Chemical Components of Aqueous Extracts of *Melia azedarach* Fruits and Their Effects on The Transcriptome of *Staphylococcus aureus*

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Abstract

*Staphylococcus aureus* is the causative agent of numerous and varied clinical infections. Crude aqueous extracts of *Melia azedarach* fruits inhibit the planktonic growth and initial biofilm formation of *S. aureus* in a dose-dependent manner. Moreover, the biofilm topologies became sparse and decreased as the concentration of the aqueous extracts increased. RNA-Seq analyses revealed 532 differentially expressed genes (DEGs) after *S. aureus* exposure to 0.25 g/ml extracts; 319 of them were upregulated, and 213 were downregulated. The majority of DEGs were categorized into abundant sub-groups in the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Finally, untargeted UHPLC-MS/MS analyses of the aqueous extracts of *M. azedarach* fruits demonstrated a highly complex profile in positive and negative electrospray ionization modes. The extracts primarily consisted of lipids and lipid-like molecules, organic acids and their derivates, phenylpropanoids, polyketides, organoheterocyclic compounds, and benzenoids annotated by abundant lipid maps and KEGG pathways. Overall, this study provides evidences that the aqueous extracts of *M. azedarach* fruits can control *S. aureus* infections and sought to understand the mode of action of these extracts on *S. aureus*.

Graphical abstract

The planktonic growth (A) and biofilm formation (B) of *Staphylococcus aureus* can be inhibited by aqueous extracts of *Melia azedarach* fruits in a dose-dependent manner.

Key words: *Staphylococcus aureus*, *Melia azedarach* fruits, differentially expressed genes, biofilms, UHPLC-MS/MS

Introduction

*Melia azedarach* (chinaberry) tolerates a wide range of adverse environmental settings so that it is commonly found in tropical, subtropical, and warm temperate areas as an ornamental plant, shade tree, and a source of fuel (Khan et al. 2011). In the past decades, various components have been extracted from chinaberry leaves, bark, fruits, and roots with different approaches. The active ingredients exhibited antimicrobial
activities, which might be affected by the solvents used in the extraction and the parts of this plant that the extracts were from (Khan et al. 2011; Zahoor et al. 2015). Chinaberry leaves, roots, and bark extracted with methanol, petrol, dichloromethane, and ethyl acetate exhibited a broad spectrum of antibacterial activity, but the dichloromethane fraction of the bark was the most effective (Khan et al. 2001). A methanol extract of chinaberry flowers demonstrated potential antibacterial effects on *Staphylococcus aureus* when evaluated with a rabbit skin infection model (Saleem et al. 2002).

The antibacterial activities of chinaberry leaves extract in aqueous or chloroform on two Gram-positive bacteria, *Bacillus subtilis* and *S. aureus*, and three Gram-negative bacteria, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*, were assessed using the agar well diffusion method. The extracts exhibited a relatively higher zone of inhibition at 75 µl/ml than at concentrations of 25 and 50 µl/ml, and the aqueous extracts were more effective at inhibiting the bacteria than those extracted in chloroform at the specific concentrations tested. It suggests that the leaf extracts caused inhibition in a dose-dependent manner, and the solvents used in extraction also influenced the activities (Suresh et al. 2008).

However, another study found that the alcoholic extracts of chinaberry leaves were more potent than those extracted with methanol, petroleum ether, and water when tested against eight human pathogens, including *E. coli*, *P. aeruginosa*, *B. cereus*, *S. aureus*, *Fusarium oxysporum*, *Aspergillus niger*, *Rhizopus stolonifera*, and *Aspergillus flavus* (Sen and Batra 2012). Antibacterial activities of the crude methanolic extracts of chinaberry were measured using an agar well diffusion method, but *E. coli* was highly resistant to the extracts at all doses tested (Zulqarnain et al. 2015). It suggests that the antibacterial activity of crude extracts of chinaberry varies with the microorganisms’ species.

In addition, the antibacterial spectrum of the same chinaberry extracts was affected by the solvents used in the extraction process. The antimicrobial, antioxidant, and cytotoxic activities of chinaberry bark extracted with various solvents were assayed. The results showed that the chloroform extract was active against both *Enterobacter aerogenes* and *Proteus mirabilis*, while both *n*-hexane and butanol were the most effective at inhibiting *E. aerogenes*. Simultaneously, aqueous and methanolic extracts were the most effective against *P. mirabilis*, and ethyl acetate was the most effective against *P. aeruginosa* (Zahoor et al. 2015).

Bacterial biofilms are complex, multi-species bacterial communities that are usually highly resistant to antimicrobial agents (Mah 2012; Zhou et al. 2015). However, chinaberry extracts have been shown to have strong antimicrobial effects against the microorganisms producing recalcitrant biofilm, including *Acinetobacter guillouiae*, *Alcaligenes faecalis*, *Bacillus pumilus*, *Bacillus safensis*, *Brevundimonas alba*, *Microbacterium lacticum*, *Staphylococcus equorum*, and *Staphylococcus saprophyticus*, which were isolated from dental plaques (Khalid et al. 2017).

In addition to their antibacterial activities, the bioactive phytochemicals were also isolated and identified from chinaberry extracts. The human pathogens *Enterococcus faecalis*, *E. coli*, *P. aeruginosa* and *Klebsiella oxytoca* were susceptible to extracts from chinaberry leaves, particularly the petroleum ether fraction that consists of secondary metabolites, such as alkaloids, terpenes/sterols, saponins, tannins and anthocyanins (Rojas Sierra et al. 2012). A variety of compounds have been found in the methanolic extracts from chinaberry, including propanedioic acid, butanedioic acid, diethyl ester, 2-pyrrolidinyl-methylamine, 2-piperidimethanamine, and trichloromethane (Al-Marzoqi et al. 2015). In addition, a substantial number of different ingredients were also isolated and identified from chinaberry by different research groups, which repeatedly demonstrated that the components of extracts varied with the extracted parts, solvents, and methodology.

Taken together, the antimicrobial activities and bioactive agents of extracts of chinaberry have been widely studied by different laboratories. However, the underlying modes of action of these extracts on bacteria remain elusive. Thus, in this study, we tried to extract components from chinaberry fruits using simple boiling water methods, and the differentially expressed genes (DEGs) were detected with RNA-Seq using *S. aureus* as a model strain. The results demonstrated that various growth and metabolism-related genes and pathways were affected by the aqueous extracts of chinaberry fruits. Moreover, lipids and lipid-like molecules, organic acids, and derivatives were relatively abundant in these extracts.

### Experimental

#### Materials and Methods

**Bacterial strains and chemicals.** The bacterial strain *S. aureus* ATCC 6538 was purchased from the American Type Culture Collection (ATCC) and was cultivated in Luria Bertani (LB) medium that consisted of 5 g/l yeast extract (Oxoid, Hampshire, England), 10 g/l NaCl (Sigma-Aldrich, St. Louis, MO, USA) and 10 g/l peptone (Oxoid) without any antibiotics at 37°C in a shaking incubator. All the chemicals used in this study were of analytical grade and supplied by Sigma-Aldrich (St. Louis, MO, USA) unless indicated otherwise.
**Extractions of chinaberry fruits with water.** The chinaberry fruits purchased in Sichuan (China) were first washed with pure water to remove dust, dried to a constant weight at 65°C, and then crushed into powders using a grinder (Tianchuang Powder Technology Co., Ltd., Changsha, China). An aliquot of 100 g of the ground powders was weighed and dissolved in 1,000 ml of distilled water, sonicated with an ultrasound instrument (Anpu Experimental Technology Co., Ltd., Shanghai, China) for 30 min at room temperature, heated, and boiled for 30 min, and sonicated again for another 30 min. The samples were filtered with 200-mesh gauze to obtain the crude extracts. The filter residues were subsequently added to 500 ml of distilled water, boiled for another 30 min, filtered with 200-mesh gauze, and the extracts obtained were combined with the previous extracts and centrifuged at 13,000 rpm for 10 min. The supernatants were then transferred into a new plastic tube, filtered through a 400-mesh nylon filter using a gas-liquid diaphragm vacuum pump (Huankai Microbiology Technology Co. Ltd., Guangzhou, China), and the filtered samples were re-filtered through a 0.45-µm membrane using the same vacuum pump. Finally, the samples that had been filtered twice were placed in a rotary evaporator (Ailang Instrument Co. Ltd., Shanghai, China) with a vacuum degree of –0.1 ~ –0.08 MPa to concentrate the liquid to 100 ml under reduced pressure to ensure that the final concentration of the bioactive reagents was approximately 1.00 g/ml. After sterilization at 121°C for 20 min, the concentrated extracts were stored in a refrigerator at 4°C for future use.

**Semi-quantitative measurements of *S. aureus* biofilms.** The biofilm biomass formed by *S. aureus* was measured using a crystal violet staining method in flat-bottomed polystyrene microtiter plates (Corning Inc., Corning, NY, USA), as previously described with minor modifications (Stepanović et al. 2000; Zhou et al. 2019). Briefly, each well of one column of a 96-well microtiter plate was inoculated with 150 µl of *S. aureus* suspensions with an optical density (OD$_{600}$) of approximately 0.05 and 50 µl of aqueous extracts of chinaberry fruits to final concentrations of 0, 0.25, 0.33, 0.50, and 1.00 g/ml. Moreover, the negative controls contained only LB medium. The inoculated plates were then placed in a static incubator with a constant temperature at 37°C and cultured for 48 h. Before staining, the OD$_{600}$ of planktonic cells was detected with a Multiskan GO plate reader (Thermo Fisher Scientific, Waltham, MA, USA). Moreover, the clean data were acquired by removing low-quality reads and the reads that contained adapter and ploy-N from raw data. Differential expression analyses of the two conditions/groups were calculated using the DESeq R package (1.18.0). Moreover, the approach of Benjamini and Hochberg was used to adjust the resulting p-values to control the false discovery rate. DEGs were assigned with an adjusted value of p < 0.05 and |fold-change| > 1.5. Gene Ontology (GO)
enrichment analyses of the DEGs were processed using the GOseq R package, in which the gene length bias was synchronously corrected. Moreover, GO terms with corrected p-values less than 0.05 were considered to be significantly enriched by DEGs. In addition, the KOBASE software was utilized to test the statistical enrichment of DEGs in KEGG pathways (https://www.kegg.jp/kegg/pathway.html). In addition, the clean transcriptomic sequencing data were deposited in the NCBI Sequence Read Archive (SRA) under BioProject ID PRJNA723959.

Chemical composition of the aqueous extracts from chinaberry fruits. The chemical composition of aqueous extracts from chinaberry fruits was analyzed as previously described (Liu et al. 2020; Wright Muelas et al. 2020; Grabowska et al. 2021). A Vanquish UHPLC system (Thermo Fisher Scientific) equipped with an Orbitrap Q Exactive series mass spectrometer (Thermo Fisher Scientific) was utilized to analyze the chemicals from the LC-MS/MS chinaberry fruit extracts. The treated and filtered samples were transferred into a Hypersil Gold column (100 × 2.1 mm, 1.9 µm; Thermo Fisher Scientific) operating at a column temperature of 50°C. The samples were eluted at 0.4 ml/min flow rate over 15 min with 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol (solvent B). Moreover, 5 mM ammonium acetate at pH 9.0 (solvent A) and methanol (solvent B) were used as eluents in the negative polarity mode. The solvent gradient was performed as follows: 2% B, 1.5 min; 2–100% B, 12.0 min; 100% B, 14.0 min; 100–2% B, 14.1 min; and 2% B, 17 min. The Q Exactive series mass spectrometer (Thermo Fisher Scientific) was operated in a positive or negative polarity mode with a capillary temperature of 320°C, a spray voltage of 3.2 kV, an auxiliary gas flow rate of 35 arb. The raw data files obtained by UHPLC-MS/MS were then processed with Compound Discoverer CD3.1 software (Thermo Fisher Scientific) to align the peaks and picks and quantify each metabolite. The main parameters in the analyses were set as follows: retention time tolerance, 0.2 min; signal intensity tolerance, 30%; actual mass tolerance, 5 ppm; minimum intensity, 100,000 and signal/noise ratio, 3; respectively. Subsequently, the acquired peak intensities were then normalized to the total spectral intensity. The acquired normalized data were continuously used to predict the molecular formula based on the molecular ion peaks, additive ions, and fragment ions. Finally, the peaks were processed to match the mzVault 2.1, mzCloud (https://www.mzcloud.org/), and MassList databases to obtain accurate and relative quantitative results. Statistical analyses in this section were performed with the statistical software R (version: R-3.4.3), Python (version: 2.7.6), and CentOS (CentOS release 6.6). Furthermore, the HMDB (http://www.hmdb.ca/), LipidMaps (http://www.lipidmaps.org/), and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (http://www.genome.jp/kegg/) were utilized to conduct the annotation of all metabolites identified.

Statistical analysis. All the data obtained were expressed as the mean ± standard deviation (SD) and were then subjected to a one-way analysis of variance (ANOVA) followed by comparing multiple treatment levels with the control using the Fisher’s LSD test. Moreover, when p-value < 0.05 was considered as significant. The statistical analyses were calculated using the data processing system (DPS) software (Tang and Feng 2007).

Results

Aqueous extracts of chinaberry fruits exhibit inhibitory effects on both planktonic growth and biofilm formation of *S. aureus*. In this study, the active components were extracted from chinaberry fruits using a simple water boiling method. The aqueous extracts were then used to test their effects on planktonic growth and the initial *S. aureus* biofilm formation in a static condition in 96-well microtiter plates. As expected, the planktonic growth of *S. aureus* was inhibited by the aqueous extracts of chinaberry fruits in a dose-dependent manner (Fig. 1A). In the presence of 0.25, 0.33, 0.50, and 1.00 g/ml aqueous extracts, the planktonic growth of *S. aureus* was repressed by approximately 17.63%, 76.05%, 89.32%, and 97.27%, respectively, compared with the controls (Fig. 1A). Furthermore, the biofilm formation of *S. aureus* treated with the same concentrations of aqueous extracts of chinaberry fruits was assayed with the crystal violet staining method. The results demonstrated that the aqueous extracts also inhibited the biofilms of *S. aureus* in a dose-dependent manner (Fig. 1B), which is like the efficiencies of the inhibition of planktonic growth. When treated with 0.25, 0.33, 0.50, and 1.00 g/ml aqueous extracts, the biofilm formation of *S. aureus* was repressed by approximately 51.68%, 77.83%, 93.22%, and 98.30%, respectively, compared with the controls (Fig. 1B). These results revealed that aqueous extracts of chinaberry fruits can inhibit not only planktonic growth but also the *S. aureus* biofilm formation in a dose-dependent manner.

Biofilm topographies of *S. aureus* can be influenced by the aqueous extracts of chinaberry fruits. As shown in Fig. 1B, since the initial biofilm of *S. aureus* formed on a polystyrene surface can be influenced by the aqueous extracts of chinaberry fruits, we sought to determine whether the biofilm topographies of this strain built on a glass surface could be affected by these extracts. Therefore, the initial *S. aureus* biofilm
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was first allowed to be formed on glass cover slips, and then stained with the fluorescent dye SYTO9, and finally observed under CLSM. The results demonstrated that when cultured for 2 days, a typical *S. aureus* biofilm could be formed on the surface of the glass slips (Fig. 2A). However, the biofilms on these slips decreased and became sparse with the increasing concentrations of the aqueous extracts (Fig. 2B-2E). Furthermore, the biofilms-related parameters were also calculated using the COMSTAT based on the images obtained from CLSM. The results showed that the average thickness and total biomass, with the exception of maximum thickness, of

![Graph showing bacterial growth and biofilm formation](image)

**Fig. 1.** Effect of aqueous extracts of *Melia azedarach* fruits on planktonic growth (A) and biofilm formation (B) of *Staphylococcus aureus* ATCC 6538. *S. aureus* ATCC 6538 was cultured on LB media supplemented with different concentrations of aqueous extracts of chinaberry fruits at 37°C for 48 h. The *S. aureus* planktonic cells and biofilms stained with crystal violet were measured at OD₆₀₀ and OD₅₉₀ respectively, using a Multiskan GO plate reader.

![Representative CLSM images](image)

**Fig. 2.** Representative CLSM images of *Staphylococcus aureus* ATCC 6538 biofilms grown in the presence of different concentrations of aqueous extracts of *Melia azedarach* fruits and stained with SYTO9. The biofilms were cultivated on the surface of glass coverslips for 48 h at 37°C. The constructed biofilms were stained with the fluorescent dye SYTO9 and then observed under a Zeiss LSM 710 CLSM. Scale bar = 50 µm. CLSM, confocal laser scanning microscopy.
the formed biofilms that formed when cultured without aqueous extracts treatments were always higher than those harvested in the presence of various concentrations of aqueous extracts (Table I).

**RNA-Seq results and DEGs.** Since both bacterial growth and the initial biofilm formation of S. aureus could be inhibited by aqueous extracts of chinaberry fruits, our next goal was to elucidate the underlying modes of action of the aqueous extracts on S. aureus cells. Therefore, the samples of S. aureus planktonic cells treated with or without 0.25 g/ml aqueous extracts were harvested and subjected to RNA-Seq to acquire their transcriptome sequence data using an Illumina NovaSeq platform. The sequencing results identified 15,306,690; 16,228,122; 14,710,464; 15,424,438; 15,052,636; and 14,875,448 clean reads for the controls and treatments with three replicates, respectively. Moreover, the DEGs between controls and treatments were also analyzed with the DESeq R package (1.18.0). A total of 532 DEGs, of which 319 were activated, and 213 were inhibited, were identified when S. aureus planktonic cells were treated with 0.25 g/ml aqueous extracts of chinaberry fruits based on the following criteria identified: DESeq2 $p_{adj} < 0.05$ and $|\log2\text{Fold-Change}| > 1.5$.

**Various GO and KEGG pathways can be affected by the aqueous extracts of chinaberry fruits.** In addition, GO analyses were also conducted to classify functions of the DEGs found in the RNA-Seq experiments. Based on the sequence homology, the DEGs were categorized into three classes that included biological process, cellular component, and molecular function, which have been shown as the first ten subgroups in each category (Fig. 3). Gene expression, membrane, and oxidoreductase activity were the largest subgroups enriched for the downregulated DEGs in biological
process, cellular component, and molecular function, respectively (Fig. 3). Moreover, the oxidation-reduction process, membrane, and oxidoreductase activity were the most significant subgroups enriched for the up-regulated DEGs in biological process, cellular component, and molecular function, respectively (Fig. 3).

To further elucidate these DEGs’ functions and explore the enriched metabolic or signal transduction pathways, the DEGs were also mapped to the terms from the KEGG pathway database (Fig. 4). Among the top 20 enriched KEGG pathways, the DEGs were significantly enriched for ribosome (KEGG pathway ID: 03010). The right y-axis represents the KEGG pathway. The x-axis exhibits the enrichment factor, which denotes the ratio of the DEG numbers to the annotated gene numbers enriched in a specific pathway. DEG, differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes.

### Table I

| Parameters | Control | 0.25 g/ml | 0.33 g/ml | 0.50 g/ml | 1.00 g/ml |
|------------|---------|-----------|-----------|-----------|-----------|
| Maximum thickness (µm) | 10 ± 0.00  | 10 ± 0.00  | 10 ± 0.00  | 10 ± 0.00  | 10 ± 0.00  |
| Average thickness (µm) | 7.75 ± 0.10  | 6.68 ± 0.36  | 3.37 ± 0.17  | 2.11 ± 0.19  | 1.11 ± 0.03  |
| Total biomass (µm³/µm²) | 9.81 ± 1.78  | 6.97 ± 0.08  | 2.98 ± 0.25  | 1.40 ± 0.24  | 0.22 ± 0.02  |

Different letters marked in the same row represent the significant differences among different treatments (p < 0.05)
Table II
Identification results of the top 25 metabolites from aqueous extracts of *Melia azedarach* fruits in a positive mode.

| No. | Compound_ID   | Name                                           | Formula         | Molecular Weight | Retention Time (min) |
|-----|---------------|------------------------------------------------|-----------------|------------------|----------------------|
| 1   | Com_2_pos     | D-Pyrrolidine-2-Carboxylic acid                 | C_8H_11NO_4     | 115.0634         | 1.382                |
| 2   | Com_3_pos     | DL-Arginine                                     | C_8H_13N_2O_3   | 174.1115         | 1.425                |
| 3   | Com_6_pos     | Choline                                         | C_8H_12NO       | 103.0999         | 1.275                |
| 4   | Com_49_pos    | L-Glutamic acid                                 | C_4H_7NO_4      | 147.0529         | 1.434                |
| 5   | Com_28_pos    | Nicotinic acid                                  | C_6H_9NO_2      | 123.0321         | 1.773                |
| 6   | Com_44_pos    | Perillartine                                     | C_7H_11NO_2     | 165.1152         | 7.342                |
| 7   | Com_30_pos    | 6-Hydroxyisocitonic acid                        | C_6H_11NO_3     | 139.0268         | 1.988                |
| 8   | Com_59_pos    | Maltol                                          | C_6H_13O        | 126.0318         | 5.271                |
| 9   | Com_21_pos    | Isoamylamine                                     | C_7H_14N        | 87.10517         | 4.875                |
| 10  | Com_75_pos    | 5-oxoproline                                    | C_6H_9NO_2      | 129.0426         | 1.45                 |
| 11  | Com_71_pos    | Ethyl 4-amino-2-(methylsulfanyl)-1,3-thiazole-5-carboxylate | C_13H_21N_2O_5  | 218.019          | 1.265                |
| 12  | Com_77_pos    | DL-Tryptophan                                    | C_6H_12N_2O_4   | 204.0897         | 6.801                |
| 13  | Com_98_pos    | Egonine methyl ester                            | C_10H_16NO_4    | 199.1206         | 8.349                |
| 14  | Com_103_pos   | Scopoletin                                       | C_13H_9O_2      | 192.0421         | 8.708                |
| 15  | Com_105_pos   | Gamma-Aminobutyric acid                         | C_9H_15O_2      | 103.0637         | 1.292                |
| 16  | Com_85_pos    | Kinetin                                         | C_12H_15O_2     | 237.0635         | 2.048                |
| 17  | Com_104_pos   | N-Acetyl-DL-serine                               | C_9H_17N_2O_2   | 147.0529         | 1.284                |
| 18  | Com_116_pos   | Pipencolinic acid                               | C_8H_13NO_2     | 129.079          | 1.734                |
| 19  | Com_119_pos   | 2-Methylenesuccinic acid                        | C_7H_13N_2O     | 130.0263         | 1.284                |
| 20  | Com_168_pos   | 2-Picolinic acid                                 | C_7H_13NO_2     | 123.0321         | 1.523                |
| 21  | Com_165_pos   | 6-O-(2-Methylbutanoyl)-a-D-glucopyranosyl a-D-glucopyranoside | C_22H_41O_12  | 443.1997         | 8.133                |
| 22  | Com_135_pos   | (+/-)12(13)-DiHOME                              | C_10H_15O_2     | 296.2345         | 14.142               |
| 23  | Com_171_pos   | Corylifol A                                     | C_6H_10O_2      | 390.1823         | 10.9                 |
| 24  | Com_78_pos    | Oleoyl ethylamid                                | C_14H_28NO      | 309.3025         | 15.542               |
| 25  | Com_114_pos   | Cytidine                                        | C_6H_11N_2O_5   | 243.0893         | 8.013                |

**Functional annotation of the metabolites from aqueous extracts of chinaberry fruits.** Three databases of HMDB, lipid maps, and KEGG pathways were used to annotate the functions of the metabolites from the aqueous extracts of chinaberry fruits. The annotation results demonstrated that a total of 242 and 148 metabolites under positive and negative ion modes were classified into 11 and 9 HMDB groups, respectively (Fig. 5). Among all the metabolites, 8.08% and 15.04% of the positive and negative ion modes referred to lipids and lipid-like molecules, respectively. The detailed information, including the total ion intensity of the metabolites identified in chinaberry fruits’ aqueous extracts, is shown in supplementary Excel files S1 and S2.

**Chemical components of the aqueous extracts of chinaberry fruits.** Since *S. aureus* planktonic growth, biofilm formation, and the related GO and KEGG pathways could be influenced by the aqueous extracts of chinaberry fruits, we sought to determine the chemical components composed of these extracts. Thus, the chemical compositions of the aqueous extracts were characterized using a UHPLC-MS/MS-based non-targeted metabolomics approach to explain which active factors played roles in these biological processes. The results demonstrated that 681 and 339 metabolites were successfully identified under positive and negative ion modes, respectively. The basic characteristics of the top 25 metabolites in both the positive and negative ion modes are shown in Tables II and III, respectively. The detailed information, including the total ion intensity of the metabolites identified in chinaberry fruits’ aqueous extracts, is shown in supplementary Excel files S1 and S2.

...and valine, leucine, and isoleucine biosynthesis (pae00290).

**pae03010** and valine, leucine, and isoleucine biosynthesis (pae00290).

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of metabolites that are members of different groups from the positive and negative ion modes (Fig. 6).

In addition, the three main KEGG pathways of metabolism, genetic information processing, and environmental information processing were enriched for approximately 184 and 137 metabolites in the positive and negative ion modes, respectively (Fig. 7). In a positive mode, most metabolites take part in global and overview maps, biosynthesis of other secondary metabolites, and amino acid metabolism (Fig. 7). In contrast, in the negative mode, the pathways of global and overview maps, amino acid metabolism, and carbohydrate metabolism occupy most of the metabolites (Fig. 7).

**Discussion**

*S. aureus* is frequently found in the respiratory tract, human nose, and on the skin, so that it has been regarded as the most common cause of nosocomial infections (Archer 1998). However, it has been reported that the growth of *S. aureus* could be inhibited by the extracts of chinaberry leaves, barks, fruits, flowers, essential oils, and seeds (Kaneria et al. 2009; Liu et al. 2010; Kharkwal et al. 2015; Zulqarnain et al. 2015; Hadadi et al. 2020). Moreover, ordinary water was used as a solvent to extract active physiochemical ingredients from chinaberry. The antibacterial activity of chinaberry leaves was evaluated using an agar well diffusion method by measuring the inhibitory diameter of bacterial growth zones with 25, 50, and 75 µl of aqueous and solvent leaf extracts. The results indicated that the growth of *S. aureus* was inhibited by phytochemical compounds of aqueous extracts of chinaberry leaves, which is better than that extracted by chloroform in the three concentrations tested (Suresh et al. 2008).

In this study, we found that the crude aqueous extracts of chinaberry fruits inhibited *S. aureus* planktonic growth in a dose-dependent manner (Fig. 1A), although only water was used to extract the bioactive agents from the chinaberry fruits. Similar assays were also conducted by Sen and Batra (2012). However, they found that the antimicrobial activity of the aqueous extracts of chinaberry leaves to *S. aureus* exhibited

| No. | Compound_ID | Name | Formula | Molecular Weight | Retention Time (min) |
|-----|-------------|------|---------|------------------|----------------------|
| 1   | Com_2_neg   | D-Saccharic acid | C6H12O6 | 210.0372         | 1.174                |
| 2   | Com_6_neg   | Palmitic acid   | C16H32O2 | 256.2397         | 14.6                 |
| 3   | Com_4_neg   | Citric acid     | C3H6O3 | 192.0267         | 1.214                |
| 4   | Com_11_neg  | N-Acetyleneuraminic acid | C13H21N5O11 | 309.1055 | 1.296                |
| 5   | Com_17_neg  | Elaidic acid    | C17H30O2 | 282.2553         | 14.704               |
| 6   | Com_15_neg  | Sucrose         | C12H22O11 | 402.137 | 1.39                 |
| 7   | Com_22_neg  | D(-)-L-lyxose   | C6H12O5 | 150.0526         | 1.268                |
| 8   | Com_21_neg  | Gluconic acid   | C8H8O4 | 196.058         | 1.256                |
| 9   | Com_29_neg  | DL-Malic acid   | C4H4O4 | 134.0214         | 1.186                |
| 10  | Com_23_neg  | 4-Oxoprolin     | C9H9NO3 | 83.03709 | 1.293                |
| 11  | Com_31_neg  | Pyruvic acid    | C4H4O4 | 88.01595         | 1.186                |
| 12  | Com_25_neg  | D(-)-Fructose   | C6H12O6 | 180.0632         | 1.307                |
| 13  | Com_48_neg  | Glutaconic acid | C7H14O4 | 130.0265         | 1.172                |
| 14  | Com_56_neg  | 2-Furoic acid   | C4H4O4 | 112.016         | 1.155                |
| 15  | Com_47_neg  | Toosendanin     | C20H18O11 | 574.2414 | 11.083               |
| 16  | Com_57_neg  | α-Eleostearic acid | C33H56O12 | 278.2241 | 14.157               |
| 17  | Com_50_neg  | α-Lactose       | C6H10O5 | 388.1213        | 1.373                |
| 18  | Com_71_neg  | Nomilin         | C6H10O4 | 514.2193        | 10.413               |
| 19  | Com_54_neg  | 4-Acetamidobutanoic acid | C7H12O4N2 | 145.0739 | 1.439                |
| 20  | Com_59_neg  | D(+)-Glucose    | C6H12O6 | 180.0632         | 1.484                |
| 21  | Com_70_neg  | 6-Sialyllactose | C21H30NO12 | 633.2127 | 1.319                |
| 22  | Com_78_neg  | Purine          | C3H6N2 | 120.0422        | 1.274                |
| 23  | Com_77_neg  | 2-C-methyl D-erythritol 4-phosphate | C7H12O5P | 216.0399 | 1.27               |
| 24  | Com_72_neg  | Azelaic acid    | C9H8O4 | 188.1047        | 5.304                |
| 25  | Com_81_neg  | 16-Hydroxyhexadecanoic acid | C30H56O2 | 254.2242 | 14.26               |
comparatively lower efficiencies than those extracted by ethanol based on the agar well diffusion method or the minimum inhibitory concentrations. In addition, the petrol, benzene, ethyl acetate, methanol, and aqueous extractions of the mature seeds of chinaberry demonstrated inhibitory activities on *S. aureus* growth depending on the concentrations of the extracts, and it was also found that the most effective crude extract was...
not aqueous but ethyl acetate (Khan et al. 2011). The above findings, including ours, suggest that the anti-
*S. aureus* efficiencies of extracts vary with the extracted parts of chinaberry. In other words, different parts of
chinaberry may possess different bioactive agents that inhibit *S. aureus*.

Furthermore, *S. aureus* biofilms are cellular communities encased by self-produced three-dimensional
polymers of hydrated extracellular polymeric substances (EPS) consisting primarily of water, polysaccharides, nucleic acids, proteins, and lipids (Flemming and Wingender 2010). It has been proven that biofilm-associated *S. aureus* is the pathogen of many human diseases, including osteomyelitis, endocarditis, and other infections (Götz 2002). Moreover, the construction of *S. aureus* biofilms increases its resistance to antimicrobial agents and poses a severe burden in healthcare settings (Suresh et al. 2019). Many studies have primarily focused on exploiting novel therapeutic strategies or approaches to eradicate infections associated with *S. aureus* biofilms (Lister and Horswill 2014; Bhattacharya et al. 2015; Abreu et al. 2016). In this study, we found that planktonic cells and the biofilms of *S. aureus* could be inhibited by aqueous extracts of chinaberry fruits (Fig. 1A and 2), which predicts a function for the aqueous extracts of these fruits to control *S. aureus* infections. Moreover, there is a consistent decrease in the trends of planktonic growth and biofilm formation of *S. aureus* in the presence of different concentrations of aqueous extracts of chinaberry fruits. Thus, we hypothesized that the decrease in *S. aureus*
biofilm formation in the presence of aqueous extracts of chinaberry fruits might be induced by the decreases in planktonic growth (Fig. 1).

Subsequently, we sought to determine how the aqueous extracts of chinaberry fruits inhibit the growth of *S. aureus*. Therefore, the transcriptome of *S. aureus* exposed to aqueous extracts of chinaberry fruits was explored using the RNA-Seq method. The results indicated that the genes of the ribosome (paef03010), peptidoglycan biosynthesis (paef00550), DNA replication (paef03030), and phenylalanine, tyrosine, and tryptophan biosynthesis (paef00400), which are crucial for *S. aureus* protein synthesis, metabolism, and survival, were primarily inhibited by the aqueous extracts of chinaberry fruits (Fig. 3 and 4), which partially explained the mode of action of these extracts on the growth of *S. aureus*.

In addition, the crude extracts from chinaberry were evaluated for their antimicrobial efficiencies. A substantial number of active components were also separated and identified from different chinaberry parts, such as leaves, bark, fruit, and seeds. A substantial number of compounds extracted by methanol were found using GC-MS in chinaberry leaves, and the results demonstrated that the extracts contained trichloromethane, 2-pyrrolidinyl-methylamine, propanedioic acid, diethyl ester, and 2-piperidimethanamine, among others.
(Al-Marzoqi et al. 2015). The ethanol extracts of chinaberry leaves exhibited significant antimicrobial activity to several bacteria, including *S. aureus*. Further isolation and identification using TLC chromatograms and GC/MS demonstrated that the compound 5,6,7,8-tetrahydro-1,2,4-benzotriazine-3-amine might be responsible for the antimicrobial activity (Kathiresan et al. 2019). A GC-MS analysis of the hexane extracts of chinaberry leaves showed a highly complex profile that contained ketones, fatty acid derivatives, ethers, 1,3-dipalmitate, methyl esters, 7,8-dihydrocaptopester, and 2-undecanol (Habib et al. 2017). The methanolic fraction of the aerial parts of chinaberry contained steroids, anthraquinones, phenolics, flavonoids, and tannins (Malar et al. 2020). The metabolites of chinaberry flowers included branched and n-hydrocarbons, fatty acids, aromatics, fatty acid methyl esters, polyisoprenoids, and fatty alcohols (Muhammad et al. 2015). Taken together, there are various compounds extracted from various parts of chinaberry with different solvents. The extracted methods and solvents also simultaneously influenced the constituents of chinaberry extracts.

Herein, we also sought to explore the bioactive agents in the aqueous extracts of chinaberry fruits extracted in this study. Using UHPLC-MS/MS analyses, we found that the aqueous extracts from chinaberry fruits contain lipids and lipid-like molecules, organic acids and derivatives, phenylpropanoids and polyketides, organoheterocyclic compounds, and benzenoids (Fig. 5), which play an important role in inhibiting planktonic growth and biofilm formation (Fig. 1 and 2). In particular, the water extractions of chinaberry fruits also contained a substantial proportion of toosendanin, which is a potential treatment for parasites in the digestive tract and agricultural insecticides (Shi and Li 2007). It also exhibited significant inhibitory effects on the growth of *S. aureus* (data not shown). In contrast, the major components extracted from chinaberry fruits with hexane were methyl palmitate (18.8%), methyl linolenate (16.1%), and methyl linoleate (9.8%) (Hadiakhoondi et al. 2006). A sequential extraction from chinaberry fruits was performed in four steps with scCO₂, a scCO₂/ethanol mixture, ethanol, and an ethanol/water mixture. As a result, linoleic, palmitic, and myristic fatty acids were identified in the supercritical extracts; moreover, caffeic, and malic acid were found in other extracts (Bitencourt et al. 2014).

This study demonstrated that the active components from chinaberry fruits, which inhibited the planktonic growth and biofilm formations of *S. aureus*, could be extracted using water with ultrasonic and boiling methods that are environmentally friendly. The main active components in the aqueous extracts of chinaberry fruits are lipids and lipid-like molecules, organic acids and derivatives, phenylpropanoids, polyketides, organoheterocyclic compounds, and benzenoids, which affect the gene expression of protein synthesis, metabolism, and transcriptional regulation at various levels. The results obtained in this study may help clarify the mode of action of aqueous chinaberry extracts on the growth of *S. aureus* and facilitate the further development and utilization of these extracts.

**Supporting information**

The detailed information, including the total ion intensity of the identified metabolites in the aqueous extracts of *M. azedarach* fruits, are available in Supplementary Excel S1 (positive mode) and S2 (negative mode).

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**Conflict of interest**

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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Supplementary materials are available on the journal’s website.