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Purification of bioactive fractionations of industrial importance from *Citrus reticulata* Blanco waste peel

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Abstract

In the present study, essential oil from *Citrus reticulata* Blanco waste peel is exploited for its commercially important components like limonene, geraniol and citral as well as the methodology for the production of its bioactive fractions has been comprehensively described. Extraction of oil was carried out at different temperatures (110-120°C) followed by fractionational distillation and low temperature chilling. Maximum essential oil yield was obtained at 115°C. Gas chromatographic mass spectrometric (GC-MS) analysis showed that limonene, geraniol and citral were present in major concentration in citrus peel oil. Limonene, geraniol and citral have important role as flavoring and fragrance components. These were separated from all other minor components present in the citrus peel oil to determine their collective antioxidant and antifungal activities. Antioxidant activity of *Citrus reticulata* Blanco peel oil and its fractions separated by fractional distillation and chilling were also determined. Antioxidant assays were carried out to determine total phenolics, flavonoids, flavonols, hydrogen peroxide and DPPH scavenging activities. Disc diffusion method was used to determine antimicrobial activities of essential oil against *Penicillium notatum* and *Aspergilus niger.*

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1. Introduction

Essential oils are strong aromatic and hydrophobic liquids having volatile nature. Because of the presence of a definite scent they are called essential. Essential oils are composite mixtures of hydrocarbons, aldehydes, alcohols, ketones, organic acids and esters. Chemically these oils are different from cooking oils because these are not esters of glycerides [1]. Essential oils are generally extracted from non-woody plant parts through the distillation, solvent extraction or cold pressed methods. Oil composition could be affected by ripeness of fruit, vegetation period of plant, storage situation and extraction process [2]. Essential oils are used in different industries and as flavoring agents in food. Essential oils are also used medically since ancient times [1]. Currently essential oils are very commonly used in aromatherapy because they are accepted as having remedial property. It also confirmed that they have many biological properties e.g antioxidant, anti-inflammatory, antiviral, antibacterial, stimulator of nervous system etc [3]. Cold pressing method is conventional method for the extraction of essential oil as in this method watery emulsions are formed which then centrifuged to separate essential oil from water. Distillation method is preferred over cold pressing in some countries because it is economically cheap and more oil yield obtained. In distillation method, steam or boiling water is applied to citrus peel which evaporates essential oil along with steam, followed by separation from water. Citrus fruit peel is important byproduct, which has two tissues called the flavedo and albedo. Flavedo is present in outer layer of peel and due to disappearance of chlorophyll its color changes from green to orange during ripening, essential oil is present in it. Albedo contains important nutrients. Citrus peel contains water up to 65%. By the process of fractionation these components could be separated. Fractionation of essential oil involves the separation of the components present in oil. The separated components individually have different applications in different industries and can be utilized in a better way. In a previous study, citrus fruit peel oil was found to have more than 50 chemical components present whereas limonene was a major component [4]. The present study was aimed to describe the utilization of waste *Citrus reticulata* Blanco essential oil, its fractions and purified components to enhance its commercial importance.

2. Material and Methods

2.1. Sample collection and essential oil extraction and fractionation

Mature fruits of *Citrus reticulata* Blanco were collected from citrus garden, University of Agriculture, Faisalabad, Pakistan. The voucher specimen number assigned to *Citrus reticulata* Blanco was CH0002M. The fruit was peeled off and peel was washed using de-ionized distilled water (DDW) to remove the dust particles and other impurities. Citrus peel was cut into small pieces before further progressing. Citrus peel essential oil was extracted using steam distilation. The essential oil was extracted under different extraction temperature (110, 115 and 125°C). Citrus peel essential oil was dried using anhydrous sodium sulphate and stored in tightly closed vials at 4°C until analysis. The yield of essential oil extracted from peel was determined as ml of oil per kg of peel. Fractionation of citrus peel oil was carried out using fractionation distillation using a fractionating column and by chilling process.

2.2. Gas chromatography- Mass spectrometric (GC-MS) analysis

Essential oil samples were analyzed by Gas Chromatographic-Mass spectrometric analysis (GC-MS) (Perkin Elmer Gas Chromatograph Model # Clarus 600). The oven temperature with a hold up time of 2 min was programmed from 60-280˚C at a rate of 3˚C/min. Carrier gas used was helium at a flow rate of 1ml/min. Mass spectra was recorded from 40-500 m/z. The MS operating parameters were as follows: ionization voltage, 70ev; scan rate, 500amu/s. Each compound was identified to a comparison of its spectral data with reference spectra in the data base (Wiley AccessPak V7, May 2003 and NIST2005 version 2.1.0).

2.3. Antioxidant activity

Antioxidant activity of citrus peel oil extract was assessed by measuring total phenolics content, DPPH, reducing power ability, hydrogen peroxide and flavonol content. Determination of total phenolic contents and DPPH scavenging activity was done by according method described by [5]. Quantification of total flavonols and hydrogen scavenging activity was performed by following procedure defined by [6]. The measurement of reducing power ability (RPA) was done according to the method published previously [7].

2.4. Antifungal activity

The antifungal activity in terms of inhibition zone of the essential oil was tested against two fungal strains, including *Penicillium notatum* and *Aspergilus niger*. Fungal strains were obtained from microbial bank of the Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan. For determination of inhibition zone, 10µL of *Citrus reticulata* Blanco peel oil or its fraction was applied on paper discs placed on solid agar medium. Each of the plate accommodated five discs without unacceptable overlapping of zones. The treated Petri dishes were then incubated at 30°C for 48 hrs. The zone of Inhibition was measured with digital zone reader.

3. Results and Discussion

3.1. *Effect of extraction temperature*

In the present study extraction of citrus peel essential oil was carried out at three different temperatures (110, 115and 125°C) through the steam distillation shown in table 1. Essential oil yield was affected by the extraction temperature. The maximum citrus essential oil yield (0.29%) was obtained at 115°C while minimum citrus essential oil yield (0.24%) was obtained at 100°C by direct steam distillation. Tue *et al*. (2002) observed considerable variation among the yield of essential oil from citrus species i.e, *C. reticulata*, *C. sinensis* and *C. paradisi*. The highest amount of oil was found in *C. reticulata* (0.30%) while lowest amount in *C. paradisi* (0.20%). About 0.24% oil yield was found in *C. sinensis*. Their results showed that the yield of citrus essential oils was differing with individual plant species ranging in most of the cases from 0.2-2.0%. This is also in agreement with [8] who reported a yield of 0.03%, 0.13% and 0.25% for fresh peels of pummelo, orange and tangerine, respectively. In a previous study yield of 0.71% and 0.79% for Colombian mandarin and orange peel oils was reported. The hydro-distilled essential oil content from fresh, ambient, and oven-dried peels of *C. reticulata*, *C. sinensis* and *C. paradisii* ranged from 0.30-0.50, 0.24-1.07 and 0.20-0.40 g/100 g, respectively. However, effect of temperature on citrus essential oil extraction was not found in literature. [9].

3.2. Fractionation of citrus peel oil.

Essential oils are complex mixture of hundreds of chemical ingredients. This complex can be broken into simpler components or single separated chemical constituents. Which are raw material for further industrial chemical ingredients and are used as blocks to introduce a partial flavor or aroma into another product, such as pharmaceutical products, perfumes and toiletries, hygiene products and pesticides [10]. The volatile and non-volatile fractions of essential oil were separated during the process of fractionation are shown in table 2.

3.3. Gas chromatographic mass spectrometric analysis (GC-MS).

Citrus oil was fractionated into volatile and non-volatile fractions using fractional distillation and low temperature. Essential oils are constant boiling isotropic mixtures. That might be the reason being the presence of limonene, geraniol and citral in all fractions. The components present in varied concentrations in different fractions of essential oil obtained.

3.4. Antioxidant activity

Folin ciocalteu reagent assay was used to determine the total phenolic content, diphenyl-2-picrylhydrazyl (DPPH) was used to study DPPH scavenging activity, Potassium ferocyanide was used in reducing power ability and hydrogen peroxide was used for hydrogen peroxide scavenging activity. It is reported that orange peel is one of the important dietary sources of antioxidant phenolics. Flavanones, flavones and flavonols are three types of flavonoids that occur in Citrus fruit [11].

Table1: Effect of extraction temperature on citrus peel essential oil yield

Extraction Temperature (°C)	Essential oil yield (v/w) %
110	0.25±0.15
115	0.292±0.22
120	0.257±0.18

Table 2: Fractionation of essential oil for the separation of volatile and non-volatile fractions

Extraction temperature (°C).	Fractionation process using fractional distillation (°C)	Fractionation temperature using chilling (°C)
110	243±0.20	-23.0±0.15
115	250±0.18	-21.4±0.25
120	241±0.35	-22.3±0.30

Table 3: Gas chromatographic analysis of citrus peel essential oil

Essential oil extraction temperature (°C)	sample	D-limonene (%)	Citral (%)	Geraniol (%)
120	Essential oil	60.1	24.6	14.2
	Volatile fraction	62.1	13	17
	Non-volatile fraction	63	22.2	2.2
115	Essential oil	50.9	22.8	22.6
	Volatile fraction	32.8	27.7	2.9
	Non-volatile fraction	28.8	14.7	14.8
110	Essential oil	51.2	29.9	3.4
	Volatile fraction	48.3	25.3	7.9
	Non-volatile fraction	62.5	21.2	4.4

Table 4: Antioxidant activity of citrus peel essential oil and its fractions

Extraction temperature (°C)	**sample**	**TPC's (mg/L)**	**TFC's (mg/L)**	**DPPH's (mg/L)**	**RPA (mg/L)**	**H_2O_2 (mg/L)**
120	Essential oil	37.30±0.36	51.64±0.32	18.82±0.52	32.41±0.15	53.54±0.07
	Volatile fraction	45.35±0.24	33.76±0.30	67.06±0.09	18.52±0.21	61.12±0.18
	Non-volatile fraction	1.08±0.22	57.89±0.51	27.05±0.32	7.8±0.45	11.92±0.12
115	Essential oil	51.21±0.12	33.12±0.23	40.0±0.25	27.78±0.05	55.28±0.14
	Volatile fraction	35.01±0.21	28.15±0.45	29.41±0.45	34.08±0.25	62.13±0.05
	Non-volatile fraction	6.62 ±0.30	52.32±0.25	28.92±0.27	33.29±0.08	48.19±0.06
110	Essential oil	87.82±0.21	32.20 ±0.12	67.05±0.09	33.08±0.09	26.12±0.18
	Volatile fraction	2.57±0.25	41.19±0.35	38.82±0.13	32.27±0.08	22.19±0.07
	Non-volatile fraction	46.58±0.15	34.49±0.10	42.64±0.12	30.98±0.12	49.71±0.25

Table 5: Antifungal activity of citrus peel essential oil

Extraction temperature (°C)	**Zone of inhibition against *Penicillium notatum* (mm)**	**Zone of inhibition against *Aspergillius niger* (mm)**
110	17.8±0.25	2.11±0.40
115	13.0±0.50	1.30±0.35
120	15.8±0.45	1.78±0.25

In the present study, total phenolics of *Citrus reticulata* Blanco were determined by applying total phenolic content essay, and it was observed that the total phenolic contents of various fractions of essential oil were different. Citrus peel essential oil showed very good antioxidant activity and five different antioxidant essays were performed. It was observed that *C. reticulata* peel essential oil showed notable results about antioxidant activity. Free radical scavenging activity is greatly influenced by phenolic composition of the sample [5].

3.5. Antifungal activity of citrus peel oil.

The antifungal activity of citrus peel essential oil and its fraction was tested against two fungal strains i.e. *Penicillium notatum* and *Aspergilus niger*. Essential oils and its fractions act as a fungicidal agent because it is able to form a charge transfer complex with an electron donor to fungal cells, which results in fungal death. The germination of *Penicillium digitatum* conidia was stimulated by certain combination of the volatiles such and CO_2. This might be due to the mechanism developed by some fungal pathogens using the secondary metabolites as a signal to initiate germination [2]. Because of the presence of such chemical compounds in the citrus peel essential oil it has very good antifungal activity. Essential oil extracted on different conditions of temperature showed considerable difference in their antifungal activity.

Conclusion

The maximum peel oil yield was obtained on 115°C temperature. GCC-MS analysis showed the presence of 3 major chemical constituents D-limonene, citral and geraniol in citrus peel oil. The highest concentration of D-limonene (63%) was in the volatile fraction of the essential oil (E.O) extracted at 120°C, citral (29.9%) in the E.O extracted on 110°C and geraniol (22.6%) in the E.O extracted on 115°C. The highest TPC's were found in essential oil extracted at 110°C, TFC's in non-volatile fraction of E.O extracted at 120°C, DPPH's in the E.O extracted at 110°C, RPA in the volatile fraction of the E.O extracted at 115°C & hydrogen peroxide scavenging activity in the volatile fraction of the E.O extracted at 115°C. Maximum antifungal activity against *Penicillium notatum* and *Aspergilus niger* was shown by the essential oil extracted at 110°C.

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