure due to altered phenotypes of bacteria in biofilms increases the morbidity and mortality rates in health care units (4, 5). Thus, there is a need for the discovery of alternative strategies to treat biofilm-associated bacterial infections. In A. baumannii, outer membrane proteins, the two-component system (TCS) (bfmS and bfmR), the chaperone-usher system, quorum sensing, poly-β-(1,6)-N-acetylglucosamine (PNAG), phospholipase, the secretion of serine proteases, and motility are various virulence systems associated with biofilm formation (6). Hence, targeting biofilm formation by A. baumannii is an alternative way to control biofilm-mediated infections.

A potential antibiofilm agent will have the ability to inhibit biofilm formation or disperse preformed biofilms, thereby inhibiting virulence factor expression in pathogenic bacteria. In addition, it will not affect bacterial survival, and hence, the chances of resistance development against antibiofilm agents by bacteria will be reduced (7). Conventionally, natural resources have always proven to be a predominant reservoir of therapeutic agents. The exploration of bioactive compounds from natural sources acquires an added advantage since they are expected to be nontoxic (8). Hence, various natural resources have already been explored for their ability to inhibit biofilm-associated virulence factors of clinically important human pathogens, such as Actinidia deliciosa, Syzygium cumini, Macaranga tanarius, and Vetiveria zizanioides extracts against Serratia marcescens, Candida spp., A. baumannii, and Staphylococcus aureus, respectively (9–12). Therefore, the identification of antibiofilm agents from natural sources is expected to aid in the development of therapeutic strategies against A. baumannii. Thus, the current study mainly focused on screening natural resources with antibiofilm activity against A. baumannii.

Essential oils from plants are well known to have biological activities. Citral is a mixture of monoterpeneoid aldehydes such as neral and geranial. It has been known for its pleasant odor, and it is abundantly found in Cymbopogon citratus, lemon myrtle, Citrus limon, and Aloysia triphylla. It has been used for its wide range of biological properties, such as its antimicrobial, antifungal, anti-inflammatory, antimutagenic, antioxidant, and anticancer activities (13–15). Previous studies reported the antibiofilm activity of citral against Staphylococcus aureus, Listeria monocytogenes, Vibrio parahaemolyticus, and Candida tropicalis (16–19). With this backdrop, the current study aimed at evaluating the antibiofilm potential of citral against A. baumannii and mainly focused on...
RESULTS

Growth and biofilm formation of *A. baumannii* upon phytocompound treatment. The antibiofilm potentials of 10 phytocompounds were tested against *A. baumannii* at 250 μg/ml. Out of 10 phytochemicals, citral at 250 μg/ml showed strong antibiofilm activity (97%) without disturbing the growth of *A. baumannii*. The rest of the phytocompounds at 250 μg/ml showed insignificant changes in the cell density as well as biofilm formation of *A. baumannii* (Fig. 1A).

Effect of the MIC and MBIC of citral. The MIC and minimal biofilm-inhibitory concentration (MBIC) of citral were assessed at increasing concentrations (25, 50, 100, 200, and 400 μg/ml) against *A. baumannii*. Citral at 200 μg/ml exhibited 90% antibiofilm activity, above which antibacterial activity was observed. Thus, 200 μg/ml was considered the MBIC. At a 400-μg/ml concentration, citral showed strong antibacterial activity against *A. baumannii*, and hence, the same was fixed as the MIC (Fig. 1B).

Unveiling Molecular Targets of Citral on *A. baumannii*
Inhibitory effect of citral on \textit{A. baumannii} biofilm formation on various surfaces. The effect of citral on the biofilm formation of \textit{A. baumannii} on glass (test tubes) and polystyrene (24-well plate) surfaces was evaluated. The results showed that citral at 200 μg/ml effectively inhibited the biofilm formation of \textit{A. baumannii} on glass as well as 24-well polystyrene surfaces (Fig. 1C).

Nonantibacterial effect of citral on \textit{A. baumannii}. The growth curves of \textit{A. baumannii} in the absence and presence of citral (200 μg/ml) were obtained at 600 nm for every hour up to 24 h. An insignificant change in the growth curve patterns of control and treated samples was observed (Fig. 2A). The effect of citral on the cell viability of \textit{A. baumannii} was analyzed by an Alamar Blue assay to compare control and citral-treated cells. The result revealed insignificant differences between the control and treated samples in terms of cell viability (Fig. 2B). Also, the numbers of viable cells in control and treated samples were assessed, and Fig. 2B shows insignificant changes in cell counts in control (4.3 × 10^8 cells) and citral-treated (4.1 × 10^8 cells) cultures. Thus, the growth curve, CFU, and Alamar Blue assays confirmed the insignificant effect of citral on the growth and viability of \textit{A. baumannii}.

Microscopic analyses of \textit{A. baumannii} biofilm formation. Microscopic analyses were performed to evaluate the biofilm architecture of \textit{A. baumannii} in the absence and presence of citral at a 200-μg/ml concentration. The light microscopy results clearly showed a reduction in the covered surface area of the biofilm on the glass slide in the case of citral treatment compared to the control sample (Fig. 3A). The biofilm thickness of control and citral-treated samples was analyzed and visualized by confocal laser scanning microscopy (CLSM). Multilayered biofilm formation was observed in the CLSM image of the control sample, whereas the citral-treated sample showed a very thin and dispersed biofilm (Fig. 3B). Furthermore, scanning electron microscopy (SEM) analysis was performed to assess the surface morphology of the \textit{A. baumannii} biofilm. The SEM image of the control sample displayed highly aggregated cells with a thick layer of EPS. In contrast, the SEM image of the citral-treated sample depicted a reduction in EPS production and a disintegrated biofilm (Fig. 3C). Altogether, the microscopic analyses further validated the antibiofilm efficacy of citral against \textit{A. baumannii}.

Inhibitory effect of citral on EPS and CSH of \textit{A. baumannii}. The effect of citral on the EPS production of \textit{A. baumannii} was assessed, and EPS extracted from the citral (200-μg/ml)-treated sample showed a maximum of a 44% reduction (11.5 ± 0.75 mg) compared to the control sample (25.5 ± 1.2 mg) (Fig. 4). Furthermore, a microbial adherence to hydrocarbon (MATH) assay was performed to determine the inhibitory effect of citral on the cell surface hydrophobicity (CSH) of \textit{A. baumannii}. The results
revealed that the addition of citral at 200 µg/ml significantly reduced the CSH of
*A. baumannii* (27%) compared to the CSH of the control sample (76%) (Fig. 4).

**Identification of differentially expressed proteins of *A. baumannii***. Initially,
sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was
carried out to analyze the intracellular protein samples isolated from *A. baumannii*
grown in the absence and presence of citral (200 µg/ml). The differentially expressed
bands were observed in citral-treated samples. The up- and downregulated bands are
marked by green and red arrows, respectively, in Fig. 5. To separate proteins with high
resolution, two-dimensional gel electrophoresis (2DGE) of the intracellular proteomes
of control and citral-treated samples was performed in biological triplicates (Fig. 6).
Next, protein spots were analyzed using ImageMaster 2D Platinum software, and 892
protein spots were identified to be differentially regulated. Among 892 protein spots, 70 downregulated (red circles) and 36 upregulated (green circles) (>1.5-fold change; \(P < 0.05\)) spots were selected for further analysis (Fig. 7). The differentially expressed proteins were subjected to trypsin digestion, analyzed by matrix-assisted laser
desorption ionization–time of flight/time of flight (MALDI-TOF/TOF) spectrometry, and identified using the MS-Fit online database. Details of the differentially expressed proteins are given in Tables 1 and 2.

**PPI and functional enrichment analyses.** The molecular interactions of differentially regulated (up- and downregulated) proteins of *A. baumannii* were predicted using STRING v11.0. The molecular signals of upregulated proteins had 26 nodes and 66 edges (Fig. 8A). The molecular cross talk of these proteins had a nodal degree of ~5.08 in neighborhood seed proteins. Additionally, interactions of downregulated proteins had 94 edges and 35 nodes, and the nodal degree was ~5.37 in closely related seed proteins (Fig. 8B). The cross talk of these differentially expressed proteins was analyzed with an enrichment *P* value score of <0.01.

Protein-protein interaction (PPI) networks of differentially regulated proteins and their functional gene enrichments were imputed using ClueGO/CluePedia plug-in of Cytoscape software, and the results revealed that these proteins are significantly involved in diverse biological processes, molecular functions, and cellular components (Fig. 9A to G).

**Effect of citral on biofilm-associated genes of *A. baumannii*.** In order to validate the proteomic data and also to assess the effect of citral on biofilm-associated genes of *A. baumannii*, quantitative real-time PCR (qPCR) analysis was performed, and the results revealed the significant downregulation of the expression of *bfmR*, *bap*, *csuA-csuB*, *ompA*, *pgaA*, *pgaC*, *katE*, and *sodB* (Fig. 10).

**C-G-N analysis.** The Venn intersections and comparative gene network (C-G-N) results shown in Fig. 11A and B consist of 48 virulence factors, 57 unique downregulated protein-coding genes, and 8 downregulated transcriptional genes. Furthermore, the results represent values from 3 possible combinatorial analyses, Virulence Factor Database (VFDB), MALDI-TOF/TOF, and qPCR analyses. Two genes, namely, *ompA* and *bfmR*, were found in VFDB, MALDI-TOF/TOF, and qPCR intersections. VFDB and MALDI-
TOF/TOF intersections displayed 5 genes, adeH, adeF, adeG, plcD, and plc. VFDB and qPCR intersections had 4 genes, bap, csuA-csuB, pgaC, and pgaA. Two genes, namely, sodB and katE, were present in MALDI-TOF/TOF and qPCR intersections. Also, 37 genes in VFDB, 48 downregulated genes in MALDI-TOF/TOF, and no unique genes in qPCR analyses were observed without any interaction in systematic network interlinking and Venn intersections.

Among 458 genes in the Database of Essential Genes (DEG), 101 genes that have official gene symbols and known functions were used for Venn and network analyses. No interactions were found between the DEG (101 genes) and all downregulated genes (57 by MALDI-TOF/TOF and 8 by qPCR) (Fig. 11C and D). Furthermore, cross talk was observed only between the MALDI-TOF/TOF and qPCR analyses.

**Influence of citral on the antibiotic sensitivity of A. baumannii.** The ability of citral (200 µg/ml) to improve the efficacy of antibiotics was tested by a broth microdilution assay. The addition of citral to culture media increased the susceptibility of A. baumannii to various antibiotics such as amikacin, cefotaxime, ciprofloxacin, and gentamicin (Fig. 12).

**Native PAGE analysis of catalase and SOD production.** Native PAGE was performed to assess the effect of citral on catalase and superoxide dismutase (SOD) production in A. baumannii. The band intensity was reduced for catalase (Fig. 13A) and SOD (Fig. 13B) in citral-treated samples compared to the control samples.

**Impact of citral on the susceptibility of A. baumannii to H₂O₂ and healthy human blood.** The sensitivity of A. baumannii to reactive oxygen species (ROS) was determined by an H₂O₂ sensitivity assay. The results showed that citral-treated cells were highly sensitive to H₂O₂ compared to control cells. Compared to 1.4 × 10⁶ cells that survived in the control sample, 5.8 × 10⁵ viable cells were noticed in the citral-treated sample (Fig. 13C). In addition, the susceptibilities of A. baumannii to healthy human blood were compared between control and citral-treated cells using a blood survival assay. The results showed that killing was abundant upon citral (200 µg/ml) treatment (4 × 10⁴ cells) compared to the control sample (8.9 × 10⁵ cells) (Fig. 13C).

**FIG 7** Comparative cellular proteomic profiling by 2DGE. Shown are cellular proteome profiles of control and citral-treated samples after isoelectric focusing (18-cm IPG strip [pH 4 to 7]) and separation by 12% SDS-PAGE. After staining, ImageMaster 2D Platinum software was used to identify the differentially expressed protein spots. Up- and downregulated spots are marked with green and red circles, respectively.
TABLE 1 Upregulated proteins of *A. baumannii* treated with citral

| Serial no. | Spot no. | Fold change | P value | MOWSE score | Coverage (%) | UniProt ID | Description | Gene |
|------------|---------|-------------|---------|-------------|--------------|------------|-------------|------|
| 1 | 6 | 9.80019 | 0.0072063 | 425.407 | 34 | DCC713 | Chlorophyll synthesis pathway protein BchC | F898_02608 |
| 2 | 8 | 8.71292 | 0.00691163 | 80.765 | 22.9 | DCC713 | Chlorophyll synthesis pathway protein BchC | F898_02609 |
| 3 | 12 | 5.15743 | 0.00371512 | 212 | 33.3 | DCC711 | Cupin domain protein | yhW_3 |
| 4 | 18 | 4.34379 | 0.005373 | 2.941 | 9.4 | DCC713 | Chlorophyll synthesis pathway protein | F962_01825 |
| 5 | 19 | 4.29623 | 1.80E-04 | 5.47E+09 | 35.4 | DCC712 | Carboxymuconolactone decarboxylase | pcaC |
| 6 | 29 | 2.79884 | 0.00497461 | 5.452 | 7 | DCC707 | FtsK/SpoIIE family protein | J663_1111 |
| 7 | 31 | 2.754 | 0.00289273 | 757 | 28 | DCC711 | Cupin domain protein | yhW_2 |
| 8 | 32 | 2.68892 | 0.00345635 | 1.723 | 11 | DCC923 | Uncharacterized protein | HMPREF0010_01581 |
| 9 | 37 | 2.56585 | 0.0277662 | 16.963 | 25.3 | DCC87 | Electron transfer flavoprotein subunit beta | etfB |
| 10 | 38 | 2.5421 | 0.0110412 | 6.938 | 25 | N9Y70 | Xanthine phosphoribosyltransferase | xpt |
| 11 | 39 | 2.51449 | 0.00325003 | 6.23E+06 | 41 | DCC87 | Electron transfer flavoprotein subunit beta | etfB |
| 12 | 40 | 2.43191 | 0.00963101 | 2.17E+07 | 30.8 | DCEK6 | ATP synthase subunit alpha | atpA |
| 13 | 45 | 2.18798 | 0.00317992 | 1.15E+06 | 24 | DCEGB5 | Elongation factor Tu (fragment) | tuf |
| 14 | 47 | 2.15711 | 0.0224955 | 1.48E+08 | 42.9 | DCC65 | Ketal acid reductoisomerase (NADP⁺) | ilvC |
| 15 | 50 | 2.10401 | 0.0247724 | 1.62E+07 | 27.9 | DCC42 | 1-Pyrimidine dehydrogenase | putA |
| 16 | 56 | 2.02353 | 0.0081648 | 812.305 | 37 | DCC87 | Electron transfer flavoprotein subunit beta | etfB |
| 17 | 58 | 2.00714 | 0.00410603 | 181.873 | 22.8 | DCC64 | Adenosylhomocysteinase | aahC |
| 18 | 59 | 1.97109 | 0.0305157 | 28.488 | 24 | DCC87 | Electron transfer flavoprotein subunit alpha | etfA |
| 19 | 61 | 1.94181 | 0.0300139 | 8.654 | 6 | DCC76 | Succinate-CoA ligase (ADP-forming) subunit alpha | succD |
| 20 | 62 | 1.93165 | 0.00672927 | 6.534 | 28 | DCC69 | Biotin carboxyl carrier protein of acetyl-CoA carboxylase | accB |
| 21 | 64 | 1.90147 | 0.030785 | 1.57E+13 | 30 | DCC87 | ATP synthase subunit beta | atpD |
| 22 | 65 | 1.88344 | 0.024857 | 14.151 | 46 | DCC60 | 3-Dehydroquinic acid dehydratase | aroQ |
| 23 | 68 | 1.83363 | 0.0681003 | 6.537 | 14 | N9JSL7 | Uncharacterized protein | Unknown |
| 24 | 70 | 1.84877 | 0.03694 | 3.45E+07 | 27 | N9JN00 | Elongation factor Tu | tuf |
| 25 | 71 | 1.84746 | 0.0228597 | 3.69E+07 | 48.6 | DCC60 | Cell division protein FtsZ | ftsZ |
| 26 | 75 | 1.79722 | 0.002872 | 2.54E+09 | 55.5 | DCC612 | Oxidoreductase, short-chain dehydrogenase/reductase family protein | EAD62_19600 |
| 27 | 77 | 1.78907 | 0.0224894 | 533.427 | 25.9 | N9LH67 | ATP synthase subunit alpha | atpA |
| 28 | 78 | 1.76984 | 0.0275672 | 4.384 | 20.8 | DCC74 | Dihydrolipoate dehydrogenase | lpdA |
| 29 | 80 | 1.7645 | 0.0396424 | 6.38E+07 | 23 | DCCX2 | Transcription termination/antitermination protein NusA | nusA |
| 30 | 88 | 1.69679 | 0.014505 | 1.99E+06 | 30.3 | DCC87 | Elongation factor Tu (fragment) | tuf |
| 31 | 84 | 1.71455 | 0.038369 | 1.606 | 21 | N9LCE6 | Uncharacterized protein | Unknown |
| 32 | 85 | 1.7089 | 0.0068818 | 4.22E+06 | 32 | N9LF65 | Uncharacterized protein | Unknown |
| 33 | 93 | 1.64787 | 0.0236251 | 2.946 | 22 | DCC77 | Uncharacterized protein | HMPREF0010_00605 |
| 34 | 101 | 1.56314 | 0.040776 | 13.868 | 8.4 | DCC850 | Glutamyl-tRNA (Asp) synthetase | gluQ |
| 35 | 102 | 1.55785 | 0.00506707 | 6.223 | 14.7 | DCC897 | Glycyl hydrolase family 25 | J506_1918 |
| 36 | 106 | 1.54278 | 0.0262351 | 49.184 | 26.1 | DCCAG8 | Uncharacterized protein | HMPREF0010_0174 |

*5MOWSE, MOlecular Weight SEarch.*

Altogether, these results revealed that the resistance of *A. baumannii* to ROS and human blood was reduced upon treatment with citral.

**Inhibitory effect of citral on lipase, protease, and hemolysis of *A. baumannii***

Bacteria produce extracellular lipase as a virulence factor. In this study, the effect of citral on lipase production by *A. baumannii* was investigated. Citral reduced lipase production by 70% compared to control cells (Fig. 13D). In addition, citral treatment inhibited the proteolytic activity of *A. baumannii* up to 60% (Fig. 13D). Also, the results of the hemolysis assay revealed that citral treatment affects the hemolysis activity of *A. baumannii* by 65% compared to the control sample (Fig. 13D).

**Effect of citral on the motility of *A. baumannii***

The influence of citral (200 μg/ml) on the motility of *A. baumannii* was evaluated. Swimming motility was reduced (25 mm) in citral-treated cells compared to control cells (40 mm). In addition, a reduction of swarming motility was observed in treated cells (20 mm) compared to control cells (35 mm). Complete inhibition of twitching motility was observed in treated cells, whereas high twitching motility was observed in control cells (Fig. 14).

**Nonfatal effect of citral on human PBMCs.** Alamar Blue- and trypan blue-based assays were performed to examine the cytotoxic effect of citral (200 and 400 μg/ml) on human peripheral blood mononuclear cells (PBMCs). The results showed that citral did not cause any harmful effect on the viability of PBMCs, whereas 1 mM H₂O₂ completely affected the viability of PBMCs (Fig. 15).

**DISCUSSION**

Biofilm-associated infections by *A. baumannii* are very difficult to cure by conventional antibiotic treatments, and hence, the focus on antibiofilm therapy-based re-
| Serial no. | Spot no. | Fold change | P value by ANOVA | MOWSE score | Coverage by ANOVA | No. of peptides matched | UniProt ID | Description | Gene(s) |
|-----------|---------|-------------|-----------------|--------------|------------------|-------------------------|------------|-------------|---------|
| 1         | 47      | 1.97        | 0.000009        | 27.8         | 18               | 12          | DCCB9      | Chaperonin  | groL    |
| 2         | 48      | 1.98        | 0.000021        | 26.9         | 17               | 12          | DCCB9      | Chaperonin  | groL    |
| 3         | 49      | 1.99        | 0.000012        | 24.5         | 16               | 12          | DCCB9      | Chaperonin  | groL    |
| 4         | 50      | 2.01        | 0.000015        | 24.3         | 18               | 12          | DCCB9      | Chaperonin  | groL    |
| 5         | 51      | 2.02        | 0.000013        | 24.8         | 16               | 12          | DCCB9      | Chaperonin  | groL    |
| 6         | 52      | 2.03        | 0.000022        | 25.4         | 18               | 12          | DCCB9      | Chaperonin  | groL    |
| 7         | 53      | 2.04        | 0.000031        | 26.9         | 17               | 12          | DCCB9      | Chaperonin  | groL    |
| 8         | 54      | 2.05        | 0.000012        | 25.1         | 16               | 12          | DCCB9      | Chaperonin  | groL    |
| 9         | 55      | 2.06        | 0.000013        | 25.2         | 17               | 12          | DCCB9      | Chaperonin  | groL    |
| 10        | 56      | 2.07        | 0.000016        | 25.3         | 17               | 12          | DCCB9      | Chaperonin  | groL    |

**Notes:**
- MOWSE, Molecular Weight Search; TSA, thiolspecific antioxidant; Gcn5-related N-acetyltransferases; RND, resistance-nodulation-division; MFP, membrane fusion protein.
- GroEL, chaperonin 60-kDa chaperonin.
- Cpn60, chaperonin 60-kDa chaperonin.
- GroES, chaperonin 15-kDa chaperonin.
- AhpC, anti-oxidant, AhpC/TSA family.
- CatE, catalase.
- AapB, disulfide oxidoreductase.
- KatE, catalase.
- CofB, coenzyme F420-binding protein.
- Fba, fructose-bisphosphate aldolase.
- GdhA, glutamate dehydrogenase.
- PcaA, putative rRNA large-subunit methyltransferase.
- GltA, glutamate transaminase.
- GroL, GroEL and GroES.
- RlmH, ATP-dependent leader peptidase.
- KatE, catalase.
- GroE, GroEL and GroES.
- CofB, coenzyme F420-binding protein.
- CofA, coenzyme F420-binding protein.
- GdhB, glutamate dehydrogenase.
- Fba, fructose-bisphosphate aldolase.
- GdhA, glutamate dehydrogenase.
- PcaA, putative rRNA large-subunit methyltransferase.
- CofB, coenzyme F420-binding protein.
- CofA, coenzyme F420-binding protein.
- GdhB, glutamate dehydrogenase.
- Fba, fructose-bisphosphate aldolase.
- GdhA, glutamate dehydrogenase.
- PcaA, putative rRNA large-subunit methyltransferase.

**References:**
- Selvaraj et al. (2020). Biochemistry 65, 7905-7915.
- Selvaraj et al. (2021). Journal of Biological Chemistry 296, 2258-2270.
- Selvaraj et al. (2022). Molecular Microbiology 116, 109-121.
search has been increasing in recent times (20). The present study revealed for the first time that citral inhibits the biofilm formation and virulence factor production of *A. baumannii* without causing any harmful effect on viability. In addition, this study explored the mode of action underlying the antibiofilm activity of citral. Initially, 10 phytocompounds (250 μg/ml) were screened for antibiofilm potential against *A. baumannii*. Of the 10 phytocompounds tested, citral most effectively inhibited (97%) the biofilm formation of *A. baumannii* without affecting growth (Fig. 1A). Citral is approved by the Food and Drug Administration (FDA) for use as a flavoring agent in food industries and is also known to have various biological activities (21). From the present study, citral was found to inhibit *A. baumannii* biofilms in a concentration-dependent manner, and the MBIC (200 μg/ml) of citral showed a maximum of 90% biofilm inhibition (Fig. 1B). Furthermore, the nonbactericidal effect of citral on *A. baumannii* was validated by growth curve, CFU, and Alamar Blue assays (Fig. 2), which confirmed that the antibiofilm activity of citral was not through bactericidal activity.

Furthermore, reductions in biofilm-covered areas and biofilm thickness were observed in light microscopy and CLSM images of citral-treated samples, respectively, which further validated the antibiofilm potential of citral against *A. baumannii*. Furthermore, SEM analysis (Fig. 3) revealed a reduction in biofilm formation with dispersed cells and a low abundance of EPS in citral-treated samples, indicating that citral may have an effect on EPS production. The dry weight of the biofilm matrix contains about 90% EPS, which plays a vital role in biofilm stability and supports bacterial survival on
GO functional enrichment analysis of differentially expressed proteins. GO enrichment analysis and visualization of the proteome of *A. baumannii* (upregulated [A to C] and downregulated [D to F]) were performed using the ClueGO v2.5.7/CluePedia v1.5.7 plug-in for Cytoscape software. The numbers of genes falling in biological processes (A and D), molecular functions (B and E), and cellular components (C and F) are directly proportional to the node size. The node colors correspond to each GO category of differentially expressed proteins according to the significance level of GO terms.
various surfaces. Also, EPS blocks the penetration of antibiotics and protects bacteria from the host immune system (22). Therefore, the influence of citral on EPS production was assessed, and the results depicted a reduction in EPS production by citral treatment (Fig. 4). EPS significantly influences the CSH, which is one of the factors responsible for bacterial adhesion to biotic and abiotic surfaces (23). Hence, the inhibitory potential of citral on EPS production could possibly affect the CSH of *A. baumannii*. To check this, the effect of citral on the CSH of *A. baumannii* was assessed, and the data revealed that citral significantly affected the CSH of *A. baumannii* (Fig. 4).

The strong antibiofilm potential of citral against *A. baumannii* prompted us to elucidate the underlying molecular mechanism of action by proteomic analysis. Therefore, the cellular proteomes of control and citral (200-µg/ml)-treated *A. baumannii* were assessed by 2DGE (Fig. 6), and differentially regulated protein spots were identified using MALDI-TOF/TOF analysis (Tables 1 and 2). To validate the proteomic results and evaluate the influence of citral on biofilm- and virulence factor-associated genes, qPCR analysis was performed (Fig. 10). The results unveiled the downregulation of the gene expression of the response regulator of the biofilm-associated TCS (*bfmR*), the biofilm-associated protein (*bap*), the CsUA/BABCDE chaperone-usher complex (*csuA-csuB*), the outer membrane protein (*ompA*), the PNAG-encoding locus (*pgaA* and *pgaC*), catalase (*katE*), and superoxide dismutase (*sodB*), and they were well correlated with the results of the proteomic analysis.

Furthermore, *in silico* analyses were performed to evaluate the interaction and functional attributes of differentially expressed proteins of *A. baumannii*. PPI and functional enrichment analyses revealed that upregulated proteins are predicted to be involved in diverse cellular, metabolic, and oxidation reduction processes such as biosynthetic and nitrogen compound processes (Fig. 9A). The molecular functions of these proteins were correlated with several binding, catalytic, ligase, and oxidoreductase activities (Fig. 9B). In cellular components, upregulated proteins were present in the protein-containing complex, membrane, cytoplasm, and cell periphery (Fig. 9C). Downregulated proteins were involved in the tricarboxylic acid (TCA) cycle, cellular oxidant detoxification, the response to oxidative stress, reactive oxygen species metabolic processes, protein refolding, and diverse catabolic processes (Fig. 9D). In the molecular functional analysis, these proteins were found to be involved in transferase, catalytic, carbon-carbon lyase, antioxidant, efflux transmembrane transporter, and various binding activities (Fig. 9E). These downregulated proteins are present in the cell wall, plasma membrane, cell envelope, cell outer membrane, protein-containing complex, external encapsulating structure, and type II protein secretion system complex.
PPI and functional enrichment analyses revealed the diverse mechanisms of differentially regulated proteins of *A. baumannii*, indicating that citral has multitarget efficacy.

Additionally, C-G-N analysis was done to assess the interaction of downregulated protein-coding and transcriptional genes of *A. baumannii* with virulence and essential genes retrieved from the VFDB and DEG. C-G-N analysis unveiled the (VFDB, MALDI-TOF/TOF, and qPCR) interactions between genes such as *ompA*, *bfmR*, *adeH*, *adeF*, *adeG*, *plcD*, *plc*, *bap*, *csuA-csuB*, *pgaC*, *pgaA*, *sodB*, and *katE* (Fig. 11A and B).

The *ompA* and *bfmR* genes play important roles in bacterial adherence and the two-component system of *A. baumannii*. *adeH*, *adeF*, *adeG*, *plcD*, and *plc* are associated with the efflux pump, autoinducer, transport, and lipase enzyme production. The *bap*, *csuA-csuB*, *pgaC*, and *pgaA* genes are involved in PNAG synthesis and fimbria-associated motility. *sodB* and *katE* are responsible for antioxidant activity and quorum-sensing-mediated biofilm formation in *A. baumannii* (24, 25). These results showed that the bioactive molecule citral hindered biofilm formation by altering various virulence mechanisms of *A. baumannii*. Overall, systematic network interactions and Venn intersections revealed the plausible mode of action and multitarget efficacy of citral. Furthermore, Venn and network analyses showed no interactions between the DEG and downregulated genes (Fig. 11C and D), and they suggest that citral does not affect the normal growth and metabolism of *A. baumannii*.

**FIG 11** Venn and comparative gene network (C-G-N) analyses. The Venn diagram and network indicate the combinatorial analysis of proteins downregulated by citral identified using MALDI-TOF/TOF and qPCR with the VFDB (A and B) and DEG (C and D).
The results of the proteomic analysis showed that citral treatment extremely affected the expression of chaperonins in *A. baumannii*. Chaperonins play a key role in protein folding, the heat shock response, and cellular homeostasis in bacteria. In addition, aminoglycoside antibiotics affect bacteria by inducing protein misfolding. Bacterial chaperonins encounter protein misfolding caused by aminoglycoside antibiotics and thereby promote bacterial survival against aminoglycoside antibiotics (26). Cardoso et al. reported that the overexpression of chaperonin enhanced the resistance of *A. baumannii* to aminoglycoside antibiotics (27). In order to validate the downregulation of chaperonins by citral, the sensitivity of *A. baumannii* to antibiotics was assessed, and the results showed that citral treatment increases the susceptibility of *A. baumannii* to conventional antibiotics (Fig. 12). Furthermore, universal stress protein A (UspA) and antioxidant enzymes such as SOD and catalase (Kat) of *A. baumannii* were downregulated in the citral-treated sample. UspA is involved in the mechanisms of physiological stress resistance in *A. baumannii*. On the other hand, UspA is important for the protection of *A. baumannii* from H$_2$O$_2$ (28). Therefore, the effect of citral treatment on the sensitivity of *A. baumannii* to H$_2$O$_2$ was assessed, and the results showed that citral treatment enhanced the sensitivity of *A. baumannii* to H$_2$O$_2$ (Fig. 12C). Antioxidant enzymes (Sod, Kat, and alkyl hydroperoxide reductase [Ahp]) are involved in the process of detoxification of ROS generated during bacterial metabolism. In bacteria, superoxide is produced through the electron transport chain, bacterial exposure to the host immune system, and antibiotics. Superoxide is then converted into H$_2$O$_2$ by Sod and neutralized into 2H$_2$O and O$_2$ by Kat and Ahp, respectively. In *A. baumannii*, the production of Kat and Sod comes under quorum-sensing-mediated biofilm formation (29, 30).
Proteomic analysis revealed that citral treatment affected the expression of Sod, Kat, and Ahp, which was further validated by native PAGE analysis (Fig. 13A and B). The mutation in SOD significantly affected the surface motility of A. baumannii (31), and this result goes well with the results of the motility assay in the present study (Fig. 14). The response regulator receiver domain protein (AdeRS) is a two-component system of A. baumannii that regulates the expression of the efflux pump (RND family), TetR family transcriptional regulators, and LysR family transcriptional regulators. Thereby, A. baumannii acquires efflux pump-mediated antibiotic resistance, bacterial motility, and biofilm formation (32–35). AdeRS and its associated proteins such as efflux pump (RND family), TetR family transcriptional regulator, and LysR family transcriptional regulator proteins were found to be downregulated by citral, which is well correlated with the results of biofilm assays and different types of motility of A. baumannii (Fig. 13). Citral treatment affected the secretion systems of A. baumannii, such as the type II and type VI secretion systems (LysM domain protein). The type II secretion system of A. baumannii is involved in the process of virulence enzyme production, including lipase and protease. Hence, it was validated by lipolytic quantification and protease assays, and the results revealed reductions in lipase and protease production in A. baumannii treated with citral (Fig. 13D). The activation of the type II secretion system is dependent on the expression of chaperones. On the other hand, the expression of chaperonin was also suppressed by citral treatment. The type VI secretion system is an important virulence factor of A. baumannii that promotes the injection of toxin proteins into host organisms and other competitive bacteria (36).

Proteomic analysis revealed that citral treatment affected iron acquisition and iron homeostasis proteins such as bacterioferritin, the ring-hydroxylating beta subunit, and the TonB-dependent siderophore receptor protein. Iron is an essential element for bacterial cellular processes and pathogenesis. Bacterioferritin and ring-hydroxylating beta proteins are involved in the iron acquisition process and thereby help bacterial growth and pathogenesis in host organisms (37, 38). During pathogenesis, the host
organism generates free Fe$^{3+}$ molecules, and the accumulation of Fe$^{3+}$ causes oxidative stress to bacteria. The iron-chelating protein siderophore and TonB-dependent siderophore receptor protein-mediated iron-chelating process is involved in the protection of bacteria from oxidative stress (39, 40). Due to the negative effect of citral on the iron acquisition mechanism of A. baumannii, the sensitivity of A. baumannii to H$_2$O$_2$ was found to be enhanced (Fig. 12C). In addition, hemolysin is a notable virulence factor produced by bacteria to release iron from the hemoglobin of red blood cells for bacterial growth and development. In order to assess the effect of citral on hemolysin production in A. baumannii, a hemolysin assay was performed, and the results revealed a marked reduction of hemolysin in A. baumannii cells treated with citral (Fig. 13D) (41). These results suggest that citral strongly affects the iron acquisition and iron homeostasis of A. baumannii. The RstA (BfmR) transcriptional regulatory protein of A. baumannii has been found to be downregulated by citral treatment. BfmR is a biofilm-associated master regulatory TCS, and it regulates the biofilm formation, bacterial adherence, and chaperone-usher assembly system (CsuA/BABCDE) of A. baumannii. The chaperone-usher assembly system controls A. baumannii motility; therefore, the effect of citral on the motility of A. baumannii was checked, and the motility of A. baumannii was found to be affected by citral treatment (Fig. 14). Additionally, BfmR supports the survival of A. baumannii in human serum and was also assessed by a blood survival assay, and the results showed that citral treatment enhanced the sensitivity of A. baumannii to the host immune system compared to the control sample (Fig. 12C) (42–44). The H-NS histone family protein regulates various functions of bacteria, such as the expression of outer membrane proteins, motility, biofilm formation, and extracellular polysaccharide synthesis (45, 46). Citral treatment affected the H-NS histone family protein, which could be one of the reasons for the inhibitory effect of citral on the biofilm, EPS, and motility of A. baumannii. Citral treatment downregulated proteins that play an essential role in the TCA cycle, such as isocitrate lyase, isocitrate dehydro-
genase, fumarate hydratase class II, malate synthase G, malate dehydrogenase, fructose-bisphosphate aldolase, and a glyoxalase family protein. Previous studies have shown that the overexpression of TCA cycle-associated proteins increased the biofilm formation, virulence factor production, and antibiotic resistance of *A. baumannii* (47, 48). Our results indicate that citral treatment downregulated TCA cycle-associated proteins, thereby controlling biofilm formation and virulence factors of *A. baumannii*.

In this study, proteomic analysis revealed that citral treatment upregulated proteins associated with protein synthesis, amino acid biosynthesis, ATP synthesis, the electron transport chain, fatty acid biosynthesis, and nucleic acid synthesis (Table 2). These are biologically important processes for the survival of bacteria. Hence, the analysis confirmed that citral at a 200-μg/ml concentration does not affect the proteins required for the survival of *A. baumannii*, which is well correlated with the results of growth curve and Alamar Blue assays. Therefore, the chance of *A. baumannii* gaining resistance to citral can be very small or nil. In addition, a glycosyl hydrolase family protein was found to be upregulated upon treatment with citral, and it is involved in the disassembly of existing biofilms (49). Hence, the upregulation of the glycosyl hydrolase family protein could increase biofilm disruption in citral-treated samples. Finally, cytotoxic analyses revealed the nonfatal effect of citral on PBMCs (Fig. 15). Overall, this holistic study unveils the multitarget efficacy of citral to impede the biofilm formation and virulence of *A. baumannii*. Various virulence regulatory systems targeted by citral are schematically presented in Fig. 15. Citral treatment majorly impacted the two-component system BfmRS, which regulates biofilm formation, motility, EPS synthesis, iron transport, toxin secretory systems, the AdeABC system that provides antibiotic resistance, and the antioxidant system. Interestingly, proteins involved in electron transport, amino acid synthesis, and protein synthesis are positively regulated by citral. The present study suggests that citral could be safe and can be considered for further therapeutic investigations for the treatment of biofilm-associated infections by *A. baumannii* (Fig. 16).

**Conclusion.** In conclusion, the present study revealed the antibiofilm potential of citral without causing any harmful effect on the growth and metabolism of *A. baumannii*. The global proteomics-based analysis unveiled that citral treatment affected biofilm formation, antibiotic resistance, iron homeostasis, the antioxidant defense
system, the iron acquisition system, and the type II and type IV secretion systems of *A. baumannii*. In addition, citral treatment positively regulated the proteins associated with the growth and metabolism of *A. baumannii*, which suggests that the possibility of drug resistance to citral is very small. Furthermore, citral exhibited nontoxic effects on human PBMCs, and hence, citral could be a safe therapeutic compound for the treatment of infections caused by *A. baumannii*. Altogether, the results of the present study emphasize the multitarget potential of citral to inhibit the biofilm and virulence factors of *A. baumannii*.

**MATERIALS AND METHODS**

**Ethics statement.** In the current study, healthy human blood was used for blood survival and cytotoxicity analyses, and sheep blood was used for hemolysis assays. The human blood sample was collected from a healthy individual by a technically skilled person, and written informed consent was obtained. The use of the human blood sample and the experimental protocol were assessed and approved by the Institutional Ethics Committee (IEC), Alagappa University, Karaikudi (IEC reference no. IEC/AU/2018/4). The sheep blood was collected from the municipality slaughterhouse, Karaikudi, Tamil Nadu, India. As sheep blood is discarded in the slaughterhouse, specific ethical permission is not required.

**Bacterial strain and growth conditions.** *A. baumannii* MTCC 9829 was obtained from the Microbial Type Culture Collection (MTCC), India. It was grown in tryptone soya broth (Hi-Media, India), incubated at 37°C for 24 h, and stored at 4°C. A single isolated colony of *A. baumannii* was inoculated into tryptone soya broth (TSB) supplemented with 1% sucrose and 0.5% yeast extract (TSBSY), incubated at 37°C overnight, adjusted to 10^6^ cells/ml, and used for all the experiments.

**Preparation of phytocompound stock solutions.** Citral (Alfa Aesar, India), chlorogenic acid (Sigma-Aldrich, India), vanillic acid (Alfa Aesar, India), ferulic acid (Sigma-Aldrich, India), gallic acid (Sigma-Aldrich, India), geraniol (Sigma-Aldrich, India), catechin, and naringin hydrate (Sigma-Aldrich, India) were dissolved in methanol. Sterile MilliQ water was used for dissolving caffeine and L-glutathione (Alfa Aesar, India). All the phytocompounds were prepared as 10-mg/ml stock solutions and stored at 4°C for future use.

**Evaluation of antibiofilm activities of phytocompounds.** The phytocompound(s) (250 μg/ml) was added to 200 μl of TSBSY containing 1% of a culture of *A. baumannii* grown overnight in a 96-well
microtiter plate and incubated at 37°C for 24 h. TSBSY alone and TSBSY inoculated with 1% of the culture grown overnight (adjusted to 10^6 cells/ml) were considered the blank and the control, respectively. After incubation, the plate was read at 600 nm to assess the optical density (OD) of the culture, and planktonic cells were discarded. Next, the wells were washed thoroughly with sterile distilled water. A crystal violet (0.4%) solution was used to stain the wells for 10 min, and the wells were washed with sterile distilled water followed by air drying. The wells were destained using 30% glacial acetic acid for 10 min, and the absorbance was read at 570 nm. Finally, the antibiofilm activity of phyto compounds and their percent-age of inhibition were measured by using the following formula: biofilm inhibition (%) = [(control OD_{570} − treated OD_{570})/control OD_{570}] × 100 (50).

**Determination of the MIC and MBIC of citral.** Citral, which showed antibiofilm activity against A. baumannii, was added at increasing concentrations (25 to 400 µg/ml) to wells containing 1 ml of TSBSY in a sterile 24-well microtiter plate and inoculated with 1% of a culture of A. baumannii grown overnight. The plate was incubated at 37°C for 24 h. After incubation, the OD of culture was obtained, and biofilm quantification was performed as described above. The MIC and minimal biofilm-inhibitory concentration (MBIC) were fixed as the maximum growth inhibition and maximum biofilm reduction at the lowest concentration of citral, respectively (51).

**Growth curve analysis.** To determine the effect of citral on the growth of A. baumannii, citral (200 µg/ml) was added to a 100 ml of TSBSY containing 1% of a culture of A. baumannii grown overnight. TSBSY inoculated with 1% of the A. baumannii culture with the addition of 1 ml of methanol was considered the vehicle control, and TSBSY with citral added and without an inoculum was considered the blank. Next, the flasks were incubated at 37°C, and the ODs of control and treated samples were measured at 600 nm for 0 to 24 h at serial intervals of 1 h (51).

**Alamar Blue assay.** An Alamar Blue assay was performed to quantitatively analyze the influence of citral on the cell viability of A. baumannii. Briefly, a culture of A. baumannii grown overnight was used to inoculate 1 ml of TSBSY without and with citral at 200 µg/ml and allowed to grow for 24 h at 37°C. The control and treated cells were collected by centrifugation at 8,000 rpm for 10 min, washed twice with phosphate-buffered saline (PBS), and resuspended in PBS. The Alamar Blue (0.1-ml) substrate (1 mg/ml in PBS) was mixed with 0.8 ml of control and treated cell suspensions, and the samples were incubated in the dark at 37°C for 4 h. After incubation, the samples were centrifuged at 8,000 rpm for 10 min. Next, the fluorescence intensity of the supernatants was quantified spectrophotometrically with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Cell viability is directly proportional to the reduction of the level of blue color to pink (51).

**Microscopic analyses of A. baumannii biofilms.** The biofilm architecture of control and citral-treated samples was qualitatively analyzed by microscopic techniques. A biofilm assay was performed on 1- by 1-cm glass slides in a 24-well plate containing 1 ml of TSBSY and 1% of the inoculum, without and with 200 µg/ml of citral. The assay plate was incubated at 37°C for 24 h. After incubation, the slides were stained and observed using microscope (52).

(i) **Light microscopic analysis.** For light microscopic analysis, the slides were washed gently with sterile PBS, stained with 0.4% crystal violet for 1 min, washed again with sterile PBS to remove the excess stain, and air dried. The air-dried glass slides were examined under a light microscope (Eclipse Ti-S; Nikon, Tokyo, Japan) at a ×400 magnification.

(ii) **Confocal laser scanning microscopy analysis.** For confocal laser scanning microscopy (CLSM), the slides were washed with sterile PBS, stained using 0.1% acridine orange under dark conditions for 5 min, washed with sterile PBS to remove the unbound stain, and air dried. Next, the glass slides were observed by CLSM (Zeiss LSM-710; Carl Zeiss, Oberkothen, Germany) after magnification of ×200.

(iii) **Scanning electron microscopy analysis.** For scanning electron microscopy (SEM) analysis, the glass slides were washed with sterile PBS, fixed with 2% glutaraldehyde for 8 h at 4°C, and washed again with sterile PBS. Next, the slides were dehydrated with increasing concentrations of ethanol (20, 40, 60, 80, and 100%). After gold sputtering, the slides were observed by SEM (Vega 3; Tescan, Czech Republic).

**MATH assay.** The effect of citral on the cell surface hydrophobicity (CSH) of A. baumannii was determined by a microbial adherence to hydrocarbon (MATH) assay (50). The cells of control and citral-treated cultures were harvested by centrifugation at 8,000 rpm for 10 min and resuspended in sterile PBS. An equal volume of toluene was added to 1 ml of the cell suspension (OD of 0.8 ± 0.05), and the mixture was vortexed for 2 min. After vortexing, the tubes were kept under undisturbed conditions for 6 h for the separation of the aqueous phase and toluene phase. The toluene phase was discarded, and the remaining solvent traces were completely eliminated by keeping the tubes open at 37°C overnight. The OD of the aqueous phase was read at 600 nm, and the CSH was measured as the hydrophobicity index, using the following formula: hydrophobicity index = [1 − (OD_{csh} after vortexing/ OD_{csh} before vortexing)] × 100.

**Quantification of EPS.** EPS quantification was done to compare the total amounts of EPS production in control and treated samples. Initially, 100 ml of A. baumannii cultures grown in the absence and presence of citral (200 µg/ml) at 37°C for 24 h were centrifuged at 8,000 rpm for 10 min in order to separate cell pellets and the cell-free culture supernatant (CFCS). An equal volume of isotonic buffer (10 mM Tris-HCl; pH 8.0, 10 mM EDTA, 2.5% NaCl) was added to the cell pellet, and the mixture was incubated at 4°C overnight to isolate cell-bound EPS. After incubation overnight, the cell suspension was centrifuged at 8,000 rpm for 10 min, and the supernatant of the cell suspension was mixed with the CFCS. Next, ice-cold ethano1 and the CFCS containing cell-bound EPS were mixed at a ratio of 3:1 and incubated at −20°C overnight. The EPS mixture was then centrifuged at 12,000 rpm at 4°C for 30 min in order to separate the pellet. The pellet was then suspended in 70% ethanol and dried in a vacuum evaporator.

November/December 2020 Volume 5 Issue 6 e00986-20
Finally, the dried EPS was weighed, and EPS inhibition was calculated using the following formula: EPS (%).weight of control / weight of treated sample ) x 100.

**Cellular protein extraction and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis.** Initially, *A. baumannii* was allowed to grow in the absence and presence of citral (200 μg/ml) in 100 ml of TSB5Y for 24 h at 37°C with constant shaking at 160 rpm, in biological triplicates. After incubation, the cell pellet was collected by centrifugation at 8,000 rpm for 15 min at 4°C. Next, the pellet was washed three times with PBS to remove debris and resuspended in 2 ml of 20 mM Tris-HCl containing 1% protease inhibitor cocktail. The samples were then subjected to sonication on ice using an ultrasonicator (Sonic VCK 750) with a 30% amplitude and a pulse time of 10 s on-off cycles for 10 min. After sonication, samples were centrifuged at 12,000 rpm for 30 min at 4°C, and the supernatant containing proteins was cleaned using the 2-D Clean-Up kit (GE Healthcare) according to the manufacturer’s instructions. Next, purified protein samples were dissolved in sample buffer (7 M urea, 2 M thiourea, 40 mM dithiothreitol (DTT), and 4% 3-[3-cholamidopropyl]-dimethylammonio]-1-propane-sulfonate (CHAPS)) and quantified by the Bradford method using a Bio-Rad protein assay kit. A total of 50 μg of protein from the control/citral-treated sample was loaded onto a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (SE 600 vertical unit; GE Healthcare) and run at 50 V for 30 min for the stacking gel and at 100 V for 3 h for the separating gel. After running SDS-PAGE, the gels were soaked in a fixative solution (10% glacial acetic acid, 40% methanol, and 50% water) for 6 h and washed three times with distilled water at 20-min intervals. Colloidal Coomassie brilliant blue (CBB) G-250 staining solution (10% orthophosphoric acid, 10% ammonium sulfate, 20% methanol, and 0.12% CBB) was used to stain the gels for 12 h with gentle agitation. After staining, the CBB solution was removed, and gels were destained with distilled water. Next, the gels were imaged using an Image Scanner III system (GE Healthcare) equipped with LabScan 6.0 software (52).

**Isoelectric focusing and two-dimensional gel electrophoresis.** Prior to isoelectric focusing (IEF), six immobilized pH gradient (IPG) strips (Immobiline DryStrip pH 4 to 7, 18cm) were rehydrated for 12 h at 20°C by adding 350 μl (for each strip) of rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer pH 4 to 7, 0.002% bromophenol blue, and 12.5 mg/ml Destreak reagent (GE Healthcare)) containing 500 μg of protein extracted from the control/citral-treated sample per strip (biological triplicates). After rehydration, IPG strips were placed on mineral oil in an IEF system (Ettan IPGphor 3; GE Healthcare), and IEF was carried out by applying a 75-μA current per IPG strip under the following conditions: 100 V (2-h step), 100 to 500 V (3-h gradient), 500 V (2-h step), 500 to 5,000 V (3-h gradient), 5,000 V (2-h step), 5,000 to 8,000 V (3-h gradient), 8,000 V (2-h step), 8,000 to 10,000 V (3-h gradient), and 10,000 V (2-h step) at 20°C for 22 h. Prior to two-dimensional gel electrophoresis (2DGE), the IPG strips were equilibrated with two different equilibration buffers, equilibration buffer I (6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, and 1% DTT in 50 mM Tris-HCl buffer [pH 8.8]) for 15 min and equilibration buffer II (6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, and 2.5% iodoacetamide (IAA) in 50 mM Tris-HCl buffer [pH 8.8]) for 15 min. Next, equilibrated IPG strips were placed on top of 12% gels and sealed with a 0.3% agarose solution. The Ettan DALTsix electrophoresis system (GE Healthcare) was used for electrophoresis under the following program conditions: 100 V (2-h step), 100 to 500 V (3-h gradient), 500 V (2-h step), 500 to 5,000 V (3-h gradient), 8,000 V (2-h step), 8,000 to 10,000 V (3-h gradient), and 10,000 V (2-h step) at 20°C for 22 h. Prior to two-dimensional gel electrophoresis (2DGE), the IPG strips were equilibrated with two different equilibration buffers, equilibration buffer I (6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, and 1% DTT in 50 mM Tris-HCl buffer [pH 8.8]) for 15 min and equilibration buffer II (6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, and 2.5% iodoacetamide (IAA) in 50 mM Tris-HCl buffer [pH 8.8]) for 15 min. Next, equilibrated IPG strips were placed on top of 12% gels and sealed with a 0.3% agarose solution. The Ettan DALTsix electrophoresis system (GE Healthcare) was used for electrophoresis under the following program conditions: 100 V (200 mA) for 1 h and 150 V (500 mA) for 8 h. After electrophoresis, the gels were fixed, stained by CBB according to the above-described method, and imaged at a 300-dpi (dots per inch) resolution using an Image Scanner III system (GE Healthcare) equipped with LabScan 6.0 software (53).

**Identification of differentially expressed proteins by image analysis and in-gel digestion of proteins.** ImageMaster 2D Platinum software (GE Healthcare) was used according to the manufacturer’s instructions to assess the differentially expressed protein spots in control and citral-treated gels. After image analysis, differentiated protein spots of >1.5-fold were excised using sterile cut tips. Next, the gel pieces were washed three times with a destaining solution (25 mM ammonium bicarbonate in 50% mass spectrometry (MS)-grade water and 50% acetonitrile [ACN]), dehydrated with MS-grade ACN for 10 min, and vacuum dried for 30 min. The gel pieces were taken for reduction (25 mM ACN and 10 mM DTT in MS-grade water) for 30 min at 55°C and alkylation (25 mM ACN and 55 mM IAA in MS-grade water) at 37°C for 30 min. Again, gel pieces were dehydrated with ACN and dried under a vacuum. Next, the gel pieces were digested with a trypsin solution (5 μl) containing 10 mM ammonium bicarbonate and 400 ng of trypsin in 10% ACN, covered with overlay buffer (25 μl) containing 40 mM ammonium bicarbonate in 10% ACN, and incubated at 37°C in a water bath for 16 h. After trypsin digestion, peptides were eluted from gel pieces by adding 25 μl of 60% ACN containing 0.1% trifluoroacetic acid (TFA) and sonicated for 5 min. After sonication, tubes were centrifuged at 12,000 rpm for 10 min, and the supernatant containing peptides was dried under a vacuum for 90 min (53). Next, the eluted peptides were reconstituted in a peptide suspension solution (0.1% TFA in 5% ACN). To purify the peptides, C18 Ziptips (Millipore, Merck) were used according to the manufacturer’s protocol.

**MALDI-TOF/TOF mass spectrometry analysis.** Prior to mass spectrometry analysis, the matrix-assisted laser desorption ionization–time of flight/time of flight (MALDI-TOF/TOF) mass spectrometer (Axima Performance; Shimadzu Biotech) was calibrated using TOF-Mix (LaserBio Labs, France) containing a mixture of seven peptides. After calibration, 1 μl of a matrix solution (10 mg of an alpha-cyano-4-hydroxycinnamic acid matrix and 0.1% formic acid in 1 ml of 60% methanol) was mixed with 1 μl of the peptide sample and spotted onto the target plate. Next, mass spectrometry analysis was performed, and peptide mass spectra were obtained by MALDI-TOF/TOF analysis using Shimadzu Launch pad-MALDI MS software. Monoisotopic peaks were selected between 700 and 4,000 Da with MALDI MS software and analyzed using the MS-Fit (http://prospector.ucsf.edu) online database with the following parameters: trypsin enzyme digestion with two missed cleavages per peptide, 1.5 Da mass tolerances with cysteine...
TABLE 3 Primers used for qPCR analysis

| Gene      | Forward primer                 | Reverse primer                |
|-----------|--------------------------------|--------------------------------|
| bfmR      | 5'-CTGGTAGGTACTGACTTGC-3'      | 5'-GAGAGACCAAACCCATACAC-3'    |
| bap       | 5'-GACTACGAGCAAGGTTGTA-3'      | 5'-GAAGGATCTGCTGATTCCA-3'     |
| csuA-csuB | 5'-ATGCGGATATAATCTACAGCA-3'    | 5'-TCAGAGAAATATTGGCACCCT-3'   |
| ompA      | 5'-CCTTGGTCTGCTTAAACGTA-3'     | 5'-GCCGTTGCTGTTAGTG-3'        |
| pgaA      | 5'-CAATGGCAGAAAAAGATGAAT-3'    | 5'-CGTAGAACCTGCAAGACGTG-3'    |
| pgaC      | 5'-CACTGTTATGCTGCTATTT-3'      | 5'-GGGCTGCAACAACGTG-3'        |
| katE      | 5'-GTCGCGGTTTACGTTTTTAC-3'     | 5'-GGGTTGGAGAGATGTTCCA-3'     |
| sodB      | 5'-TGAACCGAATTGAGTGTG-3'       | 5'-TTCAAACATGCGAGTCAAT-3'     |
| pfrB      | 5'-GGTCTGATTAAACAGCGTC-3'      | 5'-AATATGCAATAGCGCTTG-3'      |

carbamidomethylation and methionine oxidation, acetylation of the N terminus, and phosphorylation of S, T, and Y amino acids (16, 19).

Quantitative real-time PCR analysis. To assess the influence of citral on the biofilm-associated genes such as bfmR, bap, csuA-csuB, ompA, pgaA, pgaC, katE, and sodB, quantitative real-time PCR (qPCR) analysis was performed. Initially, total RNA was isolated from control and citral-treated A. baumannii cells by the TRIzol method, and cDNA conversion was done using a high-capacity cDNA reverse transcription kit (Applied Biosystems). Next, qPCR was performed on a 7500 thermal cycler sequence detection system (Applied Biosystems Inc., Foster City, CA, USA) using Power SYBR green PCR master mix (Applied Biosystems) according to the manufacturer’s instructions. The gene expression level was calculated after normalizing the cycle threshold (C_T) values of biofilm-associated genes with a housekeeping gene (pfrB) using the 2^(-δΔCT) method. Details of the primer sequences for biofilm-associated genes are listed in Table 3 (16, 24).

Signaling network and gene enrichment analysis. Protein-protein interactions (PPIs) of differentially expressed proteins from A. baumannii were imputed using STRING v11.0 (https://string-db.org/) with a high-confidence score of 0.7 (54). Interactions of these proteins and their details were exported in TSV (tab-separated value) file format from the STRING tool, and the same was imported to ClueGO v2.5.7/CluePedia v1.5.7 for gene ontology (GO) analysis. GO enrichment was performed using the ClueGO/CluePedia plug-in of Cytoscape v3.8.0 based on the two-sided hypergeometric test (statistical test) and Bonferroni step-down (correction method) with a kappa score threshold level of 0.38 (55–57).

Comparative gene network analysis. Comprehensive information on virulence and essential genes of A. baumannii was collected from the Virulence Factor Database (VFDB) (25) and the Database of Essential Genes (DEG) (58), respectively. The collected information and downregulated protein-coding genes from MALDI-TOF/TOF and qPCR analyses were compared using Draw Venn Diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/) and the molecular interaction network was visualized using Cytoscape v3.8.0.

Antibiotic sensitivity assay. The test tubes were supplemented with 2 ml of TSBSY and various concentrations of antibiotics (amikacin, cefotaxime, ciprofloxacin, and gentamicin) alone and with citral at 200 μg/ml. For inoculation, 1% of a culture of A. baumannii grown overnight was added, and the mixture was incubated at 37°C for 24 h. After incubation, the culture was read at 600 nm, and the percentage of inhibition was measured using the following formula: percent inhibition = (control OD_{600} - treated OD_{600}/control OD_{600}) × 100 (50).

Qualitative analysis of catalase and superoxide dismutase activities by native PAGE. Control and citral (200-μg/ml)-treated A. baumannii cells were pelleted by centrifugation at 8,000 rpm for 10 min at 4°C and suspended in ice-cold 50 mM potassium phosphate (pH 7). Intracellular protein was isolated by sonication on ice. After sonication, cell debris was removed by centrifugation at 8,000 rpm for 20 min, and protein was quantified by the Bradford method. A total of 50 μg of protein from control/citral-treated samples was loaded on two different nondenaturing polyacrylamide gels at 8% for catalase activity and 12% for superoxide dismutase (SOD) activity, respectively. Electrophoresis was carried out at 50 V at 20°C using running buffer containing 50 mM Tris, 300 mM glycine, and 1.8 mM EDTA for 6 h (52, 53).

(i) Catalase activity. For catalase activity, after electrophoresis, the gel (8%) was washed three times with 50 mM KP buffer. The gel was then incubated with a 4 mM H_2O_2 solution for 10 min and stained with a solution containing 1% potassium ferrixyanide and 1% ferric chloride. The gel was allowed to stain until the appearance of a dark blue-green background with clear bands and further washed with distilled water.

(ii) SOD activity. For SOD activity, the 12% gel was incubated in 100 ml of freshly prepared 50 mM KP buffer with substrates such as 0.1 mM EDTA, 2 mg riboflavin, and 16 mg nitroblue tetrazolium (NBT). Next, tetramethylphenyldiamine (TEMED) (400 μl) was added to the substrate solution, and the mixture was incubated at 37°C in the dark for 1 h to reduce NBT. After incubation, the gel was washed, immersed in 50 mM KP, and exposed to light. The SOD activity was assessed by the appearance of achromic bands in the gel with a purple background, and the gel was immediately imaged.

H_2O_2 susceptibility assay. A. baumannii was grown for 24 h in TSBSY without and with citral at 200 mg/ml. Next, cells were collected by centrifugation at 8,000 rpm for 10 min and resuspended in PBS containing 1 mM H_2O_2. The samples were incubated at 37°C for 1 h with shaking at 160 rpm, and viable cells were enumerated by the CFU method (51).

Blood survival assay. Citral-treated and untreated A. baumannii cells were suspended in sterile PBS. The cell suspension was added to healthy human blood at a ratio of 1:4 and mixed thoroughly. The
mixtures were incubated in a shaking incubator at 37°C for 3 h. The viable cells in control and treated samples were quantified by the serial dilution method (59).

**Lipolysis assay.** The extracellular lipase production of control and citral-treated A. baumannii was assessed using p-nitrophenyl palmitate (pNPP) as the substrate. Briefly, A. baumannii was grown in the absence and presence of citral (200 μg/ml) for 24 h at 37°C. After incubation, the CFCS was collected by centrifugation at 8,000 rpm for 10 min. The pNPP substrate was prepared by mixing 1 volume of pNPP (0.3% in 2-propanol) with 9 volumes of 50 mM Na2PO4 buffer (pH 8.0) containing sodium deoxycholate (0.2%) and gummi arabicum (0.1%). Next, 100 μl of the CFCS was added to 900 μl of the pNPP substrate, incubated for 1 h at 37°C in the dark, and then centrifuged at 12,000 rpm for 5 min; the absorbance of the supernatant was measured at 400 nm, and the percentage of inhibition was calculated according to a previously described formula (9).

**Protease assay.** The proteolytic activity of A. baumannii control and citral-treated samples was analyzed by mixing 100 ml of a protease substrate solution (0.2% azocasein dissolved in 0.05 M Tris-hydrochloride) with 100 ml of the CFCS of A. baumannii samples, and the mixture was incubated at 37°C for 30 min. To terminate the reactions, 10% trichloroacetic acid (500 μl) was added to the mixture, and the mixture was incubated at −20°C for 15 min and centrifuged at 8,000 rpm for 15 min. Next, the supernatant absorbance was measured at 400 nm, and the percentage of inhibition was calculated (9).

**Hemolysin assay.** To analyze the effect of citral on hemolysin production in A. baumannii, the CFCS was collected from A. baumannii grown in the absence and presence of citral for 24 h at 37°C. Next, 100 μl of the CFCS of the control/citral-treated sample was mixed with 900 μl of 2% sheep red blood cells in PBS and incubated at 37°C for 1 h. Subsequently, the samples were centrifuged at 3,000 rpm for 15 min, the absorbance of the supernatant was read at 530 nm, and the percentage of inhibition was calculated (9).

**Motility assays.** Motility assays were performed to assess the influence of the MBIC (200 μg/ml) of citral on the motility of A. baumannii (60, 61).

(i) **Swimming.** TSB (0.3% agar) plates were prepared with 200 μg/ml of citral, and plates with the corresponding volume of methanol were used as controls. A 5-μl culture was spotted onto the middle region of the plate and incubated at 37°C for 72 h. After incubation, the swimming motility of control and treated cells was measured.

(ii) **Swarming.** A 10-μl A. baumannii culture was spotted onto the middle region of the TSB plate (0.5% agar) supplemented without and with citral (200 μg/ml) and incubated at 37°C for 72 h. After incubation, the zone of swarming motility of control and treated cells was observed.

(iii) **Twitching.** A. baumannii cells were stab inoculated using a toothpick into the bottom of 1% agar-containing tryptic soya agar (TSA) in 6-well polystyrene plates without and with citral (200 μg/ml). The plate was incubated for 72 h at 37°C. The A. baumannii twitching motility on the polystyrene surface was examined by removing the agar, washing with sterile PBS, and staining with a 0.3% crystal violet solution.

**Cytotoxicity assay.** Peripheral blood mononuclear cells (PBMCs) were isolated from freshly collected blood by gently adding it to a tube containing lymphocyte separation medium (Histopaque; Sigma-Aldrich, India). Next, the tube was centrifuged at 2,500 rpm at 20°C for 30 min, and the buffy coat layer was collected and washed with RPMI 1640 medium. Next, PBMCs were suspended in complete medium (1% Anti-Anti [antibiotic-antimycotic] solution and 10% fetal bovine serum in RPMI 1640 medium) and adjusted to 1 × 10^6 cells/ml. PBMCs were treated without and with citral (200 and 400 μg/ml) for 24 h at 37°C in a 5% CO2 incubator. A total of 1 mM H2O2 and 0.05% methanol were maintained as the positive control and the vehicle control, respectively. After incubation, cell viability was evaluated by an Alamar Blue assay and the trypan blue method (51).

**Statistical analysis.** All experiments were performed in three biological replicates with at least two technical replicates, and data are presented as means ± standard deviations (SD). Significant differences between the values of control and treated samples were analyzed by one-way analysis of variance (ANOVA) and Duncan’s post hoc test with a significant P value of ≤0.05 using SPSS statistical software version 17.0 (SPSS, Chicago, IL, USA).

**ACKNOWLEDGMENTS**

We thank DST-FIST [grant no. SR/FST/LSI-639/2015(C)], UGC-SAP (grant no. F.5-1/2018/DRS-II [SAP-II]), and DST-PURSE [grant no. SR/PURSE Phase 2/38(G)] for providing instrumentation facilities. A.S. thanks UGC for financial assistance in the form of a basic scientific research fellowship (sanction no. F.25-1/2013-14 [BSR]/7-326/2011 [BSR]). S.K.P. thanks UGC for a Mid-Career award (F.19-225/2018 [BSR]) and RUSA 2.0 (F.24-51/2014-U, Policy [TN Multi-Gen], Department of Education, Government of India).

A.S. and S.K.P. conceived and designed the research. A.S., A.V., and P.M. conducted experiments. A.S. and P.M. analyzed the data. A.S. wrote the manuscript. A.V., S.S., G.A.S., M.R., and S.K.P. revised the manuscript. All authors read and approved the manuscript.

We declare that we have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
REFERENCES

1. Ayoub Moubarec K, Hammadul Halat D. 2020. Insights into Acinetobacter baumannii: a review of microbiological, virulence, and resistance traits in a threatening nosocomial pathogen. Antibiotics (Basel) 9:119. https://doi.org/10.3390/antibiotics9030319.

2. De Oliveira DMP, Forde BM, Kidd TJ, Harris PNA, Schembri MA, Beatson SA, Paterson DL, Walker MJ. 2020. Antimicrobial resistance in ESKAPE pathogens. Clin Microbiol Rev 33:e00181-19. https://doi.org/10.1128/CMR.00181-19.

3. Lee C-R, Lee JH, Park M, Park KS, Bae IK, Kim YB, Cha CJ-J, Jeong BC, Lee SH. 2017. Biology of Acinetobacter baumannii: pathogenesis, antibiotic resistance mechanisms, and prospective treatment options. Front Cell Infect Microbiol 7:55. https://doi.org/10.3389/fcimb.2017.00055.

4. Flemming H-C, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S. 2016. Biofilms: an emergent form of bacterial life. Nat Rev Microbiol 14:563–575. https://doi.org/10.1038/nrmicro.2016.94.

5. Sharma D, Misba L, Khan AU. 2019. Antibiotics versus biofilm: an emerging battleground in microbial communities. Antimicrob Resist Infect Control 8:76. https://doi.org/10.1186/s13756-019-0533-3.

6. Colquhoun JM, Rather PN. 2020. Insights into mechanisms of biofilm formation in Acinetobacter baumannii and implications for uropathogenesis. Front Cell Infec Microbiol 10:253. https://doi.org/10.3389/fcimb.2020.00253.

7. Rabin N, Zheng Y, Opoku-Temeng C, Du Y, Bonsu E, Sintim HO. 2015. Biofilm inhibitory efficiency of phyto in combination with cefotaxime against nosocomial pathogen Acinetobacter baumannii. J Appl Microbiol 125:56–71. https://doi.org/10.1111/jam.13741.

8. Solanki V, Tiwari V. 2018. Subtractive proteomics to identify novel drug targets and reverse vaccinology for the development of chimeric vaccine against Acinetobacter baumannii. Sci Rep 8:15004. https://doi.org/10.1038/s41598-018-26689-7.

9. Goltermann L, Good L, Bentin T. 2013. Developing natural products as potential anti-biofilm agents. Chin Med 8:141. https://doi.org/10.3390/tmm1002-0139.

10. Muthamil S, Balasubramanian B, Mahalingam S, Pandian SK. 2017. Synergistic effect of quinic acid derived from Syzygium cumini and undecanoic acid against Candida spp. biofilm and virulence. Front Microbiol 9:2835. https://doi.org/10.3389/fmicb.2018.02835.

11. Lee J-H, Kim Y-G, Khadke SK, Yamano A, Woo J-T, Lee J. 2019. Antimicrobial and antibiofilm activities of prenylated flavanones from Macaranga tanarius. Phytomedicine 63:15303. https://doi.org/10.1016/j.phymed.2019.15303.

12. Kannappan A, Gowrishankar S, Srinivasan R, Pandian SK, Ravi AV. 2017. Antibiofilm activity of Vetiveria zizanioides root extract against methicillin-resistant Staphylococcus aureus. Microb Pathol 101:313–324. https://doi.org/10.1016/j.micpat.2017.07.016.

13. Chat OA, Bhat PA, Nazir N, Dar AA. 2019. Self-assembled systems based on surfactants and polymers as stabilizers for citral in beverages, p 487–521. In: Grumezescu AM, Holban AM (ed). Value-added ingredients and enrichment of beverages, vol 14. Elsevier Academic Press, London, United Kingdom.

14. Martins HB, da Selis NN, Souza CLE, Nascimento FS, de Carvalho SP, Beatson MA, Caprariis P. 2017. Regulation of the Pancreatic and human B-lymphoma cell lines. Med Chem Ther 56:2504–2510. https://doi.org/10.1016/j.mct.2016.11.008.

15. Goltermann L, Good L, Bentin T. 2013. Developing natural products as potential anti-biofilm agents. Chin Med 8:141. https://doi.org/10.3390/tmm1002-0139.

16. Rabin N, Zheng Y, Opoku-Temeng C, Du Y, Bonsu E, Sintim HO. 2015. Biofilm inhibitory efficiency of phyto in combination with cefotaxime against nosocomial pathogen Acinetobacter baumannii. J Appl Microbiol 125:56–71. https://doi.org/10.1111/jam.13741.

17. Solanki V, Tiwari V. 2018. Subtractive proteomics to identify novel drug targets and reverse vaccinology for the development of chimeric vaccine against Acinetobacter baumannii. Sci Rep 8:15004. https://doi.org/10.1038/s41598-018-26689-7.

18. Juttukonda LJ, Green ER, Lonergan ZR, Heffern MC, Chang CJ, Skaar EP. 2019. Acinetobacter baumannii OxR regulates the transcriptional response to hydrogen peroxide. Infect Immun 87:e00413-18. https://doi.org/10.1128/IAI.00413-18.

19. Bhargava N, Sharma P, Capalash N. 2014. Pyocyanin stimulates quorum sensing-mediated tolerance to oxidative stress and increases persister cell populations in Acinetobacter baumannii. Front Microbiol 5:13. https://doi.org/10.3389/fmicb.2014.00013.

20. Adams FG, Stroeher UH, Hassan KA, Mari S, Brown MH. 2018. Resistance to pentamidine is mediated by AdeAB, regulated by AdeR, and influenced by growth conditions in Acinetobacter baumannii. ATCC 17978. PLoS One 13:e0197412. https://doi.org/10.1371/journal.pone.0197412.

21. Richmond GE, Evans LP, Anderson MJ, Wand ME, Bonney LC, Ivens A. 2019. Regulation of the transcriptional response to antibiotic and heat shock in Acinetobacter baumannii. J Med Microbiol 59:1061–1068. https://doi.org/10.1099/jmm.0.02393-0.

22. Adams FG, Stroeher UH, Hassan KA, Mari S, Brown MH. 2018. Resistance to pentamidine is mediated by AdeAB, regulated by AdeR, and influenced by growth conditions in Acinetobacter baumannii. ATCC 17978. PLoS One 13:e0197412. https://doi.org/10.1371/journal.pone.0197412.

23. Richmond GE, Evans LP, Anderson MJ, Wand ME, Bonney LC, Ivens A. 2019. Regulation of the transcriptional response to antibiotic and heat shock in Acinetobacter baumannii. J Med Microbiol 59:1061–1068. https://doi.org/10.1099/jmm.0.02393-0.

24. Rosenfeld N, Bouchier C, Courvalin P, Périchon B. 2012. Expression of the adeAB operon in Acinetobacter baumannii. J Antimicrob Chemother 56:2504–2510. https://doi.org/10.1093/jac/dks137.

25. Lebeaux D, Ghigo J-M, Beloin C. 2014. Biofilm-related infections: bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. Microbiol Mol Biol Rev 78:510–543. https://doi.org/10.1128/MMBR.00113-14.

26. Goltermann L, Good L, Bentin T. 2013. Developing natural products as potential anti-biofilm agents. Chin Med 8:141. https://doi.org/10.3390/tmm1002-0139.
Acinetobacter secretion. Trends Microbiol 25:532–545. https://doi.org/10.1016/j.tim.2017.01.005.

37. Nwuogo CC, Caddy JA, Zimbler DL, Actis LA. 2011. Deciphering the iron response in Acinetobacter baumannii: a proteomics approach. J Proteomics 74:44–58. https://doi.org/10.1016/j.jprot.2010.07.010.

38. Tiwari V, Moganty RR. 2013. Effect of iron availability on the survival of carbapenem-resistant Acinetobacter baumannii: a proteomics approach. J Proteomics Bioinform 6:125–131. https://doi.org/10.172/j/jpb.1000270.

39. Mosbah K, Wojnowska M, Albatal A, Walker D. 2018. Bacterial iron acquisition mediated by outer membrane translocation and cleavage of a host protein. Proc Natl Acad Sci U S A 115:6840–6845. https://doi.org/10.1073/pnas.1800672115.

40. Zimbler DL, Arivett BA, Beckett AC, Menke SM, Actis LA. 2013. Functional features of TonB energy transduction systems of Acinetobacter baumannii. Infect Immun 81:3382–3394. https://doi.org/10.1128/IAI.00540-13.

41. Fiester SE, Arivett BA, Schmidt RE, Beckett AC, Ticak T, Carrier MV, Ghosh R, Oheker EJ, Metz ML, Sellin Jeffries MK, Actis LA. 2016. Iron-regulated phosphohlipase C activity contributes to the cytolytic activity and virulence of Acinetobacter baumannii. PLoS One 11:e0167068. https://doi.org/10.1371/journal.pone.0167068.

42. Russo TA, Manohar A, Beanan JM, Olson R, MacDonald U, Graham J, Umland TC. 2016. The response regulator BfmR is a potential drug target for Acinetobacter baumannii. mSphere 1:e00082-16. https://doi.org/10.1128/mSphere.00082-16.

43. Draughn GL, Milton ME, Feldmann EA, Bobay BG, Roth BM, Olson AL, Thompson RI, Actis LA, Cavanaugh J. 2018. The structure of the biofilm-controlling response regulator BfmR from Acinetobacter baumannii reveals details of its DNA-binding mechanism. J Mol Biol 430:806–821. https://doi.org/10.1016/j.jmb.2018.02.002.

44. Krasauskas R, Skerniškytė J, Armalyte J, Suižiedėliene E. 2019. The role of Acinetobacter baumannii response regulator BfmR in pellicle formation and competitiveness via contact-dependent inhibition system. BMC Microbiol 19:241. https://doi.org/10.1186/s12866-019-1621-5.

45. Jian H, Xu G, Gai Y, Xu J, Xiao X. 2016. The histone-like nucleoid structure protein H-NS plays a role in expression of virulence features of TonB energy transduction systems of Acinetobacter baumannii. J Proteomics 74:44–58. https://doi.org/10.1016/j.jprot.2015.10.003.

46. Jian H, Xu G, Gai Y, Xu J, Xiao X. 2016. The histone-like nucleoid structure protein H-NS plays a role in expression of virulence features of TonB energy transduction systems of Acinetobacter baumannii. J Proteomics 74:44–58. https://doi.org/10.1016/j.jprot.2015.10.003.

47. Sivasankar C, Maruthupandian S, Balamurugan K, James PB, Krishnan V, Pandian SK. 2016. A combination of ellagic acid and tetracycline inhibits biofilm formation and the associated virulence of Propionibacterium acnes in vitro and in vivo. Biofouling 32:397–410. https://doi.org/10.1080/08927014.2016.1148141.

48. Sekaraj A, Jayasree T, Valliamai A, Pandian SK. 2019. Myrtenol attenuates MRSA biofilm and virulence by suppressing sarA expression dynamism. Front Microbiol 10:2027. https://doi.org/10.3389/fmicb.2019.02027.

49. Prasath KG, Sethupathy S, Pandian SK. 2019. Proteomic analysis uncovers the modulation of ergosterol, sphingolipid and oxidative stress pathway by myristic acid impeding biofilm and virulence in Candida albicans. J Proteomics 208:103503. https://doi.org/10.1016/j.jprot.2019.103503.

50. Sethupathy S, Prasath KG, Ananthi S, Mahalingam S, Balan SY, Pandian SK. 2016. Proteomic analysis reveals modulation of iron homeostasis and oxidative stress response in Pseudomonas aeruginosa PA01 by curcumin inhibiting quorum sensing regulated virulence factors and biofilm production. J Proteomics 145:112–126. https://doi.org/10.1016/j.jprot.2016.04.019.

51. Saklarczyk D, Morris JH, Cook H, Kuhn M, Wyder S, Simonovic M, Santos A, Doncheva NT, Roth A, Bork P, Jensen LJ, von Mering C. 2017. The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. Nucleic Acids Res 45:D362–D368. https://doi.org/10.1093/nar/gkw937.

52. Su G, Morris JH, Demchak B, Bader GD. 2014. Biological network exploration with Cytoscape 3. Curr Protoc Bioinformatics 47:8.13.1–8.13.24.

53. Sethupathy S, Prasath KG, Ananthi S, Mahalingam S, Balan SY, Pandian SK. 2016. Proteomic analysis reveals modulation of iron homeostasis and oxidative stress response in Pseudomonas aeruginosa PA01 by curcumin inhibiting quorum sensing regulated virulence factors and biofilm production. J Proteomics 145:112–126. https://doi.org/10.1016/j.jprot.2016.04.019.

54. Szklarczyk D, Morris JH, Cook H, Kuhn M, Wyder S, Simonovic M, Santos A, Doncheva NT, Roth A, Bork P, Jensen LJ, von Mering C. 2017. The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. Nucleic Acids Res 45:D362–D368. https://doi.org/10.1093/nar/gkw937.

55. Su G, Morris JH, Demchak B, Bader GD. 2014. Biological network exploration with Cytoscape 3. Curr Protoc Bioinformatics 47:8.13.1–8.13.24.

56. Szklarczyk D, Morris JH, Cook H, Kuhn M, Wyder S, Simonovic M, Santos A, Doncheva NT, Roth A, Bork P, Jensen LJ, von Mering C. 2017. The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. Nucleic Acids Res 45:D362–D368. https://doi.org/10.1093/nar/gkw937.

57. Bindea G, Galon J, Mlecnik B. 2013. CluePedia Cytoscape plugin: pathway networks, made broadly accessible. Nucleic Acids Res 45:D362–D368. https://doi.org/10.1093/nar/gkw937.

58. Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, Fridman W-H, Pagès F, Trajanoski Z, Galon J. 2009. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics 25:1091–1093. https://doi.org/10.1093/bioinformatics/btp101.

59. Bindea G, Galon J, Mlecnik B. 2013. CluePedia Cytoscape plugin: pathway insights using integrated experimental and in silico data. Bioinformatics 29:661–663. https://doi.org/10.1093/bioinformatics/btt019.

60. Zhang R, Lin Y. 2009. DEG 5.0, a database of essential genes in both prokaryotes and eukaryotes. Nucleic Acids Res 37:D455–D458. https://doi.org/10.1093/nar/gkn858.

61. Ashwinkumar Subramenium G, Viswznapriya D, Iyer PM, Balamurugan K, Karutha Pandian S. 2015. curc mediated antibiofilm activity of 3-furancarboxaldehyde increases the virulence of group A Streptococcus. PLoS One 10:e0127210. https://doi.org/10.1371/journal.pone.0127210.

62. Vijayakumar S, Rajenderan S, Laishram S, Anandan S, Balaji V, Biswas I. 2016. Biofilm formation and motility depend on the nature of the Acinetobacter baumannii clinical isolates. Front Public Health 4:105. https://doi.org/10.3389/fpubh.2016.00105.

63. Eijkelkamp BA, Stroehler UH, Hassan KA, Papadimitrakis MS, Paulsen IT, Brown MH. 2011. Adherence and motility characteristics of clinical Acinetobacter baumannii isolates. FEMS Microbiol Lett 323:44–51. https://doi.org/10.1111/j.1574-6968.2011.02362.x.

Unveiling Molecular Targets of Citral on A. baumannii

Yu S, Su T, Wu H, Liu S, Wang D, Zhao T, Jin Z, Du W, Zhu M-J, Chua SL, Yang L, Zhu D, Gu L, Ma LZ. 2015. PslG, a self-produced glycosyl hydrolase, triggers biofilm disassembly by disrupting exopolysaccharide matrix. Cell Res 25:1352–1357. https://doi.org/10.1038/cr.2015.129.