Abstract: Oranges and mandarins in Chongqing, China, are mostly processed for juice and their peels are wasted or landfilled. To add value, it is essential to utilize the peels and extract useful materials, such as citrus essential oils (CEOs). Here, we report the metabolome analysis of the peels of *Citrus x sinensis* (CS) and *Citrus reticulata* (CR). In total, 793 metabolites were detected in the CS and CR peels, of which 242 were differentially accumulated. The metabolites were grouped into 12 compound classes. Terpenoids were the highest accumulated class of compounds followed by phenols and alcohols in both fruit peels. CR peels were rich in all types of compounds, whereas CS showed a relatively lower content of the detected compounds. The highest accumulated compounds were β-phellandrene, beta-ocimene, 1,3,6-octatriene,3,7-dimethyl, and D-limonene. Overall, CR showed higher compound diversity than CS. The CS and CR peel extracts showed antibacterial effects against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*. The peel extracts from CR and CS showed similar antibacterial effects against *E. coli* and *B. subtilis*, while CS peel extracts were more effective against *S. aureus*. Overall, our study concludes that both CS and CR peels should not be wasted owing to the CEOs and respective antibacterial activities.

Keywords: antibacterial activity; blood orange; citrus essential oil; mandarin peel; peel extract; terpenoids

1. Introduction

China’s citrus production is projected to reach 7.6 million tons by 2021–2022. Both production and consumption have been trending higher for more than two decades on growth from China, European Union, Morocco, and Turkey [1]. Among the major citrus types, blood orange (*Citrus x sinensis* L. Osbeck) is majorly cultivated in Chongqing, China. Whereas the mandarin, i.e., *Citrus reticulata* Blanco is increasingly popular with global consumers due to its simple-to-peel characteristic. China is the largest *C. reticulata* producer followed by European Union, Turkey, and Japan [1]. It is majorly produced in Guangdong province as well as Chongqing municipality, China. Due to its rich nutritional value and unique taste, it is popular among Chinese consumers [2]. As with other citrus species and varieties, both *C. x sinensis* and *C. reticulata* are powerful sources of vitamin C and natural antioxidants [3]. Nevertheless, the vitamin C content of mandarins is different (lower) than oranges [4]. Their juice contains higher quantities of polyphenols, flavonones, anthocyanins, hydroxyxycinnamic acids, alcohols, and other health-beneficial compounds [5], which offer enormous biological potential in the pharmaceutical and food industries [6].

For the detailed understanding of the applications of CEOs in food industry, readers are referred to a detailed review by Bora, et al. [7]. With the increase in consumer consciousness of the nutritional value of the fruit flesh as well as peel, the preference for natural food (and ingredients) is increasing. This has also been witnessed in the citrus essential oil market, which it is expected to grow to USD 6.31 billion by 2022 and 8.49 billion USD by 2027. This means that an overall 6.9% record growth is estimated (www.markedataforecast.com; accessed on 28 June 2022).
The increased demand for citrus essential oils (CEOs) is due to their functional properties as they are widely used as naturally fruity perfumes, flavoring agents, pharmaceuticals, and cosmetic products [8]. US Food and Drug Administration (FDA) has recognized the CEOs from most Citrus species as “generally recognized as safe (GRAS) food additive”. Additionally, CEOs offer broad-spectrum antibacterial, antifungal, and insecticidal properties [9,10]. These CEOs are composed of mixtures of the derivatives of terpenoids, non-terpenoids, and complex hydrocarbons; the derivatives consist of functional groups, including aldehydes, alcohols, ketones, esters, and organic acids [11]. The composition varies among the citrus species and varieties depending on various factors, i.e., genetic background, season, geographic location of growth, and stage of the harvested fruit [12]. However, studies have shown that major components of the CEOs are volatile compounds (containing mixtures of terpenoids and their derivatives). These volatile compounds can account for up to 85–99% of the CEOs. Most of the studies that explored C. x sinensis peels’ CEOs have reported the presence of limonene diepoxide and a large group of monoterpenoids; its content may be as high as 97% or as low as 60%. In addition to limonene, α-/β-pinene, myrcene, terpinolene, aldehyde C8, citronellol, and linalool have been reported as the main components of C. x sinensis CEO [13]. Since C. x sinensis is grown in different regions of the world, a variation in % composition of the CEO components has been reported. For example, C. x sinensis peels from Uganda and Rwanda showed the presence of unique compounds in each location [14]. Similar reports on the presence of common and unique compounds from Turkey [15], Tunisia [16], South Africa [17], Kenya [18], Mexico, and the United States of America [19]. These studies indicate that there is always need of exploration of CEOs from different growing area. Apart from area of cultivation, the content is also affected by the extraction conditions and technique [20]. These compounds are often present exclusively in C. x sinensis peels [21]. Apart from the peels, multiple studies have explored the CEO composition of C. x sinensis rind, flower, and leaf. In these organs, limonin is not the major CEO. Contrarily, linalool, β-myrcene, β-citronellol, or sabinene have been reported as major compounds in flowers and leaves [22,23]. Similar to C. x sinensis composition, there are studies that explored C. reticulata, Citrus aurantium, Citrus limon, and Citrus deliciosa Ten. CEO composition [2,24–26]. The major components in C. reticulata (from India) are limonene, geranial, neral, geranyl acetate, geraniol, β-caryophyllene, and citronellal [27]. Similarly, other studies on this species indicated that hydrocarbons were the major CEO components in peels and γ-terpinene, α-pinene, linalool, myrcene, and sabinene were common in multiple samples from France [28]. A variation in CEO composition has also been reported in other organs of the C. reticulata, i.e., flowers, leaves, and rind [15,29,30]. Regardless of the exclusive presences, studies have also reported similarities between C. x sinensis, C. reticulata, and other Citrus species [29–31].

Research on CEOs have proven that these compounds have biological activities that can be used on different scales, such as in food and health industries. These types of activities are characteristic of the compounds that CEOs are composed off, e.g., terpenes, alcohols, esters, epoxides, aldehydes, ketones, amines, and sulfides [32]. Both major and minor components in CEOs contribute towards these types of activities and neither can be ignored. The antioxidant activities of the CEOs can also be enhanced by using essential oils from other plant species, e.g., cinnamon and clove [33]. In addition to the antioxidants and antibiotic activities in food, the essential oils from some Citrus species, e.g., C. limon, have potency to control fungal diseases in plants. For example, C. limon essential oils have proven to be potent against Formitiporia mediterranea, Botryosphaeria dothidea, and Eutypa sp. in grapevine wood [34]. It is important to note that the extent of antibacterial tests on a wide range of bacterial strains through different testing methods is greater than for antifungal and insecticidal effects [35]. The CEOs affect the cell membranes of the bacteria and fungi [36]. Additionally, the plant based essential oils, including CEOs, may cause interruptions in the ion transport processes; interact with compounds within the hosts’ cells or the proteins within the cellular membranes [37], disturbing active sites of the enzymes [38]; and disrupt the mitochondrial functions in different ways [39]. Early research on C. x sinensis has also
shown that essential oils from different varieties and cultivars offer antifungal or antibacterial activities. For example, *C. x sinensis* var. Valencia can inhibit *Aspergillus flavus* [40], *Aspergillus niger*, *Penicillium chrysogenum*, and *Penicillium verrucosum* [41] and many other fungal species. Similarly, essential oil extracts for different varieties/cultivars of *C. x sinensis* have been proven to inhibit *Lactobacillus curvatus* L. sakei, *Staphylococcus carnosus*, *S. xylosus*, *S. aureus*, and *B. subtilis*, *E. coli* [42]. However, it is always necessary to explore more and more varieties growing in different geographies to identify the composition and yields of CEOs. Since China ranks second and first in terms of fresh oranges production and domestic consumption; therefore, the adjustment of the citrus industry in the country in general and in Chongqing specifically requires the utilization of the waste products. Currently, the postharvest processing and storage of blood oranges in Chongqing mainly focuses on fresh juice and canning. This process produces a large amount of peel that is wasted/landfilled as processed waste. In order to use the blood orange and mandarin peels effectively in essential oil industry, we conducted this study and determined the peel extract composition of both Citrus species growing in their natural conditions. Furthermore, we also determined the antibacterial activity of the peel extracts. This study will be helpful to extend the citrus industry chain in Chongqing in specific and in other parts of the world (that grow *C. x sinensis* and *C. reticulata*) in general and will increase added value of the citrus products.

2. Material and Methods

2.1. Plant Materials and Overview of the Test Site

Two *Citrus* species, i.e., *Citrus x sinensis* L. Osbeck ‘Tarocco’ (CS) (also locally known as “rose orange” and “blood orange”) and *Citrus reticulata* Blanco (CR), a mandarin, were used as plant material (Figure 1).

![Figure 1](image-url)  
Figure 1. Experimental site location and plant material. (a) Chongqing (highlighted red) on the mainland China’s map. (b) *Citrus reticulata* Blanco (CR), and (c) *Citrus x sinensis* L. Osbeck ‘Tarocco’ (CS). The Chongqing map was downloaded from Wikipedia (https://en.wikipedia.org/; accessed on 18 August 2022) under CC BY-SA 3.0 (https://creativecommons.org/licenses/by-sa/3.0/; accessed on 18 August 2022).

The CS originated in Italy and was introduced in China in 1992 through the efforts of the Chinese Academy of Agricultural Sciences. The rootstock for CS is *Citrus trifoliata*.

The CR is a unique citrus germplasm resource in China (mainly in Wanzhou, Chongqing) with a history of >4000 years. The sample trees of both varieties are growing at the Citrus Production Base in Ganning National Park, Wanzhou District, Chongqing city, China.

The experimental site is located in the eastern edge of the Sichuan Basin in China, Wanzhou District, Chongqing City, 30°24′ N and 107°55′ E (Figure 1). Since the area is adjacent to the Yangtze River, the climate is a typical subtropical humid monsoon, with four seasons and long summers, during which the climate is warm. The average annual temperature and precipitation is 18.2–19.8 °C and 1243 mm, respectively.
Chongqing, the place of production, is the core area of the “predominant citrus industry belt in the middle and lower reaches of the Yangtze River”. The Three Gorges Reservoir area has the advantageous geographical conditions and unique natural climate (see above). The long frost-free winters make this area advantageous for Citrus as farmers do not face damage from freezing. It is one of the three major late-ripening citrus producing areas in China. The main diseases and insect pests of C. x sinensis and C. reticulata during the growing period are red spider, aphids, sputum scales, red wax scales, blowing cotton scales, anthracnose, and foot rot. Strict comprehensive prevention and control was carried out for the selected plants to ensure insect, pest, and disease-free trees (and fruits).

2.2. Sample Preparation

The CR and CS peels were removed, 500 g of each peel were weighed, and the albedo was removed. The samples were soaked in the ascorbic acid solution for 30 min, rinsed with distilled water, dried in an oven at 45 °C, pulverized with a juicer, and ultrasonicated (100 W) for 40 min. The samples were then put into a round-bottomed flask and added distilled water (1:2 w/v). The flask was then placed on an electric stove and heated until it boiled (boiling point) for 20 min. The distilled essential oil mixture was separated from water and oil with a separating funnel. After separation, the obtained essential oil was dried by adding anhydrous sodium sulfate, placed in a brown glass bottle, and sealed in a refrigerator at 4 °C. Five replicates of each peel were processed individually; CS1-5 and CR1-5 [43,44].

2.3. Volatile Metabolome Detection

The stored samples were removed, thawed on ice, and 10 µL was diluted to 1000 times with n-hexane. The dilutions were vortexed to mix them evenly. Weigh about 0.1 mL of each sample in a 20 mL headspace vial (Agilent, Palo Alto, CA, USA), and add 10 µL (50 µg/mL) internal standard solution. The vials were sealed with crimp-top caps using TFE-silicon headspace septa (Agilent). Prior to SPME analysis, the vials were placed individually at 60 °C for five minutes, followed by exposing a 120 µm DVB/CWR/PDMS fiber (Agilent) to the headspace of the sample for 15 min at 60 °C [45].

2.4. GC–MS Conditions

The GC–MS analysis of the samples were performed by using a method described in [46] with slight modifications. Briefly, the desorption of the volatile organic compounds (VOCs) from the fiber coating was carried out in the injection port of the GC apparatus (Model 8890, Agilent). The temperature was maintained at 250 °C for five minutes and the mode was splitless. We used the Agilent mass spectrometer (Model 8890 GC and 7000D), which was equipped with a 30 m × 0.25 µm DB-5MS (5% phenyl-polymethylsiloxane) capillary column. The carrier gas was Helium, and the linear velocity was 1.2 mL/min. The injector and detector temperatures were kept at 250 °C and 280 °C, respectively. The oven temperature was programmed from 40 °C (3.5 min), which was then increased by 10 °C/min until 100 °C, by 7 °C/min to 180 °C, and by 25 °C/min to 280 °C, then held for 5 min. Mass spectra were recorded in the electron impact (EI) ionization mode at 70 eV. The quadrupole mass detector, ion source, and transfer line temperatures were set at 150 °C, 230 °C, and 280 °C, respectively. The selected ion monitoring (SIM) mode was used for the identification and quantification of analytes.

2.5. Statistical Analyses

Unsupervised principal component analysis (PCA) was performed by using prcomp in R. However, the data was unit variance scaled prior to the PCA analysis [47]. The hierarchical cluster analysis and Pearson correlation coefficient (PCC) analyses were performed in R package (version 2.11) using ComplexHeatmap [48] and cor function [49], respectively. The differentially accumulated metabolites (DAMs) were determined by variable importance in projection (VIP) ≥ 1 and absolute log2 foldchange (|log2FC| ≥ 1). The VIP scores were
taken from the orthogonal projections to latent structures discriminant analysis (OPLS-DA). The OPLS-DA was generated in the R package MetaboAnalystR by using OPLSR.Anal function [50]. The data was log-transformed (log$_2$) and mean-centered before OPLS-DA. To avoid overfitting, a permutation test (200 permutations) was performed [50].

The identified metabolites were annotated in the KEGG compound database (http://www.kegg.jp/kegg/compound/; accessed on 28 June 2022) [51], followed by their mapping onto the KEGG pathways (http://www.kegg.jp/kegg/pathway.html; accessed on 28 June 2022) [52]. The pathways to which metabolites were significantly enriched were fed into MSEA (metabolite sets enrichment analysis) and their significance was determined by the hypergeometric test’s $p$-values [53].

2.6. Antibacterial Testing

The activity of the CS and CR essential oil samples was tested against three bacteria, i.e., Escherichia coli (ATCC25922), Staphylococcus aureus (CMCC (B) 26003), and Bacillus subtilis (ATCC6633). The stock cultures of the three strains were maintained in LB broth at 4 °C. Prior to use, the pathogens were cultured in LB broth for 24 h at 37 °C. The bacterial cultures were prepared by the palate-coating method and then placed (upside down) in an incubator (HWS-80B constant temperature and humidity incubator: Tianjin Hongnuo Instrument Co., Ltd., Tianjin, China) at 37 °C for 24 h. The inhibition zone was determined by the filter paper method. Sterile filter paper discs (Whatman No. 40; 6.0 mm in diameter, Merck, Rahway, NJ, USA) were placed on the three types of bacteria-containing plates (1 filter paper disc per plate). An amount of 10 µL of the essential oil stock solution was dropped onto each filter paper. The experiment was conducted in three replicates for each strain and essential oil. An amount of 10 µL of double-distilled water was used as the control group (blank groups). The treated plates were incubated in an incubator at 37 °C for 24 h. Pictures were taken after 24 h and the diameter of the inhibition zone was measured for each replicate of each treatment. The means and standard deviation of the three replicates were computed in Microsoft Excel 2019® (Microsoft, Redmond, Washington, DC, USA).

3. Results

3.1. Volatile Organic Compound Composition of C. x sinensis and C. reticulata

The GC–MS analysis of 10 samples (five replicates of each species’ peel) resulted in the identification of 793 metabolites (Supplementary Table S1). Of these, 242 metabolites were differentially accumulated between the two fruits’ peels (Supplementary Table S1); 133 metabolites were accumulated in higher quantities in CR as compared to CS, whereas 109 had higher accumulation in CS. These metabolites were able to be grouped into 16 chemical families (Figure 2a). To observe the degree of variation between the grouped samples for difference comparison, a PCA plot was developed. The PCA plot showed that PC1 and PC2 explained 48.7% and 29.7% variation, respectively. Furthermore, the replicates of the same fruit peel were grouped together, showing that the sampling and replication were reliable (Figure 2b). Further differences between the group were explored by analyzing the metabolome data according to the OPLS-DA model. The OPLS-DA model confirmed the PCA results (Figure S1a). The reliability of the OPLS-DA model was verified as evident from the following parameters, i.e., $Q^2 = 0.99$, $R^2Y = 0.99$, and $R^2X = 0.734$ when $p < 0.005$ (Figure S1b). The highest summed content of the detected metabolites was of terpenoids followed by phenols, alcohols, esters, and heterocyclic compounds (Figure 2c), indicating that the studied Citrus species are rich in terpenoids.
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**Figure 2.** (a) Sample population cluster plot. The $x$-axis represents sample names, the $y$-axis represents the metabolite information, i.e., fruit type and the metabolite primary classes. (b) Grouped PCA plot. (c) Comparison of different compound classes accumulated in CS and CR. Where CS and CR represent *C. x sinensis* and *C. reticulata*, respectively.

### 3.2. Differential Volatile Organic Compound Accumulation between *C. x sinensis* and *C. reticulata*

We screened the differentially accumulated metabolites between CS and CR based on the criteria if the metabolites had VIP $\geq 1$ and fold change $\geq 2$ and $\leq 0.5$. As reported above, 133 and 109 metabolites were up-accumulated in CR and CS, respectively (Figure 3a). The top 10 highly accumulated metabolites in CR were classified as terpenoids, alcohols, aldehyde, heterocyclic compounds, esters, and aromatics. It is important to note that these metabolites were exclusively accumulated in CR. On the other hand, the top 10 highly accumulated metabolites in CS were classified as terpenoids (six compounds), esters (two), and phenols (two) (Figure 3b). These observations indicate that CR peels contain a relatively...
diverse set of metabolites as compared to CS. We observed that within the peels of each fruit peel type, the replicates showed high similarity, which again confirmed the PCA and OPLS-DA results (Figure 3c). This could be further observed as the differential metabolites showed a variable (−0.8 to 0.8) correlation between each other (Figure 3d).

Of the metabolites, 36 could be enriched to different KEGG pathways; all of these were related to metabolism. The highest number of the DAMs were associated with the metabolic pathways (12) followed by biosynthesis of secondary metabolites (7), and sesquiterpenoid and terpenoid biosynthesis (3) (Figure 4). It is interesting to know that the highest number of compounds could be annotated as terpenoids (73). The top accumulated compound (%) in CS was terpenoid (Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-9-methylene-) followed by epizonarene. Overall, among the top 10 (% content) accumulated compounds only two were classified as aromatics ((3-Bromo-1-methylpropoxymethyl)benzene) and ester (Benzyl tiglate). On the contrary, in CR the top (% content) accumulated compound was a terpenoid (Phenol, 2-methyl-5-(1-methylethyl)-), which was followed by phenol (Phenol, m-tert-butyl-) (Supplementary Table S1). These observations suggest that both examined fruits’ peels accumulated different quantities of metabolites and both should be individually important when considered for the extraction of CEOs. The
differential metabolomic profile observed here could be due to their species-specific genetic background.

Of the five acids, the hydrocinnamic acid and ayristoleic acid were present in higher quantities in CS as compared to CR, whereas the benzoic acid, benzenoaecetic acid, and myristoleic acid were highly accumulated in CR. A similar trend of accumulation was noted for alcohols, i.e., a higher number of alcohols were up-accumulated in CR as compared to CS. (2E,4S,7E)-4-Isopropyl-1,7-dimethylcyclohepta-2,7-dienol was unique to CR, whereas p-Cymen-7-ol was the most accumulated alcohol in CR. The four alcohols that were up-accumulated in CS are 1-Decanol, n-Tridecan-1-ol, 1,2-Benzenedimethanol, and 2-Benzylidene-1-heptanol (Figure 4). The observed differences could be due to species-specific genetics and/or different ripening times.

Six of the eighteen aldehydes were up-accumulated in CS as compared to CR. Among the 18 differentially accumulated aldehydes, 4-MethoxycinnamAldehyde was unique to CR. The second most up-accumulated compound in CR was 2,4-Decadienal, (E,E). This compound has a fatty flavor (orange-like, fatty, fried chicken-like odor, etc.) as reported in the TGSC Information System; http://www.thegoodscentscompany.com/data/rw1028121.html#torefr; accessed on 28 June 2022). Other major aldehydes that were up-accumulated in CR are trans-4-MethoxycinnamAldehyde, 4-Isopropylcyclohexa-1,3-diene carbAldehyde, and BenzAldehyde, 3-methyl- (Figure 4).

Interestingly, four of the five amines were accumulated in higher quantities in CR as compared to CS; those are benzamide, p-isopropoxylaniline, benzenamine, 2,6-diethyl-, and acetamide, N-phenyl-. The only up-accumulated amine in CS is BenzenAmine, 2,4-dimethyl- (Figure 4).

There were 11 differentially accumulated aromatics between the two Citrus types, i.e., CS and CR; ten of which were benzene-derived. The five aromatics that were present in

![Figure 4. Heatmaps of the log2 foldchange values of acids, alcohols, aromatics, aldehydes, and amines that were differentially accumulated in C. x sinensis peels vs. C. reticulata peels. The colored bars on the right side of the heatmaps represent compound classes.](image-url)
higher quantities in CS are 1,2,3-trimethoxy-5-(2-propenyl)-Benzene, Benzene, 2-methyl-1,4-bis(1-methylethyl)-, (3-Bromo-1-methylpropoxymethyl)benzene, Butylated Hydroxytoluene, and Benzene, 1,4-dimethyl-2,5-bis(1-methylethyl)-. Interestingly, styrene accumulated in CR in higher quantities as compared to CS (Figure 4).

We also found a large number of non-terpenoid esters (53) that were differentially accumulated between CS and CR; 31 were present in higher quantities in CR as compared to CS. This indicates that CR is rich in esters. Two esters, i.e., propanoic acid, 2-methyl-, octyl ester, and triacetin were exclusively accumulated in CR. Isobornyl formate was the most accumulated ester in CR. On the contrary, benzyl tiglate was the most accumulated metabolite in CS. Overall, CR peels showed higher total ester contents as compared to CS peels (Figure 5; Supplementary Table S1).

Figure 5. Heatmaps of the log2 foldchange values of esters, hydrocarbons, heterocyclic compounds, ketones, phenols, sulfur compounds, and terpenoids that were differentially accumulated in C. x sinensis peels vs. C. reticulata peels. The colored bars on the right side of the heatmaps represent compound classes.

Twenty-six heterocyclic compounds were differentially accumulated between CS and CR. Two of these, i.e., Isoquinoline, 1-butyl-3,4-dihydro- and Pyrazine, 2,5-dimethyl-3-(3-methylbutyl)- were exclusively accumulated in CR. Other than these two, 7(1H)-Pteridinone was the most up-accumulated compound in CR as compared to CS. Almost half of the DAMs (12) showed reduced accumulation in CR as compared to CS. Debrisoquine was the most down-accumulated compound in CR as compared to CS. These observations indicate that CR peel can be a better source of heterocyclic when compared with CS as obvious from sum of the concentrations of all the heterocyclic compounds. However, both CS and CRs peels can be utilized as a source of heterocyclic compounds. Furthermore, the exclusive presence of isoquinoline and pyrazine indicates CR a relatively better source of these compounds (Figure 5).

Hydrocarbons were present in both CS and CR and three of six (adamantane, 1-tridecyne, and cyclohexene, 6-(2-butenylidene)-1,5,5-trimethyl-, (E,Z)-) were up-accumulated
in CR as compared to CS. On the contrary, 1-nonen-1, 1,4,6,8-trimethyl-, dodecan-3-methyl-, and 8-hexadecyne were present in higher quantities in CS as compared to CR. These observations show that hydrocarbons diversity in Citrus CS and CR types is limited (Figure 3).

The highest accumulated ketone in CR (+2.67-fold), as compared to CS, was cyclohexanone, 2-(1-mercapto-1-methylethyl)-5-methyl-, (2S-trans). We observed that 2′-Ethoxyacetophenone contents significantly increased in CR as compared to CS, i.e., 79.3-fold. Overall, the ketone accumulation trend was same as of other metabolites, i.e., higher in CR as compared to CS. Two phenols, i.e., acetaminophen and phenol, 2-methoxy-4-(1-propenyl)- were only detected in CR, whereas other eight were detected in both varieties. The phenols were also present in higher quantities in CR as compared to CS. Similarly, all the three detected sulfur compounds were up-accumulated in CR as compared to CS (Figure 5).

Among the terpenoids, the exclusively accumulated metabolites in CR, i.e., ledol, 1,4-Methanobenzocyclodecene, 1,2,3,4,4a,5,8,9,12,12a-decahydro-, and 4,8-Methanoazulen-9-ol, decahydro-2,2,4,8-tetramethyl-, stereoisomer had shown significantly higher content accumulation. This indicates that CR must be considered a good source of these compounds. Not only because of the accumulation of these terpenoids but the overall higher content in CR regardless of the fact that 42 terpenoids were up-accumulated in CS as compared to CR (Figure 5).

3.3. Antibacterial Activities of CS and CR Peel Extracts against Three Bacterial Strains

An antibacterial test of CS and CR peel extracts against E. coli, S. aureus, and B. subtilis was conducted. The results revealed that both CS and CR peel extracts were significantly effective against E. coli when compared to CK. However, the differences between the CS and CR were not significant. On the contrary, we found that CR peel extract was not significantly effective against S. aureus as compared to CK. However, the CS peel extract was significantly effective against S. aureus. Similarly, there was a significant difference between CS and CR peel extracts regarding their antibacterial activity against S. aureus, where CS peel extract was more effective than CR. As far as the antibacterial activity of the CR and CS peel extracts against B. subtilis are concerned, our results demonstrated that both were significantly effective. However, there was non-significant difference between CR and CS peel extracts for their activity against B. subtilis (Figure 6). Overall, we conclude that the extracts of CR and CS peels are effective against the three tested bacterial strains.

![Figure 6. Antibacterial activities of the C. x sinensis and C. reticulata peels’ essential oil samples against Escherichia coli, Staphylococcus aureus, and Bacillus subtilis. The graphs represent the mean (n = 3) inhibition zone (mm) ± SD (p < 0.05). Where, * and ns indicate that the differences between the means are significant and non-significant, respectively. The black * and ns show differences of the CR and CS with CK (blank group). The blue * and ns show differences between CR and CS.](image-url)
4. Discussion

The CEOs are a by-product of the orange juice industry. Considering the fact that the Chongqing citrus industry produces juices as the major product and the peels are considered as waste and are landfilled, we explored the peel metabolite composition of *C. x sinensis* and *C. reticulata*. This is an attempt to increase the added value of the citrus products in Chongqing specifically and blood orange- and mandarin-producing areas of the world in general.

A large diversity of CEOs was observed in the CS and CR peels (Figure 2). This high diversity is comparable to the previous results from *C. reticulata*, *C. unshiu* [54], and *C. limon* [55] with 913, 76, and 340 compounds detected, respectively. The highest accumulated content of terpenoids in both CS and CR shows that our results are consistent with the earlier reports on multiple *Citrus* species ([3], and references therein). Since the terpenes and terpenoids are the main bioactive compounds of the essential oils, both the CR and CS peels can be regarded as a useful raw material for CEO extraction [56]. Other than terpenoids, the presence of phenols, alcohols, esters, and heterocyclic compounds is also consistent with earlier reports that these compounds are the most frequently reported in the flowers of *C. x sinensis* and clementine mandarins (*C. clementina*) [57] and other *Citrus* fruit rind, flowers, and leaves [3]. Thus, our results indicate that blood orange peels are a good raw material apart from the other plant parts. In most *Citrus* species, D-limonene has been reported to be the most accumulated compound in different tissues, such as flavedo and sacs [58,59]. Although we also detected D-limonene in both CS and CR, it was not differentially accumulated. Furthermore, the highest accumulated compounds were beta-phellandrene, beta-ocimene, and 1,3,6-octatriene,3,7-dimethyl, followed by D-limonene (Supplementary Table S1). However, their insignificant differences in certain metabolites between CS and CR suggests no preference over one another. Nevertheless, the highest content of these four terpenoids and their reported health benefits [60–62] present CS and CR peels as a useful raw material for CEO extraction.

The exclusively accumulated compounds in CR belonged to aromatics, esters, heterocyclic compounds, aldehydes, terpenoids, and alcohols, which suggests that this species could be a better choice than CS (Supplementary Table S1). In addition, the exclusive presence of these compounds should be studied further from the perspective of differentiating the two fruit types since similar attempts have been made for *Citrus* taxonomy [63]. Contrasting to the CR, eight of the ten top accumulated (% content) compounds were terpenoids. This indicates that CS peels have a less diverse compound composition than that of CR. Furthermore, our results propose that CR peels are a better resource for CEO extraction in terms of quantities, as CS had relatively lower concentrations than of most of the detected metabolite classes. It is important to know that the top accumulated terpenoids in CS have also been reported in other plant species, such as *Gymnotheca involucrate*, *Murraya exotica* L., *Partrinia scabiosaefolia*, and some Egyptian medicinal plants [64–67]. Thus, these compounds should be considered widely present in plant kingdom. However, from the raw material’s utility perspective, blood orange peels should be the preferred source of these terpenoids. Furthermore, the differential content differences of aldehydes, amines, aromatics, non-terpenoid esters, heterocyclic compounds, hydrocarbons, ketones, and phenols signify that CR should be a preferred choice for the extraction of CEOs.

According to the earlier reports, CEOs show variable antibacterial activities for different bacterial species. *S. aureus* is the most important food-borne, disease-causing bacteria in humans [68]. Similarly, *E. coli* is a food-borne pathogen and is an indicator of unfavorable hygienic conditions or fecal contamination [69]. Where the third tested bacteria, i.e., *B. subtilis*, has been used as a probiotic [70] to treat multiple diseases in human and animals, e.g., the treatment of the intestinal barrier function, inflammatory response, healthy human longevity, mouth rinsing in periodontitis patients, etc. [71–73]. Thus, understanding how CEOs, especially from blood orange peels, affect these bacterial strains can contribute much to control them as per food standard requirements. The results that the CR and CS peel extracts were significantly effective against *E. coli* and *B. subtilis* are very promising.
Particularly, the significant effectiveness of both peel extracts against *E. coli* is consistent with the earlier reports that lemon, orange, and bergamot essential oils show inhibitory effects [74]. An earlier investigation reported that *C. reticulata* L. essential oil can constrain the growth of *S. aureus* by affecting the permeability and integrity of its cell membrane [75]. The differential activities of the CEOs from XC and CR for the tested bacteria indicate their usability in food sector for controlling these bacteria. These activities can be due to terpenoids. We say this because terpenoids from *G. involucrate*, *M. exotica* L., *P. scabiosaefolia*, and some Egyptian medicinal plants showed the larvicidal activities against *Aedes aegypti*, antifungal properties, anticancer, antineuroinflammatory, and antioxidant activities [64–67]. These activities can also be due to non-terpenoid compounds, e.g., esters. In Mediterranean plants, the isobornyl formate is the main essential oil component that imparts antifungal activity [76]. Benzyl tiglate has also been reported from *Lippia chevalieri* and *Lippia multiflora* from Burkina Faso, which shows antibacterial properties [77]. The exclusive accumulation of 4-MethoxycinnamAldehyde in CR is interesting since this compound has been found as an active constituent of *Agastache rugosa* and inhibited human respiratory syncytial virus when tested in the human larynx carcinoma cell line [78]. Similarly, 2,4-Decadienal, (E,E)-has a fatty flavor (orange-like, fatty, fried chicken-like odor, etc.) and is used as an aroma-active compound in cooked Korean non-aromatic rice [79]. The presence of benzamide(s) as well as acetamide in the blood orange peels suggest that these CEOs may provide better bactericidal effects [80].

With this updated and detailed analysis of CEOs from *C. x sinensis* and *C. reticulata*, futures studies should explore how their contents change under different climates and growing conditions. In addition, the future studies should investigate the key transcriptomic changes that may govern the differential accumulation of CEOs in these two *Citrus* species. Finally, as known from other studies [34], efforts should be extended to further enhance the antibacterial, antifungal, and antioxidant activities of these CEOs by using different approaches.

5. Conclusions

We investigated the essential oil from CS and CR peels. Our results indicated that CEOs belonged to 12 metabolite classes and the terpenoids were the most accumulated compounds in both fruit types. Overall, CR showed higher content of all the compounds as compared to CS. Additionally, CR peel contains a relatively diverse set of metabolites. The presence of aromatics and ester in top 10 accumulated compounds in CS is interesting observation. Based on the results that both examined fruits’ peels accumulated different quantities of metabolites, we conclude that both types of peels have individual importance for the extraction of CEOs, where CR is preferred when considering quantities of the CEOs. The differences in the metabolomic profiles could predominantly be due to their species-specific genetics. The antibacterial test of the peel extracts from CS and CR were effective against *E. coli*, *S. aureus*, and *B. subtilis*. The peel extracts from CR and CS showed similar antibacterial effects against *E. coli* and *B. subtilis*, while CS peel extracts were more effective against *S. aureus*. Overall, our results propose that CR peels are relatively better source of CEOs. However, both CR and CS peels can be used for CEO extraction based on the location of the industry and the species being cultivated nearby. The antibacterial tests results suggest the CEOs from both *C. reticulata* and *C. x sinensis* peels are valuable for the food industry. Future studies should be conducted to explore the feasibility and cost analysis for CEO extractions in light of the local market demand.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8090793/s1, Figure S1: (a) OPLS-DA model-based score chart. Abscissa represents the predicted principal components, and the difference between the groups can be seen in the direction of abscissa. The ordinate represents the orthogonal principal components, and the difference within the group can be seen in the direction of the ordinate. The percentage represents the components of the data. (b) OPLS-DA verification diagram. The abscissa represents the R2Y and Q2 values of the model, and the ordinate is the frequency of the model
classification effect in 200 random permutation and combination experiments. The orange in the figure represents the randomization model R2Y, the purple represents the randomization model Q2, and the values represented by the black arrows represent the R2X, R2Y and Q2 values of the original model; Table S1: List of compounds detected in the Citrus x sinensis and Citrus reticulata peels.

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