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Research Article

Variations in chemical composition, antimicrobial and haemolytic activities of peel essential oils from three local *Citrus* cultivars

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Abstract

The present research work was carried out to appraise the variations in the chemical composition, antimicrobial and haemolytic activities of hydro-distilled essential oils from peels of three *Citrus* cultivars including *Citrus reticulata* (Kinnow), *Citrus sinensis* (Mussammi), and *Citrus x sinensis* (Red blood orange). The essential oil yield from the peels of these cultivars was found to be 0.86, 1.70 and 1.07 %, respectively. Overall, the major chemical constituents (GC-FID analysis) in the peel essential oils of *C. reticulata*, *C. sinensis* and *C. x sinensis* were identified to be limonene (46.30-54.57%), geraniol (10.02-24.00 %) and citraniol (10.05-14.00%). Among the oils tested, peel essential oil of *C. reticulata* exhibited maximum zone of inhibition against bacterial strains whereas that of *C. x sinensis* against fungal strains. The tested peel essential oils exhibited small extent of haemolytic activity (0.29 to 1.09%) indicating negligible cytotoxicity. The antibacterial, antifungal and haemolytic activities of the tested *Citrus* peel essential oils varied considerably ($p<0.05$) among cultivars depending upon the variable composition of the oils.

Keywords: Antimicrobial agents; Cell; *Citrus* Peel essential oil; GC-FID; Hydrodistillation

Introduction

Essential oils are odorous volatile components isolated from different parts of aromatic plants **[1, 2]**. Plant essential oils are often employed to impart flavor to drinks and foods as well as are used as ingredients in the formulations of pharmaceuticals, perfumes and cosmetics products **[3]**. Besides multiple industrial applications, the essential oils are recently gaining greater recognition due to their potential biological such as antioxidant, antimicrobial and bio-pesticidal properties **[4, 5]**.

The genus *Citrus*, from *Rutaceae* family, comprises 1,300 species and 140 genera. *C. sinensis* (Orange), *C. paradise* (Grapefruit), *C. reticulata* (Tangerine), *C. grandis* (Shaddock), *C. limon* (Lemon), *C. aurantium* (Sour orange) and *C. medica* (Citron) are some of the important fruit species of genus *Citrus*. *Citrus* are grown for their fruits in several countries with tropical climates such as Egypt, Pakistan,

China, Brazil, The United States, Turkey, India, Nigeria and Spain [6].

Citrus fruits are valuable food commodity which is popular across the world due to high nutritional value and medicinal benefits. Several *in-vitro* and *in-vivo* studies have indicated *Citrus* fruits to be useful against chronic diseases such as cancers and cardiovascular diseases due to the presence of high amount of vitamin C and phenolic bioactives with antioxidant potential [7].

As results of large scale *Citrus* fruit consumption, huge amount of peels are generated annually, which are often discarded as agro-waste instead of revalorized into value-added products. According to estimates, processing of *Citrus* fruit generates peels and membrane residues containing about 40-45 % of fruit mass as a waste fraction [8]. Interestingly, *Citrus* peels can be explored as a valuable source of essential oil [9]. *Citrus* peel essential oils have been investigated for their potential biological roles such as antioxidant, antimicrobial and anti-inflammatory properties [10]. The volatile chemical compounds such as α-terpineol, linalool and α-pinene, present in *Citrus* peel essential oils, can be linked to antifungal and antibacterial activities [11]. Recently, due to growing microbial drug resistance there is increasing interest in the investigation of essential oils as natural antimicrobial agents [4, 5]. Nevertheless, the oil yield and biochemical composition of plant essentials oils not only vary within the species and its varieties but also among different agro-climatic and geographical regions [4, 5]. Various studies reveal the chemical composition and potential biological activities of *Citrus* peel essential oils [3, 6, 8, 9], however rarely data is available on the inter-cultivar variations in the volatiles composition and biological properties of *Citrus* peel essential oils. This prompts the need to appraise the variations in the chemicals profile and biological attributes of peel essential oils from different local cultivars of *Citrus*.

In perspectives of value-addition, currently, there is increasing interest on the exploration of under-utilized fruit processing agro-wastes (such as peels) for isolation of high-value bioactives and volatile oils [12, 13]. In Pakistan, consumption of *Citrus* is made on large scale both in terms of whole fruit intake and fruit processing by-products. As results of wide scale *Citrus* fruit consumption, a substantial quantity of *Citrus* peels is produced annually that can be utilized for production of essential oil. Rarely efforts have been made towards studying the comprehensive compositional analysis and biological principles of peel essential oils of different *Citrus* cultivars in Pakistan. In the present research variations in the yield, chemical composition and biological activities (antifungal, antibacterial and haemolytic activities) of peel essential oils from three widely cultivated and consumed *Citrus* cultivars (*Citrus reticulata*, *Citrus sinensis*, *Citrus*×*sinensis*) were appraised.

Materials and methods

Collection of samples

Fresh fruits of three *Citrus* cultivars such as Kinnow (*C. reticulata)*, Musammi (*C. sinensis*) and Red blood orange (*C.*×*sinensis*) were collected through local market of Sargodha, Pakistan. The fruits were peeled off using a sharp steel knife. Small pieces were made from recovered peels with a knife and dried under shade in a laboratory. The shade-dried peel material was crushed using a domestic grinder and packed in polyethylene zipped bags.

Isolation of the essential oil

The essential oil was isolated via Clevenger- type apparatus using hydro-distillation technique [14]. The ground *Citrus* peels material was hydro-distilled for 3 h. Distillates of the essential oil were collected and dehydrated over anhydrous sodium sulphate (Na_2SO_4), filtered and then kept at fridge temperature (4°C) until analysed [15].

Chemical analysis of the essential oil

Analysis of *Citrus* peel essential oils was carried out using a Gas Chromatographic

system (Schimadzu) attached to an FID (flame ionization detector). Compounds were separated on DB-5 capillary column. Nitrogen (mobile phase) was flushed at a flow rate of 5 mL/min. Initial column temperature was held at 80 ˚C for 2 min and auto increased to 240 ˚C at the rate of 10 ˚C /min. The percent composition of the compounds was reported relative to total peak areas.

Antimicrobial activity

Microbial strains

Two Gram-positive bacteria such as *S. aureus* (*Staphylococcus aureus*) API Staph TAC 6736152, and *B. subtilis* (*Bacillus subtilis)* JS 2004 and two Gram-negative bacteria such as *E. coli* (*Escherichia coli)* ATCC 25922, and *P. multocida* (*Pasteurella multocida)* (local isolate) were employed to test the isolated essential oils. Four pathogenic forms of fungi including *C. albican* (*Candida albicans), M. canis (Microsporum canis), A. flavus (Aspergillus flavus)* and *F.solani* (*Fusarium solani)* were used to test the antifungal potential of the essential oils. The above mentioned pure microbial strains were acquired from the Clinical Medicine and Surgery Department, University of Agriculture, Faisalabad, Pakistan. Purity and identity were verified and authenticated by the Department of Microbiology at University of Agriculture, Faisalabad, Pakistan. The bacterial strains were cultured in Nutrient agar (NA, Oxoid) overnight at 37 ºC while fungal strains were cultured in Potato dextrose agar (PDA, Oxoid) overnight at 28 ºC.

Disc diffusion method

The antimicrobial activity of the essential oils was evaluated by disc diffusion method **[4, 5]**. Briefly, a 100 µL tested microorganism's suspension, having 10^7 colony-forming units (CFU)/mL of bacteria and 10^6 spores/mL of fungi, were spread on NA and PDA medium, respectively. The compound's solution was added in filter discs (6 mm in diameter) and employed on the agar plates which had formerly been inoculated with the tested microorganisms. Sample-less discs were employed as a negative control. For bacteria and fungi, Amoxycillin (30 µg/dish) (Oxoid, UK) and Flumequine (30 µg/disk] (Oxoid, UK) were set as positive reference to compare sensitivities of strain/isolate. After placing for 2 h at 4ºC, plates were incubated at 37ºC for about 18 h for bacteria and at 28ºC for 24 h for fungal strains. By calculating the diameters of the growth inhibition zones (zone reader) of the organisms, antimicrobial activity was evaluated and compared with the controls **[16, 17]**.

Haemolytic activity

Haemolytic activity of the tested essential oils was evaluated by a prescribed procedure. **[18, 19]**. Freshly heparinized human blood (3mL) was taken from healthy volunteers. The bold was centrifuged for 5 min at 1000 x *g*; plasma was poured off and cells were washed three times with 5 mL of chilled (4ºC) sterile isotonicphosphate-buffered saline (PBS) of pH7.4. In each assay,erythrocytes were preserved at 10^8 cells per mL. Each cultivar's essential oil (100µL) was taken and agitated with RBC (10^8cells/mL) independently. Incubation of samples was done for 35 min at 37ºC. Instantaneously, after incubating, the samples were kept in ice for 5 min and then centrifuged for 5 min at 1000 x *g*. A 100- µL supernatant was collected from each tube and diluted 10 time with chilled (4ºC) PBS. The positive and negative controls employed were Triton X-100 and phosphate buffer saline (PBS), respectively. Using µQuant (Bioteck, USA) the absorbance was observed at 576 nm and % RBCs lysis for each sample was computed.

Results and discussion

Peel portion and essential oils yield

The results regarding % peel portion and % oil yield of essential oils obtained for three cultivars of *Citrus* including *Citrus reticulata* (Musammi), *Citrus sinensis* (Kinnow), *Citrus x sinensis* (Red blood orange orange) are presented in (Table 1). The content of % peel portion from fruits of Red blood orange was higher (36.0%)

followed by Musammi (34.50%) and then Kinnow (31.67%). These outcomes are in accordance with the findings of other scientists. Weiss [20], observed that peel portions of mandarin, sweet orange and lemon were 28.0, 25.0 and 40.0%, respectively. In another work of Manthey & Grohmann [21], peel portions from citrus fruits such as orange, grape fruit and lemon generated 25.6 - 33.0, 21.5 - 38.1, 33.7 - 36.4 and 32.0 - 46.6% of peel mass, respectively. Such variations in the peel mass of selected species from Sargodha region can be attributed to changing environmental factors of area as well as the genotype. These variable morphological and genetical traits of fruits play effective role in altering the composition of peels [22].

Among the *Citrus* species, extracted through Clevenger apparatus using hydrodistillation method, Musammi (*C. sinensis*) peels exhibited maximum oil yield (1.76%) followed by Red blood orange (*C.* x *sinensis*) (1.06%) and Kinnow (*C. reticulata*) (0.86%). The results obtained were compatible with the findings of Weiss [20] who reported the peel essential oil yield from sweet orange, lemon as well as mandarin to be 0.80, 0.90 and 0.80 % respectively, while bergamot orange had 0.45-0.65% peel oil yield. Notable variations in the oil yield from different Citrus cultivars can be mainly linked to the fruit genotype. In another study, cold pressed peel oil yield from oranges, and bergamot petitgrain was 0.5% each whereas, mandarin oil had 0.2% yield, Anon [23]. According to Ahmad *et al.* [24], the highest oil yield (1.21%) was obtained from Malta peel followed by Eureka,s lemon (1.12%), Musammi (0.98%) and Grapefruit (0.73%). Kamal *et al.* [25], reported that highest oil yield was exhibited by *C. sinensis* (0.23-1.08%), followed by *C. reticulata* (0.31-0.52%) and then *C. paradise* (0.21-0.43%). Tue *et al.* [26] reported that yield of peel essential oils differed from 0.2-2.0 % among different citrus species.

Table 1. Peel portion and yield of peel essential oil from different *Citrus* cultivars

Cultivar Common Name	% Peel portion	% Oil yield
*Citrus sinensis (*Musammi)	34.50 ± 0.50	1.76 ± 0.10
Citrus x sinensis (Red blood orange)	36.00 ± 1.00	1.06 ± 0.10
Citrus reticulata (Kinnow)	31.67 ± 1.15	0.86 ± 0.05

The data are mean ±S.D. of peel essential oil from three different samples of each *Citrus* cultivar analysed in triplicate

Chemical composition of *citrus* peel essential oils

The percent composition of major chemical components identified in the essential oils from peels of three different *Citrus* cultivars is depicted in (Table 2). The data, derived from GC-FID analysis, revealed a significant qualitative and quantitative variation in the chemical profile of the oils from selected *Citrus* cultivars. Based upon matching the relative retention time with those of pure standards, major chemical constituents (>1%) detected in essential oils of peels from *C. reticulata*, *C. sinensis* and *C. x sinensis* were limonene, linalool and citranellal, respectively. Nerol, eugenol, geraniol and ethyl benzoate *β*-myrcene, and linalool oxide were also present in considerable amounts.

The present study values are in close agreement with the results of Geraci *et al.* [27] who described that the key components of these oils were myrcene, limonene,nerol, *β*-pinene and geraniol, . Lota *et al.* [28] reported two major monoterpenes: limonene and *γ*-terpinene in essential oils from peels of *Citrus reticulata* [mandarin]. According to Feger *et al.* [29], the chief component in peel oils of

Brazillian Murcot Tangerines was noted to be limonene.

A comparative study, reported on the chemical composition of peel essential oils isolated from Italian Sweet lime as well as Bergamot, revealed that both the oils have quite comparable chemical composition [30, 31]. The differences in the composition of citrus peel oils from different cultivars and regions can be attributed to the agro-climatic and genetic factors [32, 33].

Table 2. GC-FID percent chemical composition of peel essential oil from different *Citrus* cultivars

Volatile Compound	Retention Time	Musammi	Red Blood Orange	Kinnow
Ethyl acetate	1.152	0.89	0.15	0.15
Camphene	1.463	0.32	----	0.18
Acetaldehyde	1.620	3.56	3.56	3.56
Geraniol	1.960	10.02	24.0	12.67
Limonine	2.823	48.9	46.30	54.57
Myrcene	3.780	4.67	----	2.89
γ-undecalactone	3.948	5.00	----	----
Citraniol	5.545	10.05	10.40	14.00
Eugenol	6.013	7.5	12.90	8.90
Others (Unidentified)		1.48	2.70	3.08

The data are mean ±S.D. of peel essential oil from three different samples of each *Citrus* cultivar analysed in triplicate

Antimicrobial activities

The results related to antibacterial activity of peel essential oils from different *Citrus* cultivars against two Gram +ve bacteria such as *S. aureus (Staphylococcus aureus*, API Staph TAC 6736152) and *Bacillus subtilis (B. subtilis* JS 2004) and two Gram-ve bacteria: *E. coli (Escherichia coli* ATCC 25922), and *P. multocida* (*Pasteurella multocida*, local isolate) are presented in (Table 3). In the present study Kinnow (*C. reticulata*) peel essential oil revealed maximum zone of inhibitions against microbial strains i.e. *B. subtilis*, (26.16 mm) *S. aureus* (25.50 mm), *E.coli* (37.42 mm) and *P.multocida* (35.50 mm) as compared to peel essential oil of Musammi (*C. sinensis*) 17.33,15.42,30.33, 21.00 mm and Red blood orange (*C.×sinensis*) 25.55, 23.0,31.83,30.58 mm, respectively. These values revealed that *C. reticulata* peel essential oil is more active against all the bacterial strains as compared to essential oils of *C.sinensis* and *C. x sinensis* [34].

Table 3. Antibacterial activity of peel essential oils from different *Citrus* cultivars

Cultivar	Inhibition zone diameters (mm)			
	Gram-positive bacteria		Gram-negative bacteria	
	B. subtilis	***S. aureous***	***E. coli***	***P. multocida***
Citrus reticulata	$26.16 \pm 0.28^{a}{}_{A}$	$25.50 \pm 0.50^{a}{}_{A}$	$37.42 \pm 0.38^{a}{}_{B}$	$35.50 \pm 0.50^{a}{}_{C}$
Citrus sinensis	$17.33 \pm 0.58^{b}{}_{A}$	$15.42 \pm 0.58^{b}{}_{B}$	$30.33 \pm 0.58^{b}{}_{C}$	$21.00 \pm 1.00^{b}{}_{D}$
Citrus x sinensis	$25.55 \pm 1.00^{c}{}_{A}$	$23 \pm 1.00^{c}{}_{B}$	$31.83 \pm 1.04^{b}{}_{C}$	$30.58 \pm 0.52^{c}{}_{C}$
Standard drug (Amoxycillin)	$43.00 \pm 1.00^{d}{}_{A}$	$38.41 \pm 0.38^{d}{}_{B}$	$42.50 \pm 1.32^{d}{}_{A}$	$43.00 \pm 1.00^{d}{}_{A}$

Values expressed as means ± SD of three separate experiments performed (n=3 ×3). Different caps letters in subscript within the same row express significant (p<0.05) variations of means among bacterial strains. Difference of superscript letters within the same column express significant (p<0.05) differences of means among the *Citrus* cultivars

The considerable antibacterial activity of peel essential oil (EO) of Kinnow (*C. reticulata*) is in line with the results of Sultana *et al*. [35], and Javaid *et al*. [36], who reported that among *Citrus* cultivars tested, *Citrus reticulata* was highly effective against all microbial strains. Peel EO's of *Citrus* species exhibited appreciable equal antibacterial activity on both gram +ve and gram -ve bacteria [37, 38]. The variable antibacterial activity of *Citrus* EOs can be mainly attributed to the variation in the composition of constituents such as oxygenated monoterpenes and phenolics in due part to their genetic make-up. [39, 41].

Antifungal activity

The results of antifungal activity of the tested *Citrus* peel essential oils against *C. albican (Candida albicans), M. canis (Microsporum canis), A. flavus (Aspergillus flavus)* and *F.solani (Fusarium solani)* are shown in (Table 4). Peel EO of Red blood orange (*Citrus x sinensis*) presented maximum zone of inhibitions against *A. flavus* (29.0 mm) and *F.solani* (32.0 mm). But in case of *M.canis* (23.30 mm) and *C.albicans* (22.50 mm) it gave less zone of inhibitions than Kinnow (*C. reticulata*) *A.flavus* (26.00 mm), *F.solani* (31.0 mm), *M.canis* (24.17 mm), *C.albicans* (22.83mm).

Table 4. Antifungal activity of peel eessential oils from different *Citrus* cultivars

Cultivar	Inhibition zone diamtere (mm)			
	C. albican	***M. canis***	***A. flavus***	***F.solani***
Citrus reticulate	$22.83 \pm 0.76^{a}{}_{A}$	$24.17 \pm 0.29^{a}{}_{B}$	$26.00 \pm 1.00^{a}{}_{C}$	$31.00 \pm 1.00^{a}{}_{D}$
Citrus sinensis	$13.67 \pm 0.58^{b}{}_{A}$	$17.5 \pm 0.50^{b}{}_{B}$	$25.67 \pm 0.58^{b}{}_{C}$	$23.50 \pm 0.50^{b}{}_{D}$
Citrus x sinensis	$22.50 \pm 0.50^{A}{}_{A}$	$23.30 \pm 0.26^{a}{}_{B}$	$29 \pm 1.00^{c}{}_{C}$	$32.00 \pm 1.00^{c}{}_{D}$
Flumequine	$37.33 \pm 1.1^{c}{}_{A}$	$38.00 \pm 1.00^{c}{}_{B}$	$39.00 \pm 0.50^{d}{}_{C}$	$42.00 \pm 1.00^{d}{}_{D}$

Values expressed as means ± SD of three separate experiments (n=3 ×3). Various caps letters in subscript within the same row express significant (p<0.05) differences of means among different fungal strains. Difference superscript letters within the same column express significant (p<0.05) differences of means among *Citrus* species

These values of inhibition zones revealed that peel EO of Red blood orange (*C. x sinensis*) is more active against *A.flavus* and *F.solani* while that of Kinnow (*C.reticulata*) is more potent against *C.albicans* and *M.canis*. Meanwhile, Musammi (*C. sinensis*) peel EO exhibited weaker activity against *A.flavus* (25.67 mm), *F.solani* (23.50 mm), *M.canis* (17.50 mm), *C.albicans* (13.67mm) than peel EO of Red blood orange (*C. x sinensis*) and Kinnow (*C. reticulata*). *Citrus* essential oils possess considerable anti-fungal activities. The presence of chemical compounds such as α-pinene, linalool and α-terpineol can be linked to antifungal potential of the oils [42]. Citral is one of the main components of essential oils that acts as a fungicidal since it is capable of forming a charge transfer complex with fungal cells [43]. Furthermore, the variable susceptibilities of the tested organisms to citrus peel essential oils might be ascribed to the variations in the rate of essential oil constituent's penetration via the cell wall and cell membrane components of organisms [44-46]. This indicates that antimicrobial activity of the tested *Citrus* EO's are not only cultivars dependent nevertheless this potential is also dependant on the genetic make of the microbial strains [47].

Cytotoxic /haemolytic activity

Essential oils from peels of different *Citrus* cultivars exhibited negligible cytotoxicity (0.29-1.09%) as compared to positive control, Triton X-100 which offered 100% haemolytic activity as expressed in (Table 5).

Table 5. Cytotoxic activity of peel essential oils from different *Citrus* cultivars

Cultivar	Cytotoxic/Haemolytic Activity (%)
Citrus reticulate	0.29 ± 0.02
Citrus sinensis	1.09 ± 0.34
Citrus x sinensis	0.62 ± 0.12
PBS	0.00
Triton-x-100	100

Obtained results clearly depicted that *Citrus* peel essential oils have very low cytotoxic activity and can be safe to use for nutra-pharmaceutical applications **[48]**. This cytotoxic is interesting property of many saponins to bring about haemolysis i.e. the release of haemoglobin from erythrocytes as result of change in membrane permeability **[49]**.

Some other plants have also been studied for the haemolytic activity towards humans or animal erythrocytes **[50]**. Although the cytotoxicity of essential oils of *Citrus reticulata* and *Citrus sinensis* have been checked against pests, but no data is available on their haemolytic activities against human red blood cells**.**

Conclusion

Among the *Citrus* cultivars selected, Musammi (*C. sinensis)* peels exhibited greater yield of essential oil as compared to Kinnow and then Red blood orange peels. Limonene was found as a major component in three *Citrus* cultivars. All *Citrus* peel oils revealed variable volatile composition owing to differences in genetic makeup of the cultivars. The tested *Citrus* peel essential oils exhibited appreciable antibacterial, antifungal activities. Kinnow *(Citrus reticulata)* peel essential oil exhibited maximum antibacterial and antifungal activity, while other Citrus peel oils revealed relatively lesser biological activities. The tested essential oils also gave negligible level of cytotoxicity and thus can be safe to use as valuable ingredients in nutra-pharmaceutical products. Moreover, it can be depicted that hydrodistillation is an appropriate choice for isolation of essential oils from under-utilized *Citrus* peels. The hydrodistilled *Citrus* peel essential oils can be utilized as natural antioxidant and antimicrobial agents leading to value-addition in Citrus fruit processing industry.

Authors' contributions

Conceived and designed the experiments: F Anwar & T Mehmood, Performed the experiments: R Qadir, S Zahoor & M Shahid, Analyzed the data: F Anwar, T Mehmood & M Shahid, Contributed reagents/ materials/ analysis tools: F Anwar & T Mehmood, Wrote the paper: F Anwar & R Qadir.

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