VOLATILE COMPONENTS, ANTIOXIDANT AND ANTIMICROBIAL ACIVITY OF *CITRUS AURANTIUM* VAR. BITTER ORANGE PEEL OIL

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Summary

Essential oil of Citrus aurantium var. bitter orange was analyzed by GC-MS. Out of 8 components, 3 were identified from their fragmentation pattern. Among the identified constituents, limonene(98.173%) was found as a major component followed by α-pinene (0.476%) and β-pinene (0.176%).

The antibacterial activity of the essential oil of *C. aurantium* was determined by paper disc diffusion method, against Gram positive and Gram negative bacteria (*Bacillus subtilis* ATCC 6633, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Pseudomonas aeruginosa, Pseudomonas fluorescens, Staphylococcus aureus, Escherichia coli* and Enterobacter species). Maximum zone of inhibition was resulted against Gram positive bacteria i-e *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus while Gram negative strains* i-e *Salmonella typhimurium*, *Pseudomonas aeruginosa, Klebsiella pneumoniae and Escherichia coli were found resistant to C. aurantium* peel oil.
The peel oil was found to have radical scavenging effect on DPPH in the range of 37.42-94.64% with oil concentration 20-100% when compared to BHT being strong antioxidant reagent.

Key words: *Citrus aurantium,* Limonene, antibacterial activity, 2, 2-diphenyl-1-picrylhydrazyl

Introduction

Essential oils are the complex mixture of terpenic hydrocarbons and oxygenated derivates such as aldehydes, alcohols, ketones, organic acids and esters[1]. These oils are widely used as flavouring and masking agents in many food, cosmetics and pharmaceutical industries as well as in aromatherapy[2]. Essential oils have also been shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant proreties[3,4]. Some oils have been used in cancer treatment[5].

The genus Citrus of family Rutaceae includes various species of oranges, mandarins, limes, lemons and grapefruit. Citrus fruits are mainly possessed for juice leaving peels as a primary waste. These peels are a potential source of essential oil[6] and yield oil in the range of 0.5-3.0kg/tonne of fruit[7]. Citrus oil contains large amount of monoterpene hydrocarbon (70-95%) along with smaller amount of sesquiterpene hydrocarbons which are responsible for a characteristic flavour[8,9].The major use of citrus peel oil is as a flavouring agent in candies, carbonated and non-carbonated beverages, bakery products, ice-cream, cakes and biscuits, confectionery and chewing gum[10].The food and pharmaceutical in Pakistan are extensively using citrus oil and the present citrus peel oil is worth Rs. 300 million[11]so there is a dire need to develop a feasible technique for the production of essential oils from indigenous resources. 27 species of Rutaceae exist in Pakistan [12]. Out of these 27 species, 10 belong to Genus Citrus. *Citrus aurantium* var. bitter orange is one of them.
The essential oil of *Citrus aurantium* is aromatic, internally stomachic and externally tonic [13]. It has been used to add aroma to beverages and liquors and as an ingredient to give fragrance to soaps, detergents, cosmetics and perfumes [14].
The aim of the present study is to explore the chemical composition of essential oil of locally available *Citrus aurantium* var. bitter orange peel oil as well as its anti microbial and antioxidant properties.

Materials and methods

Extraction of oil:

The fruit was washed, peeled off and peels were cut into pieces. The cut pieces were subjected to hydro-distillation by using reverse Dean Stark apparatus[15]. The steam distillate was removed and dried over anhydrous sodium sulphate and stored at low temperature.

GC-MS Analysis:

The analysis of the essential oil was carried out on GC-MS of Agilent Technologies, Model 6890N, operating in EI mode at 70 ev equipped with a split-splitless injector. Helium used as a carrier gas at the flow rate of 1ml/min, while HP-5MS (30 m × 0.25 mm, 0.25 µm) capillary column was used. The initial temperature was programmed at 50-140ºC at the rate of 5ºC/min and then 100-250ºC at the rate of 3ºC/min followed by a constant temperature at 260 ºC for period of 20 minutes. Sample (2µL) was injected to column programmed at 200ºC and resolutions of components were attained. The components were identified by their retention time and peak enhancement with standard samples in gas chromatographic mode and NIST library search from the derived fragmentation pattern of the various components of the oil.

Test Organisms:

In vitro antimicrobial studies were carried out on six bacterial strains (*Bacillus subtilis* ATCC 6633, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Escherichia coli*) some of which were obtained from PCSIR laboratories complex, Lahore and other from pathological laboratory of a local hospital. The cultures of bacteria were maintained in their appropriate agar slants at 4°C throughout the study and used as stock cultures.

Antibacterial Assay:

Paper disc diffusion method as reported by Bauer *et.al* [16] was applied with slight modification to test the antimicrobial activity of *C. aurantium* peel essential oil. Discs of Whatman No.1 filter paper having a diameter of 6 mm wrapped in tinfoil were oven dried at 65ºC overnight for sterilization.

Normal strength nutrient agar medium(OXOID, England) was prepared and autoclaved at 121±1ºC for 15 minutes under 15psi for culture growth through out the study to evaluate the antibacterial activity of the oil under investigation.

For antibacterial assay 24h old bacterial cultures grown at 36±1ºC were used. Cultures were diluted 10^{-1} in sterile ringer solution[17] containing approximately 10^6CFU/mL in each case. Twenty five micro-liters of these suspensions were inoculated to plates containing sterile nutrient agar medium using a sterile cotton swab in order to get a uniform microbial growth on both control and test plates.

Filter paper discs each impregnated with 10µl, 15µl and 20µl of oils were placed on pre-inoculated culture media under aseptic conditions separately and incubated at 36±1ºC for 24h.

The zone of inhibition in each case was measured as the diameter (in millimeters) of the clear zone around the discs. All experiments were performed in duplicate. Penicillin G and Streptomycin were used as positive controls. Inhibitory effect of positive controls was tested for all microorganisms used in this study under the incubation conditions as mentioned above. The working solution of control antibiotics were prepared in appropriate amounts (0.01g/10mL) then 25µL of each antibiotic solution was dropped on paper discs which is then used during this study.

Antioxidant activity:

Antiradical activity was evaluated by measuring the scavenging activity of the examined *C. aurantium* oil on DPPH radical. The DPPH assay was performed as described by Epsin et al [(18)]. The samples (100 µl each) were mixed with 3 ml of DPPH solution. The absorbance of the resulting solutions and the blank (with only DPPH and no sample) were recorded after an incubation time of 30 minutes at room temperature against BHT as a positive control. For each sample, 3 replicates were recorded. The disappearance of DPPH was measured spectrophotometrically at 517 nm. The percentage of radical scavenging activity was calculated using the following equation;

DPPH scavenging effect (%) = $(A_0-A_1)/A_0 \times 100$

Where $A_{0\ is}$ the absorbance of the control at 30 minutes and A_1 is the absorbance of the sample at 30 minutes.

Results and Discussion

The essential oil was extracted from peels of *C. aurantium* var. bitter oil by hydro-distillation. The yield of oil was 0.622%. The yield is slightly less than as reported by Hifnaway et al[(19)].

GC-MS of the essential oil revealed the presence of 8 components. Out of which 3 have been identified from their fragmentation pattern by mass spectroscopy using MS library (Table-1).The essential oil of *C. aurantium* was found to be constituted of monoterpene hydrocarbons only. Among the constituents limonene (98.173%) was found as a chief component followed by α-pinene (0.476%) and β-pinene (0.176%).

Table 1: GS-MS Analysis of essential oil of *Citrus aurantium* peels oil.

S. No.	Components	% age	M/Z Values
1	Limonene	98.173	M^+(136,31)(121,34)(107,29)(103,3)(93,85)(79,44) (68,100)(63,4)(53,25)
2	α- pinene	0.476	M^+(136,8)(121,17)(105,13)(93,100)(80,6)(77,31) (67,6)(65,6)(55,6)(53,8)
3	β-pinene	0.176	M^+(136,5)(121,8)(107,4) (93,100) (79,19)(65,7)(53,10)(50,3)

The results are in conformity with previous GC-MS studies of *C. aurantium* peel oil[20,21,22] however the local variety of *C. aurantium* showed a relatively higher concentration of limonene i-e. 98.173%. It is used as a fragrance material in perfuming household products and as a component of artificial essential oils. The other constituents of peel oil i-e α-pinene and β-pinene are used as a starting material in fragrance and flavour industry[23].

The data pertaining to the *in vitro* antimicrobial potential of C. *aurantium* essential oil against Gram positive and Gram negative bacteria along with control antibiotic are presented in table-1. These results showed that *Bacillus subtilis* exhibited maximum antimicrobial activity at 20µL concentration of oil with an inhibition zone diameter (IZD) of 11±0.36mm. The IZD of *Staphylococcus aureus* was 12±0.28mm for discs impregnated with 15µL of essential oil whereas with higher conc. of C. *aurantium* peel oil (20µL) the IZD was also increased to18±0.35mm. In present study, the biological activity of *C. aurantium* essential oil was also evaluated against four G-ve bacteria including *Salmonella typhimurium, Pseudomonas aeruginosa, Klebsiella pneumoniae*. The results indicated that all tested G-ve bacteria were resistant to C. *aurantium* essential oil as shown in table-1. Present findings indicted that is more effective against Gram+ve bacteria which is in accordance with Kirbaslar *et al*[20] who reported strong antimicrobial activity of citrus peel oil against tested Gram+ve bacteria. While considering the efficacy of the C. *aurantium* essential oil against Gram-ve bacteria present results correlated with Quintero *et. al*[21] who reported that *Pseudomonas aeruginosa* and *E. coli* were resistant to C. *aurantium* peel oil. In case of antibiotics Streptomycin exhibited antimicrobial activity against all tested microorganisms as shown in table-1. Maximum zone of inhibition observed in case of *Bacillus subtilis* i.e 33.5±0.71mm whereas the minimum IZD was 18±0.71mm exhibited by *Pseudomonas*

aeruginosa. Penicillin G was effective against only *Bacillus subtilis* and *S.aureus* with an IZD of 15±0.71mm and 17.5±0.25mm respectively. This investigation supports the already documented fact Izzo *et al*., 1995; [24 25 26 27] that antimicrobial potential of the *C. aurantium* peel oil is depending upon its components which may vary with physiological development period, seasonal changes in plants, extraction process and the kind of bacteria used etc.

Table 2: Assessment of antimicrobial activity of *Citrus aurantium* essential oil against six microorganisms.

Test Micro-organisms	**G.staining/ Colony morphology**	**Antimicrobial activity of *C. aurantium* essential oil and some standard Antibiotics. Zone of inhibition (mm)**				
		Oil conc. 10µL/D[a]	Oil conc. 15µL/ D	Oil conc. 20µL/ D	Strepto-Mycin 25µg/D	Penicillin G 25µg/D
Bacillus subtilis ATCC 6333	G +ve rods/ White, dry surface	-	-	11 ±0.36	33.5 ±0.71	15±0.71
Staphylococcus aureus HI[b]	G +ve rods/ Light yellow, round, shiny	-	12±0.28	18 ±0.35	24±0.70	17.5±0.25
Salmonella typhimurium HI	G -ve rods/ Transparent white,	-	-	-	29±0.35	-
Pseudomonas aeruginosa HI	G -ve rods/ green, flat-spreaded	-	-	-	18±0.71	-
Klebsiella pneumoniae HI	G -ve rods/ large grey-white, mucoid	-	-	-	19.5 ±0.35	-
Escherichia coli HI	G -ve rods/ Smooth, white	-	-	-	25±0.36	-

[a]Paper disc (6mm diameter)
[b]Hospital isolated pathogen
± Standard deviation
-No inhibition zone (resistant)

Several natural compounds are known to quench free radicals[28]. In the current study (Figure 1) essential peel oil was able to reduce the stable radical DPPH to yellow-colored DPPH-H reaching 94.64% of DPPH scavenging effect. The comparison of DPPH scavenging activity of *C. aurantium* peel oil with well known antioxidant BHT showed that peel oil has equally strong antioxidant potential.

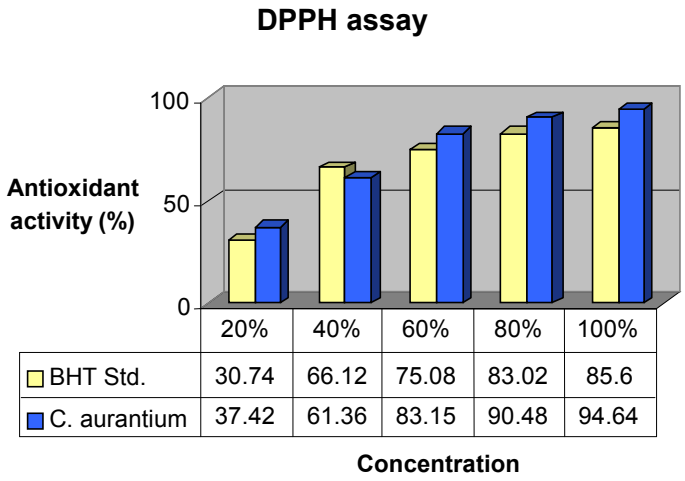

Figure1: Percentage antioxidant activity of Citrus aurantium peel oil in comparison with BHT as standard reference by DPHH assay.

Conclusion

The results of the present study recommend use of *C.aurantium* var. bitter oil peel oil in perfumery while its strong antimicrobial and antioxidant activities suggest it a possible substitute for synthetic preservative in food and medicines.

Acknowledgement

Authors are grateful to Dr. Zia-ur-Rehman, Senior Scientific Officer, PCSIR Labs Complex, Lahore for GC-MS analysis.

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