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EXTRACTION AND CHARACTERIZATION OF ESSENTIAL OILS FROM PEEL OF KINNOW (*Citrus reticulata* L.)

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ABSTRACT

Kinnow peel, a processing industry waste is a rich source of essential oils with immense antioxidant and antimicrobial properties, thus having food and pharmaceutical industry potential. In the present study, the essential oils from kinnow peel extracted using hydro-distillation produced an optimized yield of 1.22% (w/w). Physico-chemical properties of the extracted oil were typical of a water insoluble and light oil with low acid value thus presenting a good storage life. The GC-MS analysis of Kinnow peel essential oils showed the presence of monoterpenes, sesquiterpenes and their oxygenated derivatives. The essential oils had D-Limonene as the major component along with the presence of Myrscene and α- terpineol as the other major components that are typical of manadarin peel oil. The kinnow essential oils had higher EC_{50} and lower AEAC values of 42.8 µM and 0.320 than 28.19 µM and 0.485 in peel extract with ascorbic acid as positive control.

Keywords: Antioxidant, Essential oils, GC-MS analysis, Hydrodistillatio, Kinnow peel

Citrus fruits are consumed worldwide and are one of the prime fruit crops. Punjab, the second largest producer of kinnow (*Citrus reticulata* L.) in India, reported an estimated 8 lakh MT production in 2021. The fruit is produced for its juice which is rich in vitamin C, calcium and phosphorus (Kour *et al.,* 2014). The processing of kinnow produces 35-40% (by weight) of wastes in the form of peel (Mahawar *et al.,* 2020). The latter is a good source of bioactive compounds in the form of flavanoids and terpenes which also possess antioxidant and antimicrobial properties (Sidhu *et al.,* 2016). The highest amounts of flavanoids are present in peels than in other parts, and these can be extracted in the form of essential oils. The latter are comprised of volatile, secondary metabolites which are enriched with a range of terpenoids, alcohols, esters, hydrocarbons, sterols, flavanoids etc. Recently, hispiridin, a flavanone glycoside of citrus peel has been of interest because of its inhibitory effect against SARS-CoV2 replication thus having possible therapeutic activity against COVID-19 virus (Ambrosio *et al.,* 2021). In fact, there are recent reports of essential oils from a number of citrus plants (Goyal and Kaushal, 2018; Lu *et al.,* 2019; Pradhan *et al.,* 2019 and Khalid *et al.,* 2021). Kinnow mandarin is the most important fruit crop of Punjab, India and its peel wastage has not been evaluated for the production of its value added essential oils and other compounds. Hence, the present work was aimed at optimizing extraction of kinnow essential oils by hydrodistillation, their characterization and evaluate their potential as antioxidant agents.

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MATERIALS AND METHODS

Oil extraction from kinnow peel

Peeling of kinnow fruits was performed and the peels were dried at 50° C for 48 hr in hot air oven. It was ground and subjected to hydro-distillation using Clevenger apparatus for 90 min. Herein, peels get exposed to high temperature which releases oil from oil glands (Huet, 1991). Mixture of oil and water recovered during the distillation, was allowed to settle and the oil layer was separated using separatory funnel. The mixture was washed thrice with n-hexane (Kamaliroosta *et al.,* 2015). The essential oil layer containing n-hexane was transferred into round bottom flask. The solvent was eliminated by fractional distillation and the collected essential oils were stored at 4°C for further analysis. The yield of extracted oil was calculated as:

$$\%\ \text{Yield} = \frac{\text{Weight of oil extracted (g)}}{\text{Weight of sample used (g)}} \times 100$$

Physico- chemical characterization of essential oil

The different physical properties of essential oil viz. shrinkage, specific gravity, solubility of the essential oil in water, refractive index and density of essential oil were studied. It was characterized in order to be sure that the extracted liquid was actually the oil. For chemical properties, saponification value, acid value and ester values of the oil were determined by standard methods (Manual of methods of analysis of foods-oils and fats, 2021).

Polyphenols

Kinnow peel was oven dried at 50℃ for 48 hr until moisture content reduced to less than 10 %. Dried peel was ground to fine powder and subjected to maceration. Extraction from 5g of kinnow peel powder was carried out using methanol at 80 % solvent concentration with 1:15 sample/solvent ratio at 40℃ of extraction temperature in a shaking water bath for 20 hr. The extract was then passed through Whatman filter paper and spun at 5000 rpm for 10 minutes. The supernatant was collected and solvent was allowed to evaporate with a rotary evaporator to obtain extract. The extract was further filtered through 0.45μm membrane filter and the total polyphenols were estimated by the method of Malik and Singh (1971).

Analysis of volatile compounds through GC-MS

Different volatile components present in the oil were analyzed by gas chromatography-mass spectroscopy (GC-MS) at Regional instrumentation laboratory (RIL), Punjab University, Chandigarh. The GC-MS was carried out on Trace 1300 GC, TSQ 8000 Triple Quadeupole MS which was fitted with TG 5MS (30m x 0.25mm x 0.25μm) column and S/SL injector. The injector temperature, MS transfer line temperature and ion source temperature were maintained at 250℃, 300℃ and 230℃, respectively. The column temperature was in the range of 60-280℃ with increase of 10℃/min, using helium as carrier gas at carrier flow rate of 1 ml/min. Injection volume of 1.0μl was prepared in Dimethyl sulphoxide (DMSO) having split flow of 1 ml/min, the mass spectra was taken at 75 eV with mass scan range by m/z 40-500 amu. The individual components were identified by comparing their mass spectra using standards using NIST (National Institute of Standards and Technology, US Dept. of Commerce) compounds.

Determination of antioxidant activity

The free radical scavenging activities of kinnow peel oil and extract were analyzed using DPPH (2,2-diphenyl-1-picrylhydrazyl) method of Sanchez-Moreno *et al.* (1999). EC_{50} was analyzed by assaying percent inhibition of free radical generation by extracts using various concentrations. Sample concentrations with 50 % scavenging activity were selected and their graphs were drawn for all the samples of kinnow peel extract and ascorbic acid as positive control (Shimamura *et al.* 2014). The free radical scavenging activity of each analytical sample was expressed relative to ascorbic acid.

RESULTS AND DISCUSSION

Oil extraction from kinnow peel

The Clevenger apparatus using hydro-distillation involves heating mechanism which increases the oil yield during extraction from ruptured oil glands. The penetration of solvent (water) in peel tissue depends on temperature which thus influences extraction of essential oil from kinnow peel. The results revealed that the amount of oil extracted with time increases significantly till 90 minutes of time span after which further increase in time resulted in charring of oil (Table 1). Further, results presented in Table 1 reveal that the optimum weight of solid to solvent ratio is 80g peel/250 ml water. The latter produced an optimum kinnow oil extract yield of 1.22% (w/w) as with further increase in the solid to solvent ratio, bubbling occurs and results in loss of oil vapor during condensation. This provides a comparatively higher yield than reported elsewhere on essential oil yield from orange peels. In literature, oil yield of 0.29% with a hydro-distillation temperature of 122°C from kinnow peel has been reported (Sharma 2016). Goyal and Kaushal (2018) and Dwivedi *et al.*(2018) reported extraction yield of 0.3% and 0.04% from kinnow peel. Earlier, Nadeem and Asghar (2016) reported that yield of peel oil from *Citrus sinensis* (malta) was the highest (1.21 %) among all citrus fruits while Tao *et al.* (2008) reported a higher yield of 3% in peel of *Satsuma mandarin*.

Table 1. Standardization of peel weight and extraction time on recovery of essential oils from kinnow peel

*Extraction parameter	Quantity of oil (ml)
Weight of peel (g/ 250 ml water)	
50	0.6
60	0.7
70	0.8
80	1.2
100	1.1
**Extraction time (min) for 80g/250 ml	
10	0
30	0.25
50	0.55
70	0.95
90	1.20
100	1.05
*$CD_{wt.}$	0.12
**CD_{time}	0.175

Physico- chemical characterization of essential oil

The analysis of physical and chemical properties is pertinent in order to be sure that the extracted liquid is oil. The organoleptic property of the essential oil extracted in Clevenger apparatus was colorless and it had fresh tangy citrus smell (Table 2). The weight of essential oil is determined by the specific gravity which is also the most important factor in determining the quality as well as purity of essential oil. The specific gravity value of essential oil was found out to be 0.815±0.45. The essential oil extracted from the Clevenger apparatus has a specific gravity less than 1 which implies that oil is lighter than water and is insoluble in it. Similarly, a refractive index is a unique number which designates how the oil bends light. The refractive index of 1.47 is categorized as highly pure by Kumar (2014) and the refractive index of our essential oil was found out to be 1.439. The density of the oil was estimated to be 0.835 gcm^{-3}.

Table 2. Physico-chemical properties of kinnow peel essential oil extract

Physical properties	
Parameter	**Value**
Color	Colorless
Odor	Fresh tangy citrus smell
Solubiltiy	Insoluble in water
Specific Gravity	0.815±0.45
Density (g/cm^3)	0.835±0.56
Refractive Index	1.439±0.04
Shrinkage (%)	64.17±6.83
Chemical properties	
Parameter	Value
Saponification Value (mg KOH/g)	181.73±7.47
Acid Value (mg KOH/g)	4.45±6.11
Ester Value	177.28±4.60
Total Phenol Content (mg/100g)	53.77±0.69

The saponification value means the number of milligrams of KOH required to saponify fat under the specified conditions. It is the index of the mean molecular weight of the fatty acids of glycerides. The saponification value of the essential oil extracted in the present study was 181.73 mg KOH/g. The saponification value is inversely proportional to the molecular weight of the oil (Onwuka, 2005). The acid value indicates the extent of rancidity and the acid number of essential oil crude extract in present study was found to be 4.45 mg KOH/g. The low acid value of extracted oil indicated a good shelf life. The ester value of the oil was estimated to be 177.28 mg KOH/g.

GC-MS analysis of essential oil

The relative abundance of volatiles detected in essential oil extracted from kinnow peel was analyzed qualitatively through GC–MS (Table 3 and Fig. 1). Several volatile compounds from both hydrocarbon fraction and oxygenated fractions were identified. The monoterpene hydrocarbons constituted the most dominant chemical group among which limonene is most predominant and has antimicrobial properties too. Besides D-Limonene (43.89%), Myrcene (9.13%), 4-carene (2.92%) and α- Phellendrene (0.76%), which are considered to be the major class of compounds found in mandarin essential oil extract (Tao *et al.* 2008; Dwivedi *et al.* 2018; Ambrosio *et al.* 2021). The oxygenated monoterpene hydrocarbons observed were α- terpineol (7.67%), Decanal (2.53%), Curlone (2.11%), Carveol/Citral (0.56%) and Octanal (0.46%). Khaleel *et al.* (2018) earlier reported citrus peel as a sole source of α-Terpineol. Kinnow essential oil extract contained considerable amount of oxygenated Sesquiterpene hydrocarbons and sesquiterpene hydrocarbons. The oxygenated Sesquiterpene hydrocarbon were Dodecanal, (2.53%), Cyclododecanal (0.89%) and 2,6 dimethyl-10 methyli-denedodecana 2,6,11-trienal (0.75%) whereas, Sesquiterpene hydrocarbons were Cis- muurola-4,5-diene (2.09%), Cis- muurola-3,5-diene (1.85%), Humulene (0.3%), Cis-α-Farnesene (0.28%) and naphthalene derivatives (3.2%). Farnescene and naphthalene derivatives have also been observed by Tao *et al.* (2008) in *S.mandarin* and Muurola-4,5-diene has been reported in traces in peel of different citrus species (Vitalini *et al.,* 2021)

Anon (2004) studied that the major chemical components in tangerine peel essential oil are limonene and citral. Choi and Sawanura(2000) investigated Hyuganatsu oil and found that the most common components were limonene (80.35-82.39 %), α-terpinene (7.71-9.03 %), myrcene (2.11-2.28 %), linalool (1.37-2.01 %) and α-pinene (1.17-1.43 %). Our results are also in consensus with the findings of Gancel *et al.* (2000) in terms of the chemical composition of *Citrus paradisii* oil. Vekiari *et al.* (2002) reported limonene, ß-pinene, myrcene, neral, geranial, neryl acetate and ß-caryophyllene as the major components of citrus essential oils. Lota *et al*. (2001) studied essential oil extracted from peels of sour orange found that limonene and α-pinene as the main compounds. In fact, significant variations have been observed in the chemical composition of essential oils among different citrus species probaby to the difference in their genetic profile and also drying conditions of peels (Ahmad *et al.,* 2006, Ambrosio *et al.,* 2021, Vitalini *et al.* 2021). The present study thus reveals the presence of known essential oil components in kinnow peel oil thus strengthening its potential as a high value bioactive

Table 3. Volatile components analyzed in kinnow essential oil extract through GC-MS

Compound Name	Molecular Formula	Retention Time	% area
Monoterpene Hydrocarbon			
4- Carene	$C_{10}H_{16}$	10.77	2.92
α- phellandrene	$C_{10}H_{16}$	11.78	0.76
α- Myrcene	$C_{10}H_{16}$	12.22	9.13
D-Limonene	$C_{10}H_{16}$	13.18	43.89
Oxygenated Monoterpene Hydrocarbon			
Carveol/Citral	$C_{10}H_{16}O$	11.63	0.56
Octanal	$C_8H_{16}O$	12.48	0.41
α - Terpineol	$C_{10}H_{18}O$	13.34	7.67
Decanal	$C_{10}H_{20}O$	16.77	2.53
Curlone	$C_{16}H_{18}O$	25.52	2.11
Sesquiterpene Hydrocarbon			
Cis Muurola-3,5 diene/ α- Cubenene	$C_{15}H_{24}$	19.96	1.85
Cis Muurola-4,5 diene	$C_{15}H_{24}$	20.16	2.09
Cis α-Farnesene	$C_{15}H_{24}$	21.11	0.28
Humulene	$C_{15}H_{24}$	21.30	0.30
Napthelene derivatives	$C_{15}H_{24}$	22.25	3.20
Oxygenated Sesquiterpene Hydrocarbon			
Dodecanal	$C_{12}H_{24}O$	16.77	2.53
Cyclododecanal	$C_{15}H_{24}O$	20.36	0.89
2,6 dimethyl-10 methyli-denedodeca-2,6,11-trienal	$C_{15}H_{22}O$	24.74	0.75

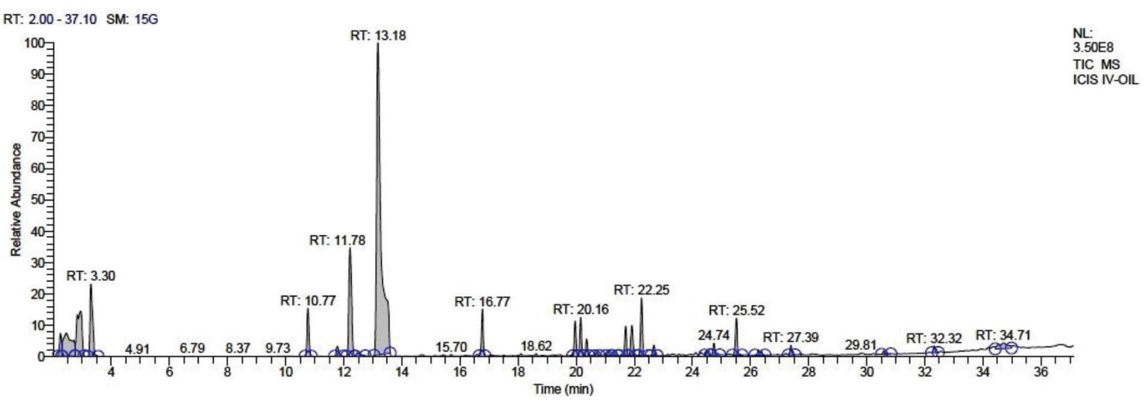

Fig 1. GC-MS chromatogram of kinnow peel extract. Column TG 5MS (30mX0.25mmX0.25um), Injector, S/SL, Column temperature: 60-280 C@ increase of 10C/min, Injector temperature: 250C, MS transfer line temperature: 300C, Ion source temperature: 230C, Mass spectra (75eV,m/z 40-500 amu).

commodity.

Polyphenol extraction and antioxidant activity of kinnow peel essential oil

Results presented in Table 2 revealed that 53.77 mg GAE/100 g total phenols were extracted from kinnow peel through maceration technique using 80 % methanol as a solvent. The DPPH scavenging activity for kinnow peel oil and extract was tested at different concentrations of 30, 70 and 100 µM, respectively for determination of EC_{50} values and AEAC values. The results revealed that concentrations of kinnow peel essential oil and extract needed for 50 % inhibition were 28.19 and 42.8 µM, respectively in comparison to the concentration of ascorbic acid taken as positive control which was13.72 µM. Hence, in terms of AEAC, these were 0.320 and 0.486 effective as equivalent to per unit (1.0) of ascorbic acid (Table 4).

Table 4. DPPH scavenging activity with respect of different concentrations of Kinnow peel extract

	Concentration (µM)	OD_{515} of samples (A_s)	% DPPH scavenging activity	EC_{50} Values (µM)	AEAC
Ascorbic acid	20	0.40	59.18	13.72	1.0
	40	0.040	95.60		
	60	0.033	96.63		
	80	0.230	97.65		
	100	0.030	99.86		
Kinnow peel extract	30	0.440	51.00	28.19	0.486
	70	0.249	74.52		
	100	0.080	91.83		
Kinnow peel essential oil	30	0.557	43.16	42.80	0.320
	70	0.384	60.81		
	100	0.263	73.16		

The correlation among total phenol contents and antioxidant activity has been widely studied in different fruit and vegetables (Klimczak *et al.,* 2007; Jayaprakash *et al.,* 2008). The antioxidant activity of citrus essential oils is due to the presence of variable phenolic compounds as the DPPH scavenging activity in hydro-distillated oils of Valencia orange, Ponkan, and Eureka lemon were 77.8, 81.7 and 54.5%, respectively (Lu *et al.,* 2019). Raffiq *et al.* (2019) reported IC50% of 73.15 ug/ml of kinnow peel extract and advocated their richness in bioactive compounds.

This study thus reports standardization of hydro-distillation technique for extraction of essential oils from kinnow peel that revealed the presence of limonene as the primary component and having a good antioxidant activity. It may thus be concluded that instead of disposing off the peel it needs to be used for producing essential oils which have wide applications in food and pharmaceutical industries for flavouring, preservation, cosmetics, etc.

Authors' contribution

Conceptualization and designing of the research work (AK, GSK); Execution of field/lab experiments and data collection (AK, GSK); Analysis of data and interpretation (AK, GSK, K); Preparation of manuscript (AK, GSK, K).

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