



OPEN Molecular networking-based GC/MS profiling of *Citrus japonica* Thunb. peel and pulp lipophilic fractions and their antimicrobial potential against diabetic foot ulcer pathogens

Nermin A. Ragab¹✉, Mona M. Marzouk², Hossam M. El-Masry³, Salwa A. Kwashty² & Reda S. Mohammed¹

Diabetic ulcers are a serious complication of diabetes, often exacerbated by microbial infections that hinder wound healing. This study investigates the antimicrobial potential of non-polar constituents derived from the peel and pulp of *Citrus japonica* (kumquat) as natural antimicrobial agents for managing microbial infections associated with diabetic ulcers. Gas Chromatography-Mass Spectrometry (GC-MS)-based molecular networking enriches the annotation of 113 and 92 compounds in the peel and pulp lipophilic fractions, respectively. Moreover, preliminary in vitro antimicrobial assays demonstrated that the pulp non-polar extract possesses weak to moderate efficacy against common diabetic ulcer pathogens, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans*, with inhibition zone diameters (mm) of 13.67 ± 0.89 , 19.33 ± 0.22 and 20.5 ± 0.17 compared to those of the standard 12.33 ± 0.22 , 19.83 ± 0.39 and 10.71 ± 0.05 as well as MIC values (mg/mL) of 3.75 ± 0.04 , 0.938 ± 0.002 and 1.88 ± 0.001 , respectively. Furthermore, Principal Component Analysis (PCA)-based GC-MS revealed notable variations in the chemical profiles of the peel and pulp lipophilic fractions, with the pulp showing higher concentrations of trimethyl citrate and azelaic acid ester, compounds related to antibacterial and antifungal properties. This finding suggests that non-polar matter, especially from the pulp, could be a promising natural antimicrobial agent for treating diabetic ulcers. Further research is required to assess its efficacy in clinical applications and to explore its mechanisms of action in promoting wound healing and preventing infections.

Keywords Chemometrics, Diabetic foot infections, Fatty acid esters, *Citrus japonica*, Unsaponifiable matters

Diabetic foot ulcers are a serious consequence of poorly controlled diabetes. Globally, 537 million people are estimated to have diabetes, and 19% to 34% are expected to develop a diabetic foot ulcer at some point in their lifetime¹. Previous studies proved that citrus essential oils are effective potential therapies for diabetic complications, including diabetic ulcers, through their antimicrobial activity against pathogenic bacteria and fungi, which are often involved in diabetic ulcer infections^{2,3}. Moreover, citrus extracts and essential oils mitigate diabetic ulcers through their wound healing, antioxidant⁴, and anti-inflammatory effects⁵. *Kumquats* are tiny oval-shaped fruits, belonging to the genus *Citrus* of the family Rutaceae⁵. They are cultivated in four primary species: *Citrus japonica* Thunb., *C. crassifolia* Swingle, *C. margarita* (Lour.) Swingle, and *C. hindsii* (Champ. ex Benth.) Govaerts⁶. *C. japonica* (syn. *Fortunella japonica* Thunb.) is indigenous to South Asia and Asia-Pacific

¹Pharmacognosy Department, Pharmaceutical and Drug Industries Research Institute, National Research Centre, 33 El Bohouth St, P.O. 12622, Cairo 60014618, Egypt. ²Phytochemistry and Plant Systematics Department, Pharmaceutical and Drug Industries Research Institute, National Research Centre, 33 El Bohouth St, P.O. 12622, Cairo 60014618, Egypt. ³Chemistry of Natural and Microbial Products Department, Pharmaceutical and Drug Industries Research Institute, National Research Centre, 33 El Bohouth St, P.O. 12622, Cairo 60014618, Egypt. ✉email: nerminmaherragab@gmail.com

territories⁷ and is becoming more widely grown in South Africa due to its compatibility with subtropical climates. Its cultivation may increase in importance as market demands evolve and local populations seek diverse and healthy food options^{8,9}.

In folk medicine, *C. japonica* has been used to treat coughs, colds, and other respiratory tract inflammation⁵. Unlike the other *Citrus* species, the kumquat fruit is usually eaten whole (peel and pulp altogether), because the composition of its peel essential oil differs from that of other citrus members¹⁰. Despite the various health benefits associated with *C. japonica* in Asian folk medicine, the phytochemical and biological properties remain notably understudied¹¹.

The lipophilic constituents of *C. japonica* exhibit a wide range of functional properties, including aromatic attributes, insect-repellent, and antioxidant effects⁶. Previous studies have suggested that essential oils of *C. japonica* peel possess potent antimicrobial properties at microgram concentrations^{6,12} and shows an inhibitory effect on the proliferation of human prostate cancer cells by stimulating apoptosis and inflammation inhibition¹³. Furthermore, *C. japonica* oils possess effective antibacterial effects against *Escherichia coli*, *Bacillus strains*, *Salmonella typhimurium*, *S. aureus*, and *Lactobacillus bulgaricus*, in addition to antifungal effects against *C. albicans*⁶. From a nutritional perspective, essential oils add an incremental value to *C. japonica* fruit, as they influence flavour attributes and play a crucial role in maintaining human health¹⁴.

However, comparative studies on the lipophilic composition and biological activities of *C. japonica* peel and pulp are scarce. The incomplete understanding of the lipophilic compositions of *C. japonica* fruit and the chemical variability between its tissues (peels and pulp) may stem from limited research attention and the use of inadequate techniques for lipophilic identification in existing studies. Recently, GC-MS has been widely used to investigate bioactive natural compounds due to its sensitivity and effectiveness in quantitative and qualitative analysis¹⁵. Additionally, although research specifically on *C. japonica* lipophilic matter and diabetic ulcers is limited, the antimicrobial and wound-healing properties of citrus-derived compounds such as fatty acids, essential oils, and flavonoids are well established. Further research should focus on isolating and evaluating the lipophilic fractions of *C. japonica* peel and pulp for the treatment of diabetic ulcers. While GC-MS profiles of various *Citrus* species exist, this is the first study to compare peel vs. pulp lipophilic metabolomes of *C. japonica* using GNPS-based molecular networking. Furthermore, this study was designed as a preliminary in vitro screening of these fractions against diabetic-ulcer-relevant pathogens (*P. aeruginosa*, *S. aureus* and *C. albicans*).

Materials and methods

Plant material

C. japonica fruits were purchased from the Agriculture Research Centre (fruit section) (GPS: 30.02189° N, 31.21083° E) and were authenticated by the Fruit Department, Agricultural Research Institute, Fig. S1, National Research Centre (NRC). The peel and pulp were pressed until dry to prepare their voucher specimens (M301 and M302, respectively). They were deposited by Dr Mona M. Marzouk (Co-author) in the CAIRC (the herbarium of NRC).

Preparation of extracts

Individually, the peel and pulp nonpolar fractions of *C. japonica* fruits were extracted at room temperature with ethanol/H₂O (70:30) till exhaustion (4 L × 3 times). Under reduced pressure at 50 °C, the combined extracts evaporated using a rotary evaporator (Heidolph, Germany), yielding the hydroethanol extract of peel and pulp. In a separating funnel, about 10 g of each extract was dissolved in distilled water and repeatedly fractionated with dichloromethane till exhaustion. The collective fractions were filtered and evaporated to yield the dichloromethane fraction of each fruit part: non-polar fraction of peel (NPFPI, 1.5 g) and non-polar fraction of pulp (NPFPP, 0.78 g). Part of the NPFPI and NPFPP (0.5 g) was subjected to GC/MS analysis of the saponifiable and non-saponifiable matter to identify their chemical composition. The other part was biologically screened for its antimicrobial activities against selected microbial strains. All solvents used are of analytical grade ADWIC (Egypt).

Chemical investigation of non-polar fractions

Preparation of saponifiable and unsaponifiable fractions from NPFPI & NPFPP

NPFPI or NPFPP (0.5 gm, each) was refluxed with 0.5 N alcoholic KOH (100 mL, 6 h,) in a boiling water bath. The procedure was conducted as reported by¹⁶ to obtain unsaponifiable fractions (USFPI & USFPP) and fatty acid residues.

Preparation of fatty acid methyl esters

The residues of fatty acids that remained after the saponification step were dissolved separately in methanol (100 mL), mixed with sulphuric acid (0.5 mL), refluxed in a water bath (3 h, 100° C), then cooled, evaporated to dryness. The procedure was completed as reported by¹⁶ to obtain fatty acid ester fractions (FAEPI & FAEPp).

GC-MS analysis

USFPI, USFPP, FAEPI, and FAEPp were subjected separately to GC/MS analysis; GC/MS system (Agilent Technologies) was equipped with gas chromatograph (7890B) and mass spectrometer detector (5977 A) at Central Laboratories Network, National Research Centre, Cairo, Egypt. GC/MS analysis was equipped through the following conditions: fused silica GC capillary column (30 m length, 0.25 µm thickness, and 0.32 mm ID). 5% phenylpolysil phenylene siloxane (TR-5MS) was used as the stationary phase. the carrier gas is Helium (1mL/min, 13 psi). The temperature program is designed to be 60° C isothermal for 3 min, increased to 60–280 °C at a rate of 5° C per minute, and then to 260° C isothermal for 10 min. A mass spectrometer is employed as the detector with an electron ionization (EI) voltage (70 eV) and ion source temperature (200° C)¹⁷. Before each sequence,

the column was baked at 280 °C for 60 min, and a procedural blank was run. The system was considered ready only when the blank showed a flat baseline ($S/N < 3$). The constituents were identified by matching their mass fragmentation spectra and retention times analysed by Gas/ Mass tools with those of the mass spectral libraries: NIST (Nat. Inst. St. Technol., USA), Wiley (Int., USA), and lipid library (<https://lipidlibrary.aocs.org>) as well as of the published data described by Adams 2019¹⁸ with similarity $\geq 80\%$.

GC/MS Molecular Networking

All raw GC-MS data files were converted to the universal .mzML open format using the MSConvert tool (Proteowizard Software, Version 3.0.19330, USA) with centroiding enabled. The converted files were then uploaded to the GNPS platform for processing with MS-Hub. Deconvolution was performed using the MS-Hub machine learning algorithm with the time unit set to minutes. All parameters related to peak symmetry and baseline adjustment were automatically determined internally by MS-Hub. After deconvolution, a spectral library search and molecular networking job was launched directly from the MS-Hub result page. The network was generated using the following GNPS parameters: fragment ion mass tolerance 0.5 Da, minimum matched fragment ions 6, score threshold 0.5, and minimum pairs cosine similarity 0.7. Spectral matching was performed against the GNPS spectral library (version 2023-11-01). Nodes with fewer than three MS/MS spectra or a cosine similarity below 0.7 were filtered out from the final network¹⁹. Two molecular networks (MNs) were established for GC/MS data of the unsaponifiable matters and the fatty acid methyl esters of the lipophilic fractions of *C. japonica* peel and pulp. Molecular networking enriches the annotation by leveraging information from interconnected nodes rather than individual components²⁰. Visualization of the spectral network was conducted using Cytoscape 3.9.1.

Principal component analysis (PCA)

PCA was applied on the feature table obtained from GC- MSHUB, PCA biplot were constructed through JMP®, JMP Statistical Discovery LLC, Cary, NC". A biplot combines loading plots and score plots, as the large databases generated from analytical results require chemometric evaluation. PCA was applied particularly to obtain low-dimensional representations of the data, and the PCA score plots were used to interpret different tissues of *C. japonica* fruits²¹.

Antimicrobial investigation of non-polar fractions

Media and microbial strains

Nutrient agar medium (NA), which comprises (gm/L) yeast extract (2.0), peptone (5.0), meat extract (1.0), NaCl (5.0), and agar (15.0) with final pH value 7.4 ± 0.2 , nutrient broth medium (NB) that has the following components (gm/L): yeast extract (2.0), peptone (5.0), meat extract (1.0), and NaCl (5.0), and final pH value 7.4 ± 0.2 for pathogenic bacterial strains, while the Sabouraud dextrose agar medium which comprises (gm/L) Dextrose (40.0), Peptic Digest of Animal Tissue (5.0), Pancreatic Digest of Casein (5.0), and agar (15.0) with final pH value 5.6 ± 0.2 at 25°C was used for *C. albicans* cultivation, All media components were purchased from Neogen Culture Media Formerly known as Lab M. Heywood, the Metropolitan Borough of Rochdale, Greater Manchester, England". Three standard pathogenic strains were tested in the current study: one Gram-negative bacterium (*P. aeruginosa*; ATCC 27853), one Gram-positive bacterium (*S. aureus*; ATCC 6538), and one pathogenic fungal yeast (*C. albicans*; ATCC 10231), All microbial strains were obtained from the Bio-Evaluation Unit for Natural and Microbial Products, Laboratory no. 174 of the National Research Centre (NRC) through purchase from the American Type Culture Collection (ATCC, Manassas, Virginia, USA).

Procedure

A stock solution and the tested samples (NPFPI and NPFPP) were prepared by dissolving them separately in DMSO to give a concentration of 50.0 mg/mL. The qualitative antimicrobial evaluations of NPFPI and NPFPP took place using the NB medium according to Hafez et al.²². The inocula of pathogenic microbial strains were prepared from fresh overnight broth cultures using NB medium and incubated at 37 °C²³. The sizes of inoculum of the tested pathogenic strains were prepared and adjusted to a 0.5 McFarland standard (1.5×10^8 CFU /mL)^{24,25}. 20.0 µL inoculum size of each microorganism strain was separately inoculated into each plate containing 25.0 mL of the sterile nutrient agar medium. After the media cooled and solidified, the two tested samples were applied to a 0.6 cm well of the inoculated agar plates, which were prepared previously using a 0.6 cm cork borer and the well-diffusion method. Each well was separately filled with 70.0 µL of each sample. Triplicate trials of these samples were used²⁶.

The previously prepared plates were placed for one hour in the refrigerator for more diffusion of these samples, then the plates were incubated at 37 °C for 24 h, and then the inhibition zones (IZ) that appeared were measured in mm²⁷. Negative control: 5% DMSO, Gentamicin 10 mcg; standard antibiotic disc, Fluconazole (50 µg/disc) a standard antifungal disc. The experiment was performed three times independently (biological triplicates), and each independent experiment included triplicate wells for each sample (technical triplicates).

The samples that gave inhibition zones were passed to another antimicrobial test for the determination of minimum inhibitory concentration value (MIC) using Nutrient Broth medium, applying the microdilution broth method^{26,28}.

The micro dilution broth method, take place using micro titer eliza plate, In this method each well of the Eliza plate was filled with 180.0 µL of sterile nutrient broth medium, then by serial dilution technique, each sample was used separately in first well by using the starting volume 100.0 µL that contains 50.0 mg/200 µL, of the used sample as a starting concentration, and by transfer of 100.0 µL from the first well to second well then transfer 100.0 µL from the second to the third, and so on to reach the final well that has sample concentration 0.78 mg of the used sample (6 dilutions were taken place), Then the wells were inoculated with the used tested pathogenic

No.	R_t (min)	Compounds	Area %		BP	MW	Mol. formula
			USFPI	USFPp			
1	7.34	3,3-Diethoxy-1-propanol	0.58	-	75	148	C ₇ H ₁₆ O ₃
2	8.45	Decane	0.002	0.34	57	142	C ₁₀ H ₂₂
3	9.28	Limonene	2.23	0.26	68	136	C ₁₀ H ₁₆
4	9.35	Allyl benzyl ether	0.12	2.4	91	148	C ₁₀ H ₁₂ O
5	9.47	1-Hexanol, 2-ethyl-	20.7	0.46	57	130	C ₈ H ₁₈ O
6	10.26	3,3-Dimethyl-Hexane	0.42	0.3	43/57	114	C ₈ H ₁₈
7	10.68	Linalool oxide	-	0.17	43/94	170	C ₁₀ H ₁₈ O ₂
8	10.86	2-Nonanol	0.16	0.98	41	144	C ₉ H ₂₀ O
9	11.25	n-Undecane	0.09	0.11	71	156	C ₁₁ H ₂₄
10	11.29	4-Methyl-octane	0.46	-	43	128	C ₉ H ₂₀
11	11.53	1-Heptanol, 3-methyl	0.35	-	43	130	C ₈ H ₁₈ O
12	11.53	Pulegone	-	0.27	67/81	152	C ₁₀ H ₁₆ O
13	12.10	2,4-Dimethyl-Heptane	1.16	0.02	57	128	C ₉ H ₂₀
14	12.35	2-Methylhept-6-en-3-one	0.16	3.76	55	126	C ₉ H ₁₄ O
15	12.86	2-Ethyl-4-methyl-1-pentanol	-	0.049	57/43	130	C ₈ H ₁₈ O
16	13.07	n-Dodecane	0.18	0.11	57	170	C ₁₁ H ₂₄
17	13.26	3-Methyl undecane	0.009	0.11	57	170	C ₁₂ H ₂₆
18	13.31	2,2-diethoxy Ethanamine	0.35	1.04	47/103	133	C ₆ H ₁₅ NO ₂
19	14.09	2,4-Dimethyl-decane	0.31	1.94	57	170	C ₁₂ H ₂₆
20	16.80	n-Tridecane	0.007	0.14	57	184	C ₁₃ H ₂₈
21	18.11	8-p-Menthadien-1,2-diol	0.61	-	71	152	C ₁₀ H ₁₆ O
22	18.14	Limonene 1,2-oxide	0.13	14.448	43	152	C ₁₀ H ₁₆ O
23	18.19	1,8-p-Menthadien-7-ol (Perillyl alcohol)	3.03	0.21	79	152	C ₁₀ H ₁₆ O
24	18.75	2,2-Diethoxy-N, N-dimethyl-ethanamine	0.56	-	75/103	161	C ₈ H ₁₉ NO ₂
25	18.76	Methyl-2-phenyl hexane	-	0.25	105	176	C ₁₃ H ₂₀
26	19.19	1-Tetradecene	1.08	0.18	55	196	C ₁₄ H ₂₈
27	20.64	1-Phenylnonane	0.03	2.04	91	204	C ₁₅ H ₂₄
28	22.29	Butylated hydroxytoluene	10.58	1.67	220	220	C ₁₅ H ₂₄ O
29	22.41	2,6-Bis(1-methylpropyl) Phenol	0.86	0.10	191	206	C ₁₄ H ₂₂ O
30	22.78	5-Phenyl decane	0.66	-	91	218	C ₁₆ H ₂₆
31	23.00	4-Phenyl decane	0.56	2.10	91	218	C ₁₆ H ₂₆
32	23.42	3-Phenyl decane	0.73	2.62	91	218	C ₁₆ H ₂₆
33	22.37	Caryophyllene	0.72	0.001	79	204	C ₁₅ H ₂₄
34	23.77	2-Phenyl decane	1.33	2.30	105	218	C ₁₆ H ₂₆
35	23.97	1-Hexadecene	1.17	-	55	224	C ₁₆ H ₃₂
36	24.26	6-Phenyl undecane	0.86	3.79	161	232	C ₁₇ H ₂₈
37	24.36	5-Phenyl undecane	1.87	0.14	91	232	C ₁₇ H ₂₈
38	24.97	4-Phenyl undecane	1.80	2.87	91	232	C ₁₇ H ₂₈
39	25.08	3-Phenyl undecane	2.10	4.66	91	232	C ₁₇ H ₂₈
40	25.51	2-Phenyl undecane	1.43	4.18	105	232	C ₁₇ H ₂₈
41	25.99	6-Phenyl dodecane	1.35	0.19	91	246	C ₁₈ H ₃₀
42	27.11	5-Phenyl dodecane	3.29	0.10	91	246	C ₁₈ H ₃₀
43	27.22	4-Butyl-indan-5-ol	-	3.87	147	190	C ₁₃ H ₁₈ O
44	28.37	5-Octadecene	1.08	-	55	252	C ₁₈ H ₃₆
45	27.82	4-Phenyl dodecane	2.98	3.80	91	246	C ₁₈ H ₃₀
46	27.92	3-Phenyl dodecane	4.58	4.58	91	246	C ₁₈ H ₃₀
47	28.61	2-Phenyldodecane	2.65	7.67	105	246	C ₁₈ H ₃₀
48	29.14	6-Phenyl tridecane	1.53	4.44	91	260	C ₁₈ H ₃₀
49	29.28	5-Phenyl tridecane	1.13	3.05	91	260	C ₁₉ H ₃₂
50	29.52	4-Phenyl tridecane	1.21	3.00	91	260	C ₁₉ H ₃₂
51	29.98	3-Phenyl tridecane	1.15	3.79	91	260	C ₁₉ H ₃₂
52	30.74	2-Phenyl tridecane	1.83	5.31	105	260	C ₁₉ H ₃₂
53	32.17	3-Eicosene	1.58	-	55	280	C ₂₀ H ₄₀
54	37.50	n-Tricosane	-	0.13	57	324	C ₂₃ H ₄₈
Continued							

No.	R_t (min)	Compounds	Area %		BP	MW	Mol. formula
			USFPI	USFPp			
55	40.64	n-Pentacosane	0.47	0.16	71	352	$C_{25}H_{52}$
Total identified compounds			82.646	94.368			
a. Oxygenated			37.28	28.647			
• Phenolic compounds			11.44	1.77			
• Oxygenated monoterpene			3.77	15.358			
• Alcohols			21.79	1.489			
• Ketones			0.16	3.76			
• Miscellaneous			0.12	6.27			
b. Non-oxygenated			44.038	62.581			
• Phenyl HC			33.07	60.63			
• Straight chain saturated HC			0.749	0.99			
• Branched saturated HC			2.359	2.62			
• Unsaturated HC			4.91	0.18			
• Non-oxygenated monoterpene			2.23	0.26			
• Sesquiterpenoids			0.72	0.001			
c. Nitrogenous oxygenated compounds			0.91	1.04			
Total non-identified compounds			16.36	5.992			

Table 1. GC/MS analysis of unsaponified fractions of *Citrus japonica* (USFPI and USFPp). BP; Base peak, Mw; Molecular weight, R_t ; retention time, HC; hydrocarbons, USFPI; unsaponifiable fraction of peel, USFPp; unsaponifiable fraction of pulp.

microbial strains separately by transfer 20.0 μ L [a 0.5 McFarland standard (1.5×10^8 CFU /mL)] of these tested strains, finally each well contain final volume 200 μ L, the microtiter plate incubate for 24 h at 37 °C, after that, the MIC was measured by using eliza reader throughout the absorption reading of this inoculated micro titer plate, where the mixture of this plate was shaken vigorously for 20 s and allowed to stand, the absorbance was then measured at 600.0 nm using a microplate multi-well reader (BioTek ELx800 Absorbance Microplate ELISA Reader, Agilent company, USA). The lower absorbance of the reaction mixture, which is equal to the absorption of the blank sample, is referred to as MIC activity^{26,28}.

Statistical analysis

Statistical analyses were performed using SPSS (Statistical Package for the Social Sciences) followed by Duncan's multiple range test was employed to evaluate the differences among groups, and results were considered statistically significant at $P \leq 0.05$. In ANOVA assumptions; Normality was checked by Shapiro Wilk test ($p > 0.05$) and homogeneity of variances by Levene's test ($p > 0.05$). Both assumptions were met for all datasets.

Duncan's multiple range test was used for post hoc comparisons because it is appropriate for pairwise comparisons of a single factor (sample type) with three or more groups; we chose Duncan's as it is more sensitive for detecting differences among close means in agricultural/biological studies. The same threshold ($p \leq 0.05$) was applied.

Results

Chemical investigation of non-polar fractions and GC/MS-based molecular networking

GC/MS analysis revealed a significant difference in the unsaponifiable matter (USFPI and USFPp) (Table 1) and fatty acid esters (FAEPI and FAEPP) of saponifiable matter (Table 2) among the studied peel and pulp extracts. Two GC/MS-based GNPS molecular networks were created. Only matches between investigated and library spectra with a score above 0.5 and at least 6 matched fragments were considered and confirmed through published data¹⁸. MN groups of interest are represented in Figs. 1 and 2, for the unsaponifiable and saponifiable matters, respectively.

The GC/MS investigation of the USFPI and USFPp (Fig. S2 & Fig. S3) revealed the identification of 49 and 46 compounds constituting total areas of 82.646 and 94.368, respectively (Table 1). The oxygenated compounds constituted 37.28% and 28.647% while the non-oxygenated compounds constituted 44.038% and 62.581%, respectively. Phenyl hydrocarbons are major compounds in two fractions, constituting 33.07% and 60.63%, respectively. Among the identified unsaponifiable compounds, butylated hydroxytoluene (10.58%) and limonene (2.23%) were present in the peel fraction in significantly higher amounts than in the pulp (1.67% and 0.26%). In contrast, limonene oxide accumulated in the pulp (14.448%), while only a trace amount was detected in the peel (0.13%).

Similarly, FAEPI and FAEPP were analyzed by GC/MS (Fig. S4 & Fig. S5), which resulted in the identification of 53 and 46 compounds constituting total area % (71.27 and 82.96), respectively (Table 2). The percentages of saturated & unsaturated fatty acids were close in both parts, as (32.920 and 30.372) & (13.522 and 11.008), respectively, for (FAEPI and FAEPP). Most of the fatty acids identified belong to dicarboxylic structures. The major compounds in both parts were hexadecanoic acid methyl ester (methyl palmitate) with area % (9.597%

No.	R_f (min)	Compounds	Area %		BP	MW	Mol. Formula
			FAEPI	FAEPp			
1	10.56	Methyl 2-furoate	-	0.844	95	126	C ₆ H ₆ O ₃
2	12.66	Butanedioic acid, dimethyl ester (Dimethylsuccinate)	-	1.024	115	146	C ₆ H ₁₀ O ₄
3	13.82	Butanedioic acid, methyl-, dimethyl ester (Dimethyl methyl succinate)	-	0.263	129/59	160	C ₇ H ₁₂ O ₄
4	14.75	Methyl benzoate	0.463	-	105	136	C ₈ H ₈ O ₂
5	15.16	Butanedioic acid, ethyl methyl ester (Methyl ethyl succinate)	0.483	-	-	160	C ₇ H ₁₂ O ₄
6	16.49	3-Methyl glutaric acid dimethyl ester	0.362	1.373	114/101	174	C ₈ H ₁₄ O ₄
7	17.22	Methyl – 10-Pentadecenoate	0.342	-	55	254	C ₁₆ H ₃₀ O ₂
8	17.33	Benzeneacetic acid, methyl ester	0.282	0.318	91	150	C ₉ H ₁₀ O ₂
9	17.43	Butanedioic acid, diethyl ester (Diethyl succinate)	0.563	-	101/129	174	C ₈ H ₁₄ O ₄
10	17.99	Propyl isobutyrate	0.101	1.205		130	C ₇ H ₁₄ O ₂
11	19.29	Hexanedioic acid, dimethyl ester (Dimethyl adipate)	0.161	0.097	114/101	174	C ₈ H ₁₄ O ₄
12	20.58	Methyl octanoate	0.553	0.083	74	158	C ₉ H ₁₈ O ₂
13	22.09	Heptanedioic acid, dimethyl ester (Dimethyl pimelate)	0.021	0.789	115/74	188	C ₉ H ₁₆ O ₄
14	23.15	2-Propenoic acid, 3-phenyl-, methyl ester (Methyl cinnamate)	1.267	0.003	131/103	162	C ₁₀ H ₁₀ O ₂
15	23.98	1-Ethyl heptanedioate (Pimelic acid monoethyl ester)	0.704	0.006	88/74	188	C ₉ H ₁₆ O ₄
16	23.98	Heptanedioic acid, dimethyl ester (Dimethyl pimelate)	0.362	-	125	188	C ₉ H ₁₆ O ₄
17	24.54	1, 2, 3-Prop-1-ene tricarboxylic acid, trimethyl ester	-	0.928	153	216	C ₉ H ₁₂ O ₆
18	24.81	Octanedioic acid, dimethyl ester	6.237	1.301	138	202	C ₁₀ H ₁₈ O ₄
19	25.36	Citric acid, trimethyl ester (Trimethyl citrate)	-	7.3	143/101	234	C ₉ H ₁₄ O ₇
20	25.74	Heptanedioic acid, diethyl ester	0.261	-	125	216	C ₁₁ H ₂₀ O ₄
21	27.23	Nonanedioic acid, dimethyl ester (Dimethyl azelate)	6.518	7.51	152	216	C ₁₁ H ₂₀ O ₄
22	28.12	9- Hexadecenoic acid methyl ester	-	0.346	55	268	C ₁₇ H ₃₂ O ₂
23	28.15	Octanedioic acid, diethyl ester (Diethyl suberate)	1.127	0.001	143	230	C ₁₂ H ₂₂ O ₄
24	28.34	Ethyl dodecanoate (Ethyl laurate)	0.744	0.166	88/74	228	C ₁₄ H ₂₈ O ₂
25	29.55	1,10-Decanedioic acid, dimethyl ester (Dimethyl sebacate)	0.785	2.907	125/74	230	C ₁₂ H ₂₂ O ₄
26	30.17	2-Propenoic acid, 3-(4-methoxyphenyl)-, methyl ester (Methyl-p-methoxycinnamate)	0.008	0.305	161/133	192	C ₁₁ H ₁₂ O ₃
27	31.67	1,11-Undecanedioic acid dimethyl ester	0.463	0.397	213/139	244	C ₁₃ H ₂₄ O ₄
28	31.87	3,4- dimethyl benzoic acid methyl ester	0.010	0.221	133	164	C ₁₀ H ₁₂ O ₂
29	32.56	Decanedioic acid diethyl ester (Diethyl sebacate)	0.342	-	97	258	C ₁₄ H ₂₆ O ₄
30	31.24	Tetradecanoic acid, methyl ester	1.247	-	74	242	C ₁₅ H ₃₀ O ₂
31	32.71	Tetradecanoic acid, ethyl ester	1.207	0.042	88	256	C ₁₆ H ₃₂ O ₂
32	33.36	Isopropyl tetradecanoic acid methyl ester (Tetradecanoic acid, 1-methyl ethyl ester)	0.301	-	74	270	C ₁₇ H ₃₄ O ₂
33	33.55	9-Hexadecenoic acid, methyl ester (methyl palmitoleate)	1.83	1.273	55	268	C ₁₇ H ₃₂ O ₂
34	35.42	Hexadecanoic acid, methyl ester (Methyl palmitate)	9.597	22.872	74	270	C ₁₇ H ₃₄ O ₂
35	35.52	13-Methyl pentadecanoic acid methyl ester	0.004	0.249	74/87	284	C ₁₈ H ₃₆ O ₂
36	36.71	Hexadecanoic acid, ethyl ester (Ethyl palmitate)	7.465	0.208	88	284	C ₁₈ H ₃₆ O ₂
37	36.81	14- Methyl heptadecanoic acid methyl ester	-	0.235	74/87	298	C ₁₉ H ₃₈ O ₂
38	37.60	Hexadecanoic acid 2-hydroxy-methyl ester (Hydroxypalmitate)	1.408	7.864	97/83	286	C ₁₇ H ₃₄ O ₃
39	38.55	Heptadecanoic acid, ethyl ester	0.664	-	88	298	C ₁₉ H ₃₈ O ₂
40	39.36	9,12,15 octadecatrienoic acid methyl ester	1.830	0.152	79	292	C ₁₉ H ₃₂ O
41	38.58	9,12-Octadecadienoic acid methyl ester (Methyl linoleate)	0.163	5.524	67/81	294	C ₁₉ H ₃₄ O ₂
42	38.74	6, 9- Octadecadienoic acid methyl ester	0.724	-	67	294	C ₁₉ H ₃₄ O ₂
43	38.78	9-Octadecenoic acid methyl ester (Oleic acid, methyl ester)	1.147	4.430	55/87	296	C ₁₉ H ₃₆ O ₂
44	38.80	9,12-Octadecadienoic acid ethyl ester (Ethyl linoleate)	1.227	0.001	67/88	308	C ₂₀ H ₃₆ O ₂
45	38.83	16-Octadecenoic acid, methyl ester	0.382	4.264	97/55	296	C ₁₉ H ₃₆ O ₂
46	39.08	9-Octadecenoic acid ethyl ester (Ethyl Oleate)	0.624	-	95	310	C ₂₀ H ₃₈ O ₂
47	39.14	Octadecanoic acid, methyl ester (Methyl stearate)	2.193	4.015	74	298	C ₁₉ H ₃₈ O ₂
48	40.29	10-Methyl octadecanoic acid methyl ester	-	0.387	74/87	312	C ₂₀ H ₄₀ O ₂
49	40.33	Octadecanoic acid, ethyl ester (Stearic acid, ethyl ester)	2.193	-	88	312	C ₂₀ H ₄₀ O ₂
50	42.54	9,10-dihydroxy octadecanoic acid, methyl ester	-	2.409	87	330	C ₁₉ H ₃₈ O ₄
Continued							

No.	R _i (min)	Compounds	Area %		BP	MW	Mol. Formula
			FAEPI	FAEPp			
51	43.04	14-Oxo-nonadec-10-enoic acid methyl ester	1.710	-	99	324	C ₂₀ H ₃₆ O ₃
52	43.62	5,8,11-Heptadecatriynoic acid, methyl ester	1.911	-	101	272	C ₁₈ H ₂₄ O ₂
53	45.78	Docosanoic acid, methyl ester	0.766	-	74	354	C ₂₃ H ₄₆ O ₂
54	45.80	Methyl-20-methyl-heneicosanoate	0.966	0.914	74/87	354	C ₂₃ H ₄₆ O ₂
55	46.76	Docosanoic acid, ethyl ester	0.362	-	88	368	C ₂₄ H ₄₈ O ₂
56	48.37	Tricosanoic acid, ethyl ester	2.052	0.941	74/87	382	C ₂₅ H ₅₀ O ₂
57	48.73	Tetracosanoic acid, methyl ester	0.905	-	74	382	C ₂₅ H ₅₀ O ₂
58	49.2	Methyl 2-hydroxydocosanoate	0.060	0.332	57/97	370	C ₂₃ H ₄₆ O ₃
59	50.61	Methyl 2-hydroxytetracosanoate	-	0.373	57/97	398	C ₂₅ H ₅₀ O ₃
60	51.49	Hexacosanoic acid, methyl ester	0.443	0.263	74	410	C ₂₇ H ₅₄ O ₂
61	52.42	Hexacosanoic acid ethyl ester	0.342	0.028	88	424	C ₂₈ H ₅₆ O ₂
62	54.79	Octacosanoic acid methyl ester	0.865	-	143	438	C ₂₉ H ₅₈ O ₂
63	56.06	Heptacosanoic acid ethyl ester	0.422	0.010	88	452	C ₃₀ H ₆₀ O ₂
Total identified compounds			71.27	82.96			
a. Fatty acid methyl esters							
• Saturated Fatty acids ester			32.920	30.372			
• Unsaturated fatty acids ester			13.522	11.008			
• Hydroxyl saturated fatty acids ester			1.469	10.979			
• Dicarboxylic fatty acid ester			18.108	13.008			
b. Carboxylic acid methyl esters							
• Mono carboxylic acid ester			2.492	3.270			
• Dicarboxylic acids ester			1.046	3.287			
• Tricarboxylic acid ester			-	11.034			
c. Miscellaneous							
• Oxo unsaturated fatty acid ester			1.710	-			
Total non-identified compounds			28.73	17.04			

Table 2. GC/MS analysis of fatty and carboxylic acids esters of *Citrus japonica* (FAEPI and FAEPp). BP; Base peak, Mw; Molecular weight, R_i; retention time, FAEPI; fatty acid methyl esters of peel, FAEPp; fatty acid methyl esters of pulp.

and 22.872%), followed by nonanedioic acid dimethyl ester (dimethyl azelate) (6.518% and 7.51%), then octadecanoic acid, methyl ester (stearic acid) (2.193% and 4.015%), respectively. The pulp and peel were rich in carboxylic acids (mono-, di-, and tricarboxylic acid). The latter acid was detected in the pulp and identified as trimethyl citrate with a relative percentage of 7.3%.

Principal component analysis

PCA is a data analysis method commonly used in chemometric metabolomic research. Dimensionality-reduction techniques such as PCA typically require investigators to determine the number of components to retain. Visual assessment of the scree plot is crucial for determining the proper number of components to keep. The scree plot is an experimental graphic method constructed of the eigenvalues (y-axis) against the components (x-axis), and it represents the maximum number of components to keep²⁹(Figs. 3A and 4A). The PCA biplot for datasets acquired from both the saponifiable and unsaponifiable fractions showed a clear segregation between the peels and pulp of *C. japonica*. Since the biplot analysis of both the saponifiable and unsaponifiable fractions demonstrated adequate variance coverage—explaining 79.1% and 20.9% of the total variance along PC1 and PC2, respectively, for the saponifiable matter, and 58.3% and 41.7% for the unsaponifiable matter (Figs. 3A and 4A)²¹.

For the unsaponifiable and saponifiable matters, component 2 (PC2) revealed a clear segregation between the peel and pulp samples and showed a distinct metabolic profile unique to each tissue. Unlike component 2, peels and pulp are pulled together at the positive side of component 1 (PC1) (Figs. 3B and 4B).

In the unsaponifiable matter, the positive side of Component 2, associated with the peels, indicates an abundance of constituents such as 1-hexanol, 2-ethyl-, butylated hydroxytoluene, and limonene. Pulp appeared rich in alkylbenzene primary compounds, 2-phenyl undecane, 2-phenyldodecane, and 5-phenyl dodecane compared to peel (Fig. 3B). In the saponifiable matter, the positive loadings of the saponifiable constituents associated with pulp are methyl palmitate, trimethyl citrate, octadecadienoic acid methyl ester, and nonanedioic acid dimethyl ester (dimethyl azelate). Controversy, the negative loadings of the saponifiable constituents associated with the peel are hexadecanoic acid ethyl ester (ethyl palmitate) (Fig. 4B). The fruit’s pulp main role is nutrient storage and aiding the seed development. Methyl palmitate and citric acid ester may participate in energy storage and metabolic activities essential for these processes^{30,31}. Whereas the peel serves as a shield

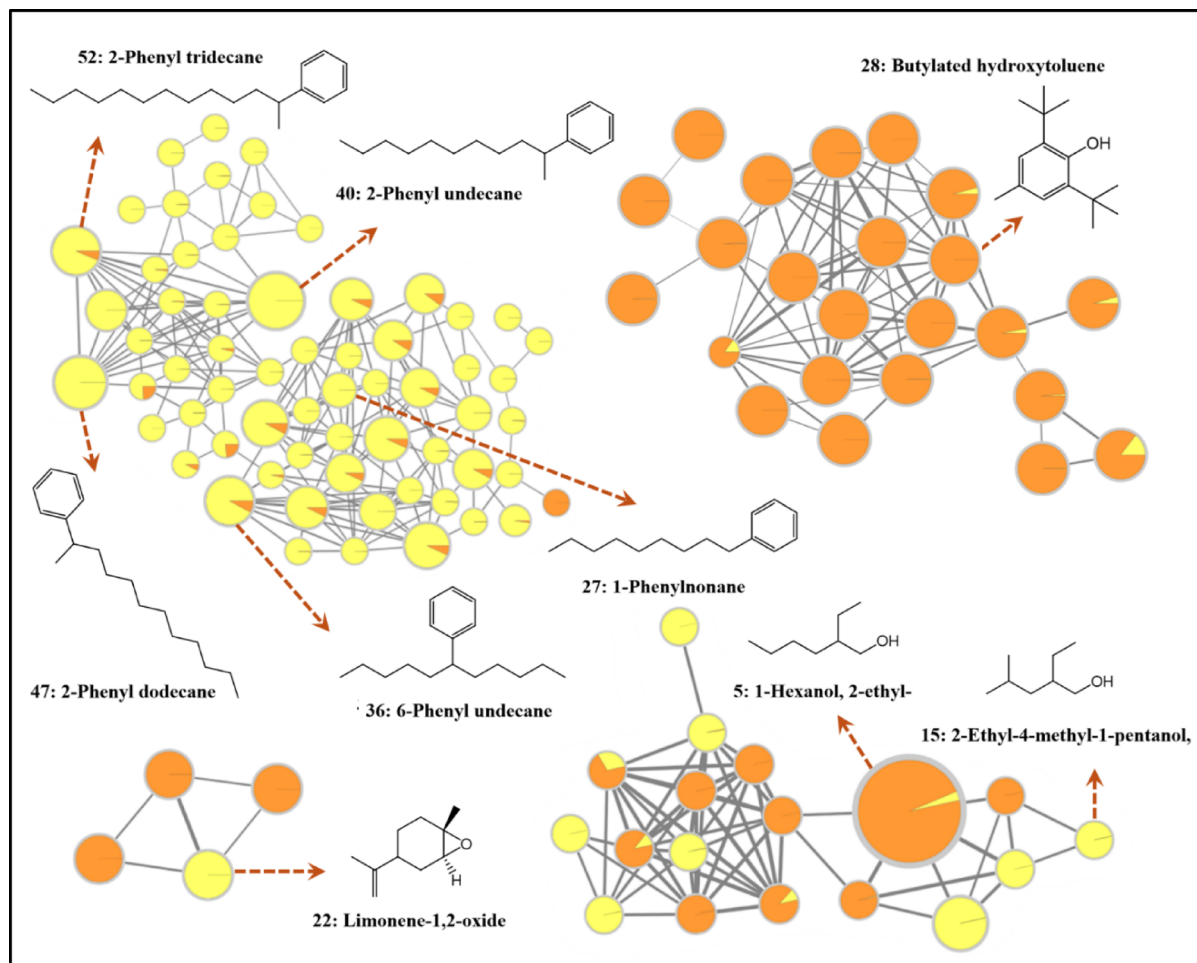


Fig. 1. Groups of interest for the GC/MS Molecular network of unsaponified fractions of *Citrus japonica*. Node color: distribution of compound among peel and pulp (USFPI with orange and USFPp with yellow). Nodes represent MS/MS spectra. Node size: total sum of intensity of the corresponding ion.

against environmental threats such as pathogens, UV radiation, and mechanical injury, ethyl palmitate, a fatty acid ester rich in peels, may help reinforce the protective wax layers of the peel³².

The PCA finding highlights the different functional roles of peel and pulp, with the peel storing limonene as a protective mechanism and pulp accumulating aromatic alkylbenzenes for nutrient storage and seed protection, potentially drawing seed dispersers^{33–35}. These metabolomic profile variations may be attributed to differences in multiple metabolic pathways establishment³⁶.

Antimicrobial investigation of non-polar fractions

The antimicrobial evaluation of NPFPI and NPFp was screened against three selected microbial strains. The positive antimicrobial effects were observed for NPFp against a Gram-negative bacterium (*P. aeruginosa*), a Gram-positive bacterium (*S. aureus*), and a fungal yeast strain (*C. albicans*) with inhibition zone diameters (mm) 13.67 ± 0.89 , 19.33 ± 0.22 , and 20.5 ± 0.17 compared to those of standard 12.33 ± 0.22 , 19.83 ± 0.39 , and 10.71 ± 0.05 , respectively (Table 3; Fig. 5). One-way ANOVA followed by Duncan's multiple range test ($p \leq 0.05$) revealed that antimicrobial inhibition zone against *P. aeruginosa*: NPFp (letter a) was significantly more active than gentamicin (letter b), whereas against *S. aureus*: NPFp and gentamicin shared the same letter (a), indicating no significant difference. The results against *C. albicans*: NPFp (letter a) were significantly more active than fluconazole (letter b).

The MIC values of NPFp were 3.75 mg/mL for *P. aeruginosa*, 0.938 mg/mL for *S. aureus*, and 1.88 mg/mL for *C. albicans*, all significantly higher than those of the positive controls (different letters, $p \leq 0.05$). No effects were observed for NPFPI.

Discussion

A diabetic foot ulcer represents one of the most serious complications of diabetes mellitus; if not properly treated, it may progress to osteomyelitis, gangrene, and ultimately limb amputation, contributing to increased mortality rates³⁷. The most frequently isolated bacterial strains include *Pseudomonas* spp. and *S. aureus*³⁸, with *Pseudomonas* reported as the predominant gram-negative organism in diabetic foot infections across several

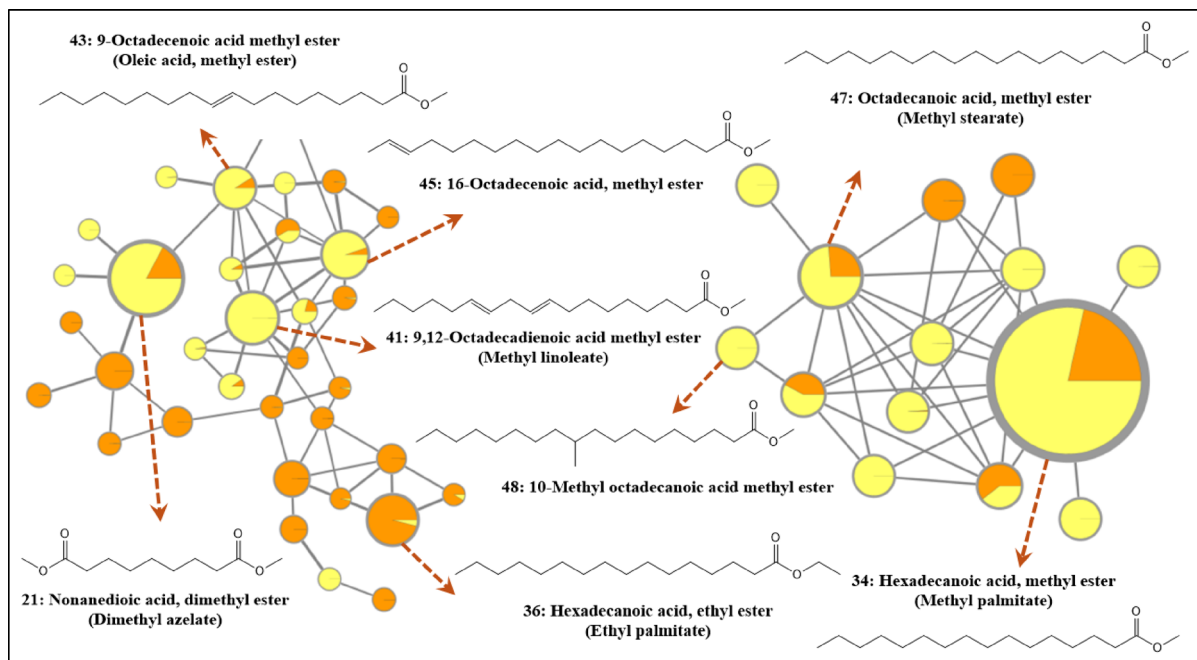


Fig. 2. Groups of interest for the GC/MS Molecular network of fatty and carboxylic acids esters of *Citrus japonica*. Node color: distribution of compound among fatty acid esters of peels and pulp (FAEPi with orange and FAEPp with yellow). Nodes represent MS/MS spectra. Node size: total sum of intensity of the corresponding ion.

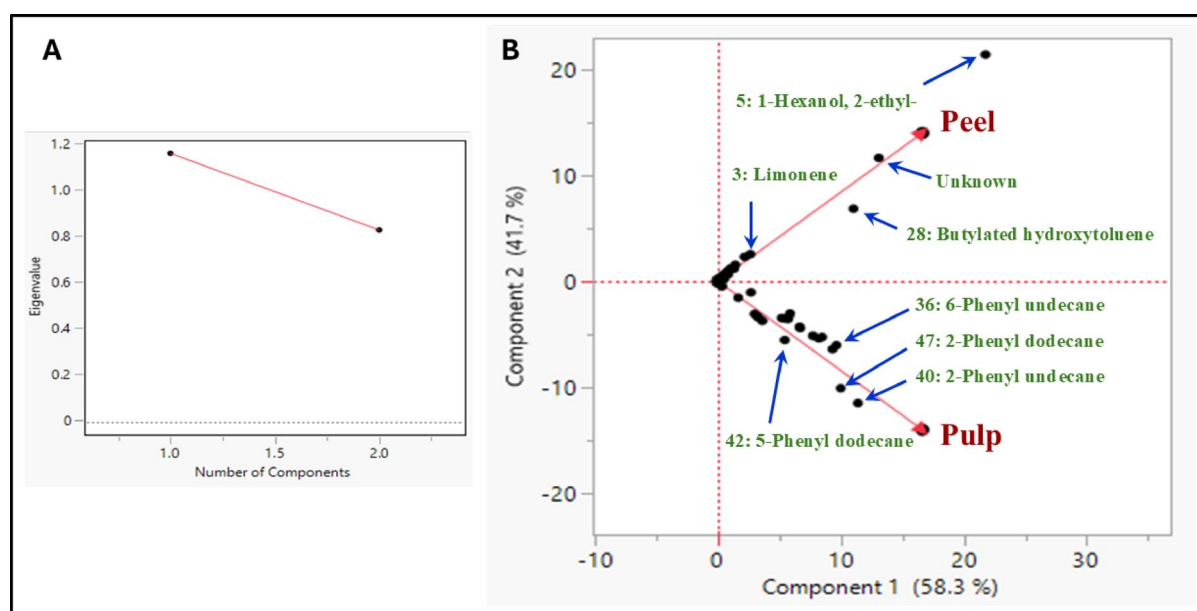


Fig. 3. **A:** Scree plot of unsaponifiable matter, **B:** Principal component analysis (PCA) biplot (combination of variables on loadings plot and samples on scores plot) of unsaponifiable matter from gas chromatography-mass spectrometry of *Citrus japonica* peels and pulps.

Middle Eastern populations³⁹. Although fungal infection is less common, *C. albicans* has been repeatedly identified among clinically relevant fungal isolates in diabetic foot infections⁴⁰.

C. japonica is globally recognized for its antimicrobial and nutritional properties¹². In this study, a metabolomic GC-MS-based approach was used to characterize non-polar metabolites in *C. japonica* fruit tissue (peel and pulp), revealing 63 saponifiable and 55 unsaponifiable compounds. Multivariate PCA analysis clearly distinguished between the two tissues, indicating distinct metabolic pathways and potential ecological roles.

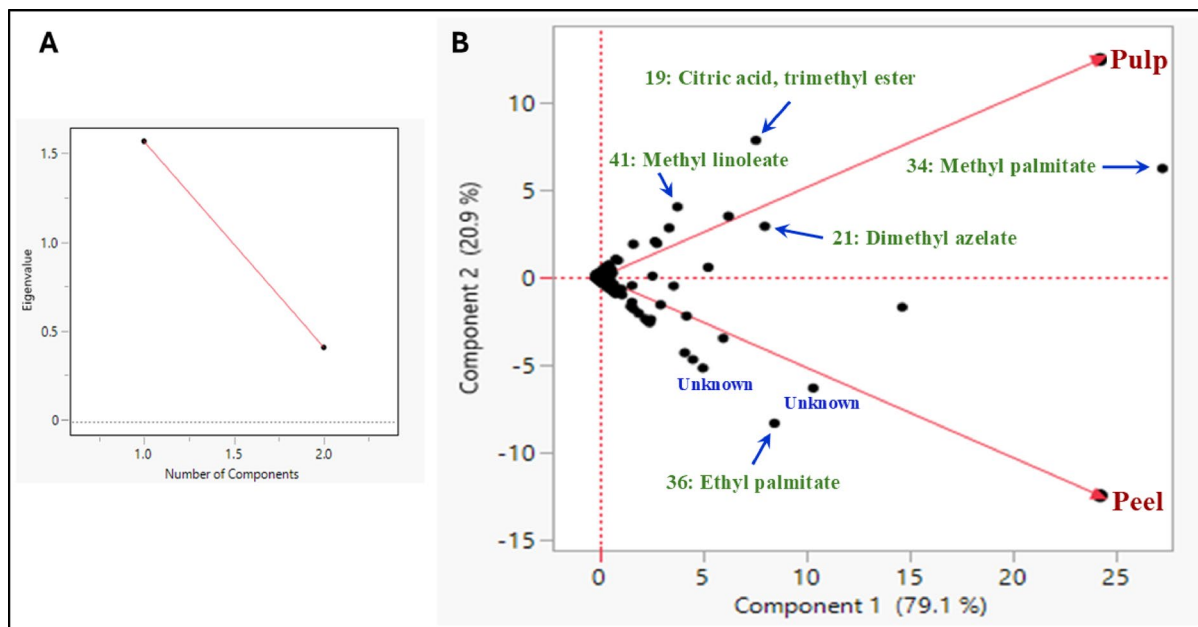


Fig. 4. **A:** Scree plot of fatty acid methyl/ethyl esters, **B:** principal component analysis (PCA) biplot (combination of variables on loadings plot and samples on scores plot) of saponifiable matter from gas chromatography-mass spectrometry of *Citrus japonica* peels and pulps.

Tested organisms	Inhibition zone diameter (mm)		MIC (mg/mL)	
	NPFPP	CN	NPFPP	CN
<i>Pseudomonas aeruginosa</i>	13.67 ^a ±0.89	12.33 ^b ±0.22	3.75 ^a ±0.04	0.125 ^b ±0.0009
<i>Staphylococcus aureus</i>	19.33 ^a ±0.22	19.83 ^a ±0.39	0.938 ^a ±0.002	0.118 ^b ±0.0003
<i>Candida albicans</i>	20.5 ^a ±0.17	10.71 ^b ±0.05	1.88 ^a ±0.001	0.06 ^b ±0.0001

Table 3. Inhibition zone diameter (mm) applying the well diffusion method and MIC (mg/mL) applying the microdilution broth method of NPFPP. Data are presented as mean ± SD. One-way ANOVA was used for data analysis ($n = 3$, $P \leq 0.05$) the NPEpp; non-polar fraction of pulp, CN; Reference sample [(Gentamicin 10 mcg; standard antibiotic disc, Fluconazole (50 µg/disc) a standard antifungal disc); MIC; the minimal inhibitory concentration.) determined by the well diffusion assay. Values represent the mean ± standard deviation. Different letters mean significant differences between the NPEpp and the standard ($p < 0.05$).

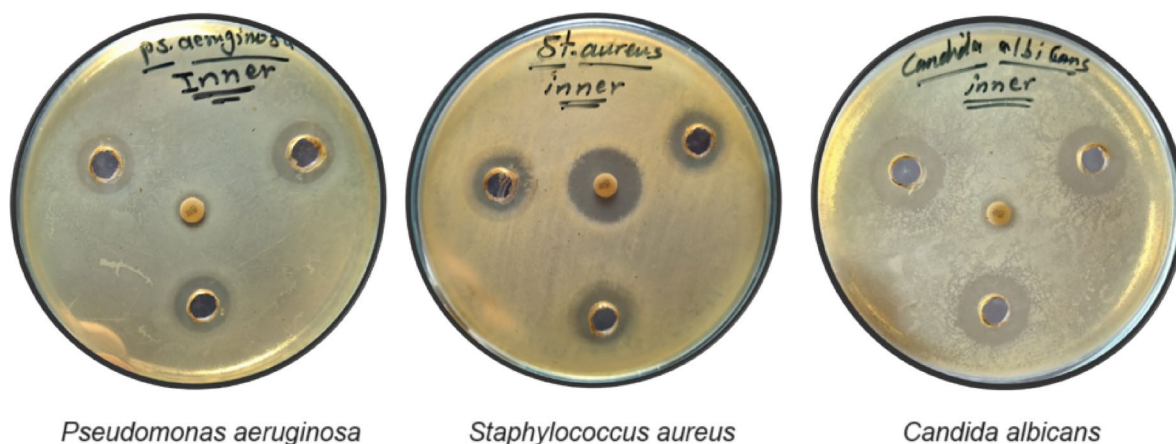


Fig. 5. Inhibition zone diameter (mm) applying the well diffusion technique of NPFPP against the tested microbial strains.

Chemometric profiling further demonstrated tissue-specific enrichment of bioactive constituents. Alkyl benzenes were present in both tissues but were markedly higher in USFPp (58.53%), where PCA highlighted phenyl decane derivatives as key discriminants (Fig. 4. B). These compounds have been previously associated with antimicrobial defense mechanisms in plants, supporting their potential role in pathogen suppression^{41,42}. Among these alkyl benzenes are phenyl decane derivatives, which showed antifungal and antibacterial activities⁴³.

Oxygenated monoterpenes, including limonene oxide, linalool, and others, were detected in both tissues, with slightly higher abundance in the peel (Fig. 4-B). These Oxygenated monoterpenes exhibited varying degrees of antibacterial activities⁴⁴. Their antimicrobial relevance has been well documented, particularly enhanced activity against Gram-negative bacteria, which has been attributed to oxygenated functional groups that increase membrane permeability⁴⁵.

More importantly, PCA-based GC-MS indicated that the pulp fraction (FAEPp) is characterized by higher levels of hexadecanoic acid methyl ester, trimethyl citrate, and dimethyl azelate (Fig. 5-B). These metabolites likely contribute collectively to the stronger antimicrobial activity observed in the pulp compared to the peel. Fatty acid methyl esters, such as hexadecanoic acid methyl ester, are known to disrupt microbial membranes^{46–48}, an acknowledged mechanism established for long-chain unsaturated fatty acids. Additionally, citrate derivatives may enhance activity through metal ion chelation, thereby destabilizing cell envelope integrity and biofilm structure^{49,50}. This may weaken bacterial defenses and increase susceptibility to other antimicrobial agents, particularly in Gram-negative strains⁵¹. Furthermore, azelaic acid derivatives further complement this effect by inhibiting key microbial enzymes involved in DNA and protein synthesis^{52,53}. Approved by the FDA, azelaic acid derivatives are widely used in dermatological treatment, exhibiting in vitro bacteriostatic activity against several bacteria, including *P. aeruginosa*, *S. aureus*, *Staphylococcus epidermidis*, and *Propionibacterium acnes*⁵⁴.

Although the observed MIC values obtained (0.938–3.75 mg/mL) indicate only moderate antimicrobial potency, they are consistent with previously reported values for other *Citrus* extracts, which typically range from 2.5 to 25 mg/mL against *S. aureus* and *P. aeruginosa*^{51,52}. This moderate activity is expected, given the complex, slightly non-purified nature of the lipophilic extracts, where multiple compounds may act synergistically or competitively, influencing overall bioactivity.

Overall, the modest but broad-spectrum antimicrobial activity of the pulp fraction is likely driven by a synergistic interplay between membrane-disrupting active fatty acid esters, citrate with metal-chelating properties, and azelate acting as an enzyme inhibitor, rather than a single dominant constituent.

Finally, this study serves as a preliminary screening of tissue-specific bioactivity in *C. japonica*. Further fractionation and isolation of pure compounds are required to establish definitive structure–activity relationships and to validate the therapeutic potential of the most active metabolites.

Conclusion

This study provides the first GC-MS-based metabolomic comparison of the non-polar fractions from the peel and pulp of *C. japonica*, supported by GNPS molecular networking and PCA chemometrics. The pulp fraction was enriched in bioactive metabolites, including trimethyl citrate, dimethyl azelate, and methyl palmitate, and exhibited weak to moderate in vitro antimicrobial activity against three pathogens commonly associated with diabetic foot infections (*P. aeruginosa*, *S. aureus*, and *C. albicans*). These findings suggest that the observed activity is driven by synergistic effects of multiple compounds affecting microbial membranes, metal ion balance, and enzymatic pathways. In conclusion, the pulp fraction of *C. japonica* represents a promising source of antimicrobial lead compounds, warranting further isolation, in vitro assays (biofilm, cytotoxicity, and wound healing), and in vivo validation.

Data availability

The datasets supporting the conclusions of this article are publicly available via the Global Natural Products Social Molecular Networking (GNPS) platform. The specific analysis job can be accessed at the following permanent link: for Unsaponifiable fractions (UCSD Computational Mass Spectrometry Website) and for fatty acids fractions (UCSD Computational Mass Spectrometry Website).

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References

- McDermott, K., Fang, M., Boulton, A. J. M., Selvin, E. & Hicks, C. W. Etiology, Epidemiology, and Disparities in the Burden of Diabetic Foot Ulcers. *Diabetes Care*. **46**, 209–211 (2023). <https://doi.org/10.2337/dci22-0043> Preprint at.
- Surboyo, M. D. C. et al. Number of macrophages and transforming growth factor β expression in citrus limon L. Tlelung peel oil-treated traumatic ulcers in diabetic rats. *Trop. J. Pharm. Res.* **18**, 1427–1433 (2019).
- Raspo, M. A., Vignola, M. B., Andreatta, A. E. & Juliani, H. R. Antioxidant and antimicrobial activities of citrus essential oils from Argentina and the United States. *Food Biosci.* **36**, 100651 (2020).
- Agarwal, P. et al. Citrus Essential Oils in Aromatherapy: Therapeutic Effects and Mechanisms. *Antioxidants* 11 Preprint at (2022). <https://doi.org/10.3390/antiox11122374>
- Ragheb, A. Y. et al. MS/MS-based molecular networking for mapping the chemical diversity of the pulp and peel extracts from *Citrus japonica* Thunb.; in vivo evaluation of their anti-inflammatory and anti-ulcer potential. *Scientific African* **20**, (2023).
- Wang, Y. W. et al. Chemical composition and antimicrobial activity of the essential oil of kumquat (*Fortunella crassifolia* Swingle) peel. *Int. J. Mol. Sci.* **13**, 3382–3393 (2012).
- Satyral, P., Paudel, P., Limbu, K. & Setzer, W. N. Leaf essential oil composition of *Citrus japonica* from Nepal. *J. Essent. Oil-Bearing Plants*. **15**, 357–359 (2012).
- Kassim, A., Workneh, T. S. & Laing, M. D. Development and Evaluation of a Small-scale In-field Integrated Postharvest Citrus Treatment Unit - Part I. *International J. Food Engineering* **13**, (2017).

9. Phala, K. et al. Inhibition of Kumquat Postharvest Fungi through Vapor Contact with Spearmint Essential Oil and Carvone. *ACS Agricultural Sci. Technol.* **2**, 330–339 (2022).
10. Liu, X. et al. The accumulation and composition of essential oil in kumquat peel. *Sci. Hort.* **252**, 121–129 (2019).
11. Lou, S. N. & Ho, C. T. Phenolic compounds and biological activities of small-size citrus: Kumquat and calamondin. *Journal of Food and Drug Analysis* vol. 25 162–175 Preprint at (2017). <https://doi.org/10.1016/j.jfda.2016.10.024>
12. Sumonrat, C., Suphitchaya, C. & Tipparat, H. Antimicrobial activities of essential oils and crude extracts from tropical Citrus spp. against food-related microorganisms. *Songklanakarin J. Sci. Technol.* **30**, 125–131 (2008).
13. Jayaprakasha, G., Murthy, K. C., Demarais, R. & Patil, B. Inhibition of prostate cancer (LNCaP) cell proliferation by volatile components from Nagami Kumquats. *Planta Médica.* **78**, 974–980 (2012).
14. Schirra, M. et al. Influence of postharvest hot water treatment on nutritional and functional properties of Kumquat (*Fortunella japonica* Lour. swingle cv. ovale) fruit. *J. Agric. Food Chem.* **56**, 455–460 (2008).
15. Al-Rubaye, A. F., Hameed, I. H., Kadhim, M. J. A. & Review Uses of Gas Chromatography-Mass Spectrometry (GC-MS) Technique for Analysis of Bioactive Natural Compounds of Some Plants. *Int. J. Toxicol. Pharmacol. Res.* **9**, 81–85 (2017).
16. Ragab, N. A. et al. Chemical characterization of *Melilotus messanensis* (L.) all.: Antioxidant, antidiabetic and antimutagenic effects in alloxan induced diabetic rats. *Biocatal. Agric. Biotechnol.* **33**, 101976 (2021).
17. Waly, D. A. et al. Avocado fruit peel and flesh as antioxidant and anti-inflammatory agents: A comparative phytochemical and in vitro study. *South. Afr. J. Bot.* **179**, 334–344 (2025).
18. Adams, R. P. *Identification of Essential Oil Components by Gas Chromatography/Quadruple Mass Spectroscopy*. (Allured Publishing Corporation, USA).
19. Liu, J., Clarke, J. A., McCann, S., Hillier, N. K. & Tahlan, K. Analysis of Streptomyces Volatilomes Using Global Molecular Networking Reveals the Presence of Metabolites with Diverse Biological Activities. *Microbiology Spectrum* **10**, (2022).
20. Aksenov, A. A. et al. Auto-deconvolution and molecular networking of gas chromatography-mass spectrometry data. *Nat. Biotechnol.* **39**, 169–173 (2021).
21. El-Sawi, S. A. et al. Principal component analysis of phenolic compounds of grape waste parts and correlations to their bioassays. *Biocatal. Agric. Biotechnol.* **51**, 102780 (2023).
22. Hafez, A. I., Ali, H. M., Sabry, R. M. & El-Masry, H. M. Abd El-Gawad, W. M. Generation of novel, hygienic, inhibitive, and cost-effective nanostructured Core-shell pigments. *Prog. Org. Coat.* **175**, 107325 (2023).
23. El-Masry, H. M. et al. Phenazine-Producing *Pseudomonas aeruginosa* OQ158909: A Promising Candidate for Biological Activity and Therapeutic Applications. *Egypt. J. Chem.* **66**, 281–303 (2023).
24. McFarland, J. The nephelometer: an instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines. *J. Am. Med. Assoc.* **49**, 1176–1178 (1907).
25. Hamoda, D. M. et al. Plasma Technique Application for Coating Non - Woven Fabric (SMS) by (CaSiO₃/CuO) Nano Particles for Biomedical Sector. *Egypt. J. Chem.* **0**, 0–0 (2022).
26. El-Anssary, A. A., Raoof, G. F. A., Saleh, D. O. & El-Masry, H. M. Bioactivities, physicochemical parameters and GC/MS profiling of the fixed oil of *Cucumis melo* L seeds: A focus on anti-inflammatory, immunomodulatory, and antimicrobial activities. *J. HerbMed Pharmacol.* **10**, 476–485 (2021).
27. Tohamy, H. A. S. & El-Masry, H. M. Fluffy-like amphiphilic graphene oxide and its effects on improving the antibacterial activity and thermal outstanding of ethyl cellulose /polyvinyl alcohol hydrogel film. *BMC Chem.* **18**, 116 (2024).
28. Saquib, S. A. et al. Evaluation and comparison of antibacterial efficacy of herbal extracts in combination with antibiotics on periodontal pathobionts: An in vitro microbiological study. *Antibiotics* **8**, (2019).
29. Ledesma, R., Valero-Mora, P. & Macbeth, G. The Scree Test and the Number of Factors: a Dynamic Graphics Approach. *The Span. J. Psychology* **18**, (2015).
30. Sébastien Baud, L. L. Physiological and developmental regulation of seed oil production. *Prog. Lipid Res.* **49**, 235–249 (2010).
31. Sweetlove, L. J., Beard, K. F. M., Nunes-Nesi, A., Fernie, A. R. & Ratcliffe, R. G. Not just a circle: flux modes in the plant TCA cycle. *Trends Plant. Sci.* **15**, 462–470 (2010).
32. Kunst, L. & Samuels, A. L. Biosynthesis and secretion of plant cuticular wax. *Prog. Lipid Res.* **42**, 51–80 (2003).
33. Soleimani, H., Mostowfizadeh-Ghalamfarsa, R., Ghanadian, M., Karami, A. & Cacciola, S. O. Defense Mechanisms Induced by Celery Seed Essential Oil against Powdery Mildew Incited by *Podosphaera fusca* in Cucumber. *J. Fungi.* **10**, 17 (2024).
34. Turlings, T. C. J. & Erb, M. Tritrophic Interactions Mediated by Herbivore-Induced Plant Volatiles: Mechanisms, Ecological Relevance, and Application Potential. *Ann. Rev. Entomol.* **17**, 14 (2025).
35. Borges, R. M., Bessière, J. M. & Ranganathan, Y. Diel Variation in Fig Volatiles Across Syconium Development: Making Sense of Scents. *J. Chem. Ecol.* **39**, 630–642 (2013).
36. Sweetlove, L. J. & Fernie, A. R. The Spatial Organization of Metabolism Within the Plant Cell. *Annu. Rev. Plant Biol.* **64**, 723–746 (2013).
37. Shaheen, M. M. A., Dahab, A., Abu Fada, S., Idieis, R. & M. & Isolation and characterization of bacteria from diabetic foot ulcer: amputation, antibiotic resistance and mortality rate. *Int. J. Diabetes Developing Ctries.* **42**, 529–537 (2022).
38. Kaimkhani, G. M. et al. Pattern of Infecting Microorganisms and Their Susceptibility to Antimicrobial Drugs in Patients with Diabetic Foot Infections in a Tertiary Care Hospital in Karachi, Pakistan. *Cureus* **10**, 6 (2018).
39. Obeid, M. et al. Epidemiology and susceptibility profiles of diabetic foot infections in five hospitals in Lebanon. *J. Infect. Developing Ctries.* **12**, 347–351 (2018).
40. Missoni, E. M. et al. Fungal infection in diabetic foot ulcers [4]. *Diabet. Med.* **22**, 1124–1125 (2005).
41. Benitez, E. et al. Bottom-up effects on herbivore-induced plant defences: A case study based on compositional patterns of rhizosphere microbial communities. *Scientific Reports* **7**, (2017).
42. EL-Hefny, M., Mohamed, A. A., Salem, M. Z. M., El-Kareem, A., Ali, H. M. & M. S. M. & Chemical composition, antioxidant capacity and antibacterial activity against some potato bacterial pathogens of fruit extracts from *Phytolacca dioica* and *Ziziphus spina-christi* grown in Egypt. *Sci. Hort.* **233**, 225–232 (2018).
43. Asghari, G., Jalali, M. & Sadoughi, E. *Antimicrobial Activities and Chemical Composition of Essential Oil from the Seeds of Artemisia aucheri* Boiss. *Jundishapur J. Nat. Pharm. Prod.* **7**, 11 (2012).
44. Kotan, R., Kordali, S. & Kadir, A. Screening of Antibacterial Activities of Twenty-One Oxygenated Monoterpenes. *Zeitschrift für Naturforschung C.* **62**, 507 (2007).
45. Guimarães, A. C. et al. Antibacterial activity of terpenes and terpenoids present in essential oils. *Molecules* **24**, 2471 (2019).
46. Dvoodbasha, M. A., Edachery, B., Nooruddin, T., Lee, S. Y. & Kim, J. W. An evidence of C16 fatty acid methyl esters extracted from microalga for effective antimicrobial and antioxidant property. *Microb. Pathog.* **115**, 233–238 (2018).
47. Desbois, A. P. & Smith, V. J. Antibacterial free fatty acids: Activities, mechanisms of action and biotechnological potential. *Appl. Microbiol. Biotechnol.* **85**, 1629–1642 (2010).
48. Shaaban, M. T., Ghaly, M. F. & Fahmi, S. M. Antibacterial activities of hexadecanoic acid methyl ester and green-synthesized silver nanoparticles against multidrug-resistant bacteria. *J. Basic Microbiol.* **61**, 557–568 (2021).
49. Nagaoka, S., Murata, S., Kimura, K., Mori, T. & Hojo, K. Antimicrobial activity of sodium citrate against *Streptococcus pneumoniae* and several oral bacteria. *Lett. Appl. Microbiol.* **51**, 546–551 (2010).
50. Lieleg, O., Caldara, M., Baumgärtel, R. & Ribbeck, K. Mechanical robustness of *Pseudomonas aeruginosa* biofilms. *Soft Matter* **7**, 3307–3314 (2011).

51. Su, L. C., Xie, Z., Zhang, Y., Nguyen, K. T. & Yang, J. Study on the antimicrobial properties of citrate-based biodegradable polymers. *Front. Bioeng. Biotechnol.* **2**, 23 (2014).
52. Petrovici, A. G. et al. A Comprehensive Review of Azelaic Acid Pharmacological Properties, Clinical Applications, and Innovative Topical Formulations. *Pharmaceuticals* **18**, 1273 (2025).
53. Sauer, N. et al. The multiple uses of azelaic acid in dermatology: mechanism of action, preparations, and potential therapeutic applications. *Adv. Dermatol. Allergol.* **40**, 716–724 (2023).
54. Feng, X. et al. Azelaic Acid: Mechanisms of Action and Clinical Applications. *Clin. Cosmet. Invest. Dermatology.* **17**, 2359–2371 (2024).

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Author contributions

N.A.R.: Data curation; Investigation; Methodology; Software; Visualization; Writing – original draft; and Writing - review & editing. M.M.M. and R.S.M.: Conceptualization; Data curation; Funding acquisition; Investigation; Methodology; Project administration; Resources; Writing - original draft; and Writing - review & editing. M.M.M.: designed figures and tables. S. A. K.: Resources; and Writing - review & editing. H. M. E.: antimicrobial section: Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Validation; Writing - review & editing.

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Declarations

Competing interests

The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to N.A.R.

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