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Chemical composition of the essential oils from some citrus species and evaluation of the antimicrobial activity

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Abstract: *Citrus oils are mixtures of volatile components as terpenes, oxygenated compounds and terpenoids. In the present study different fruits of citrus varieties were collected from the local market in Adana/Turkey. The essential oils of peels were extracted using hydro distillation extraction method. The essential oil compositions of these citrus varieties were analyzed by Gas chromatography–mass spectrometry (GC–MS). The main components of extracted oils were determined to be α-pinene, sabinene, β-pinen, β-myrcene, d-limonene, linalool, m-cymene and 4-terpineol. Based on the results d-limonene was the major component (66-93%). All of essential oils were investigated for activity against the microorganisms E. coli, B. cereus, S. aureus, S. typhimurium, L. monocytogenes and E. faecalis, using a disc-diffusion method. The essential oil from Blood orange [C. sinensis (L.) Osbeck] showed the highest antimicrobial activity on all these bacteria. However, Clementine mandarin and Sour orange showed no activity on E. coli and S. typhimurium.*

Keywords: *Citrus Oils, Antimicrobial activity, Gas chromatography–mass spectrometry, d-limonene, Linalool, α-pinene*

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I. Introduction

Essential oils that are named as volatile oil, aromatic oil, etheric oil, essence oil or spirit are one of the important components of plant chemicals. These oils originated from aromatic plants may be found in all plant organs or leaves, fruits, peel, fruit stem (3). Essential oils are generally in liquid form at room temperature and have ability to crystallize easily. These are considered as colorless lipoid mixtures and are in different structure compared to fixed oils. There are wide area of use of essential oils in the pharmaceutical industry and cosmetic industry, in addition to chemical industry such as detergent, toothpaste, soap (28, 29).

It has been reported that "essential oil" was utilized the first time in the 16th century by Paracelsus von Hohenheim, who referred to the effective component of a drug as "quinta essential" (4, 16). The essential oils have many significant features. One of the most important feature is that they have antimicrobial effects. The first research to determine the bactericidal properties of essential oils is reported by de la Croix in 1881 (6, 7). The degree of antimicrobial impacts of essential oils vary because they contain complex structures of the different components. Also, citrus oils have been generally recognized as safe (GRAS) by the Food and Drug Administration (13).

Citrus fruits, which are very rich in essential oils, belong to Rutaceae family. Citrus fruits are one of the fruit groups having the highest production in the world. In general, citrus fruits contain species belonging to the "Citrus" genus as lemon (*Citrus limon* (L.) Burm. F), orange (*Citrus sinensis* (L.) Osbeck), mandarin (*Citrus reticulata* Blanco) and grapefruit (*Citrus paradisi* Macfie.). Citrus essential oils are obtained by cold pressing or distillation from fruit peels. Citrus peels consist of two layers, called the flavedo and albedo. These essential oils consist of a mixture of more than 100 compounds, containing terpenes, oxygen saturated and non-volatile compounds. The majority of essential oils are composed of terpenes. Terpenes constitute approximately 50% of a 95% oil. A large portion (more than about 90%) of the citrus essential oil constitutes d-limonene. D-limonene is an important terpene that has the specific aromatic smell of citrus (14, 21).

The aim of the present study is to obtain the essential oils from peels of various citrus species, to determine the antimicrobial activity on several bacteria and to evaluate the components of the essential oil by GC-MS.

II. Materials and methods

1.2. Plant material

The ripe fruit of different citrus species were collected between October and April 2015 from the different local markets in Adana/Turkey. Satsuma mandarin (*C. unshiu* Marc.), Clementine mandarin (*C.*

reticulata Blanco), Meyer lemon (*C. meyeri*), Interdonato lemon (*Citrus x limon* L. Burmf), Washington Navel orange [*C. sinensis* (L.) Osbeck], Star Ruby grapefruit [*C. paradisi* (Macf.)], Sour orange (*C. aurantium*) and Moro blood orange [*C. sinensis* (L.) Osbeck] were used as plant materials.

1.3. Essential Oil Extraction

The healthy and mature fruits were washed carefully with tap water to remove dust and foreign materials. The rind of citrus fruits were removed and cut into small pieces with the help of a knife and 50 g peels were weighed and added into a blender cup containing 200 mL of distilled water. These plant materials were pulverized to puree using a blender. The rind puree was subjected to hydrodistillation in a 500 mL round bottom flask fitted to a Clevenger apparatus. After 3 hour distillation, the distillate was stored in air-tight brown bottle at 4 °C until analysis.

1.4. Gas chromatography–mass spectrometry analysis

Gas chromatography–mass spectrometry (GC–MS) analyses were performed using an Agilent 6890 with selective mass detector Agilent 5973. A capillary column of HP-5MS (30 m x 0.25 mm i.d. x 0.25 µm film thickness) and an automatic injection system (Agilent 7683 series injector) were used. The analysis was carried out at 280 ºC, the oven at initial 50 ºC for 5 minutes increasing 5 ºC per minute until 120 ºC was reached. Later, the temperature was set to 180-230 ºC for 2 minutes increasing 30 ºC per minute until 280 ºC was reached. The injector temperature was set to 200 ºC. Helium was used as a carrier gas at a flow rate of 1.0 mL/min. Before analysis, the samples were diluted 1:100 ratio with hexane. Individual GC peaks and Mass spectra were identified by a computer. Identification of components was based on computer matching with commercial libraries (NIST and WILEY) (1).

1.5. Detection of Antimicrobial Activity

Antimicrobial activities from hydrodistillated-essential oils from fresh citrus peels were performed against various microorganisms by agar disc diffusion method (10). Susceptibility tests were performed by Bauer-Kirby (5) disc diffusion by using Mueller Hinton Agar (Merck 1.05437). *E. coli* (ATCC 25922), *B. cereus*, *S. aureus* (ATCC 25923), *S. typhimurium* (ATCC 14028), *L. monocytogenes* (ATCC 7644) and *E. faecalis* (ATCC 29212) were used as test microorganisms. These standard strains were inoculated into Luria-Bertani (LB) broth (Merck) and incubated for 18 h at 37 ºC in a shaking incubator. Then, 100 µL of each bacterial solution revived in this way were evenly spread on Muller Hinton Agar plates by using sterilized cotton swabs. The plates were left at room temperature for 15-20 min to allow the agar surface to dry. Sterilized blank antibiotic susceptibility discs (0,6 cm in diameter) were arrayed on the plates dish using a disc dispensing system (Oxoid). Steril blank discs were impregnated with 10 µl of the oil. Plates were incubated at 37 °C, for 18-24 h in an incubator.

The results were expressed as susceptible/resistant according to the criteria developed by National Committee for Clinical Laboratory Standards (23) and Manual of Antimicrobial Susceptibility Testing guidelines (9, 10, 24, 26, 27). The antibacterial activity was evaluated by measuring the diameter of the inhibition zone formed around the disc. The diameter of the inhibition zones was measured in mm. All tests were performed in triplicate. The antimicrobial activities were compared with standard Tetracycline (10 µg/disc) and Cefazolin (10 µg/disc) antibiotic discs.

1.6. Statistical Analyses

Experiments were carried out in triplicates. Means were separated by analysis of variance and the LSD test was performed to examine significant differences among zone diameters and components of essential oils by using JMP 8 (SAS Institute Inc., NC, USA) statistical package program.

III. Results and Discussion

3.1. Instrumental Analysis

Chemical components of the essential oils obtained by hydrodistillation of citrus fruit peels were detected by GC-MS analysis. The chromatograms were evaluated and most important compounds were presented in Table 1. Based on these results, the d-limonene amount was highly dominant (66-93%) in all citrus essential oils. It was remarkable that d-limonene amount of Moro blood orange oil was observed to be higher (93.32%) than other cultivars. On the other hand, Mayer lemon is determined as second highest being 75.50%, while Interdonat lemon has the lowest amount of d-limonene with 66.58%. The other main components of oils were determined to be α-pinene, sabinene, β-pinen, β-myrcene, linalool, m-cymene and 4-terpineol in addition to d-limonene. Most studies have emphasized that amount of limonene is high in citrus essential oils (30, 11). Hosni et al. (17) studied different citrus peel oils with GC-MS analysis, and showed that the ratio of limonene was risen up to 97.30% in citrus oils. They reported that limonene and β-pinene were major components in citrus oils. Lota et al. (20) investigated the leaf and peel oils of 30 sour orange cultivars, belonging to four different species. The chemical compositions of oils were investigated using capillary GC, GC–MS and ^{13}C-

NMR. They reported that limonene and α-pinene were major compounds for peel oils of Sour orange. Karoui and Marzouk (18) investigated the aroma compounds in peel and juice of *C. aurantium* by GC-MS. Twenty-seven components were identified in the *C.aurantium* peel essential oil, amounting to 99.48% of the total oil. Limonene was determined to be the major volatile compounds of fruit peel (90.25%) and fruit juice (91.61%).

Table 1. GC-MS profile[a] (%) of the components of peel essential oils from several citrus varieties obtained by hydrodistillation.

	Satsuma Mandarin	Clementine mandarin	Meyer lemon	Interdonato lemon	Washington navel orange	Blood orange	Star Ruby grapefruit	Sour orange	LSD
α-pinene	1,28 (6,51) PQR	0,45 (3,86) UVWX	1,93 (7,98) MNO	2,03 (8,20) MNO	0,90 (5,46) RST	0,90 (5,45) RST	0,92 (5,51) RST	0,79 (5,12) STU	1,48***
Sabinene	0,21 (2,67) XYZ	0,54 (4,24) TUVW	0,32 (3,25) WXYZ	1,56 (7,18) OPQ	0,92 (5,50) RST	0,79 (5,11) STU	2,39 (8,89) LM	0,25 (2,91) WXYZ	1,23***
ß-pinene	0,44 (3,83) UVWX	0,03 (1,07)	1,06 (5,91) QRS	9,50 (17,95) H	0,05 (1,33)	0,05 (1,27)	0,26 (2,93) WXYZ	0,80 (5,15) RSTU	1,22***
ß-Myrcene	2,30 (8,73) MN	3,09 (10,12) KL	2,24 (8,61) MN	1,67 (7,44) NOP	2,63 (9,34) KLM	2,46 (9,02) LM	2,23 (8,59) MN	2,39 (8,90) LM	2,18***
d-limonene	86,32 (68,29) E	90,88 (72,42) BC	75,49 (60,35) F	66,58 (54,69) G	92,01 (73,77) B	93,32 (75,28) A	89,82 (71,52) CD	89,29 (71,02) D	16,88***
Linalool	0,16 (2,31) YZ	0,06 (1,45)	0,41 (3,67) VWXY	0,21 (2,65) XYZ	0,44 (3,83) UVWX	0,68 (4,75) STUV	0,25 (2,87) WXYZ	3,43 (10,67) K	0,99***
m-cymene	5,13 (13,09) J	0 (0)	5,36 (13,39) J	7,29 (15,66) I	0 (0)	0 (0)	0,14 (2,19) Z	0 (0)	1,37***
4-terpineol	0,09 (1,79)	0,20 (2,61) XYZ	0,14 (2,17) Z	0,27 (2,99) WXYZ	0,08 (1,67)	0,05 (1,27)	0,25 (2,89) WXYZ	0 (0)	0,48***
Others	3,63	4,52	12,78	10,85	2,92	1,44	3,49	2,94	
Total identified	99,61	99,80	99,77	100,00	99,97	99,70	99,77	99,93	

The values given in parentheses are obtained after angle transformation. The LSD's are calculated according to the angle transformation value.
a: Data are means of three replicates expressed as percent area.
$P<0.05$*, $P<0.01$**, $P<0.001$***

3.2. Antimicrobial Analysis

All the essential oils of the citrus species, according to the disc diffusion test results are presented in Table 2. According to the results of the study, Moro blood orange oil against the tested bacteria showed strong antimicrobial activity on all strains. It has showed the highest activity especially on *E.coli* (19 mm). It was found that this activity was 11.76% higher compared to that of Cefazolin (17 mm). It showed a lower activity against the *B. cereus* with the diameter of inhibition of 10 mm. Other oils have demonstrated antimicrobial effects of different levels. Essential oils of Clementine mandarin and orange did not show any effect on *E. coli* and *S. typhimurium*. While essential oil of Meyer lemon showed the lowest activity on the *E. faecalis*, it has demonstrated highest activity on the *B. cereus* (15 mm).

Table 2. Antibacterial activity of peel essential oils of eight cultivars of citrus against different six bacteria.

	E. coli	*B. cereus*	*S. aureus*	*S. typhimurium*	*L. monocytogenes*	*E. faecalis*	LSD
Satsuma Mandarin	11 Ea	11 DEa	10 Eb	7 Ed	11 Ea	9 Cc	0,96***
Meyer lemon	10 EFcd	15 Ca	13 CDb	10 Dcd	11 Ec	9 Cc	1,49***
Sour orange	0 Gd	12 Db	14 Ca	0 Fd	11.5 DEb	10 BCc	1,23***
Clementine mandarin	0 Gd	8 Gc	12 Da	0 Fd	12 DEa	9 Cb	0,93***
Star Ruby grapefruit	14 Db	10 EFc	14 Cb	10 Dc	16 Ba	13 Ab	1,37***
Interdonato lemon	15 Da	11 DEc	13 CDb	9 Dd	14 Cab	9Cd	1,14***
Moro blood orange	19 Ba	10 EFd	12 Dc	12 Cc	14 Cb	11 Bcd	1,85***
W.N. Orange	9 Fc	9 FGc	10 Eb	0 Fd	13 CDa	9 Cc	0,79***
Tetracycline	21 Ab	33 Aa	22 Ab	18 Bc	34 Aa	11 Bd	1,34***
Cefazolin	17 Cd	25 Bb	17 Bd	20 Ac	35 Aa	14 Ae	1,81***
LSD	1,15***	1,35***	1,14***	1,17***	1,67***	1,09***	

Lowercase letters (comparison between essential oils), uppercase letters (comparison between bacteria), $P<0.05$*, $P<0.01$**, $P<0.001$***, W.N.: Washington navel

Star Ruby grapefruit showed highest activity on the *L. monocytogenes* (16 mm) when it showed the lowest activity on *B. cereus* (10 mm) and *S. typhimurium* (10 mm). The essential oil of Interdonat lemon showed the lowest activity on *S. typhimuri* and *E. faecalis* (9 mm), whereas it has the highest activity against *E. coli* with diameter of 15 mm inhibition. It showed no activity on *S. typhimurium* while essential oil of Washington Navel orange showed the highest activity on *L. monocytogenes* (13 mm). Given the results from essential oils applied to bacteria, *S. typhimurium* were determined to be the most resistant bacteria. For many years, many researchers have investigated the antimicrobial properties of essential oils derived as by-products of the citrus against bacteria. Also, they have observed that most of them have been shown to possess antimicrobial properties (7, 22, 14, 8, 19, 15, 32) Geraci et al. (15) investigated the component of orange peel oils and the antimicrobial activity of these oils. They found that limonene was the most abundant component (73.9-97.6%) in all the essential oil samples from the orange cultivars. It has been reported that 'Solarino Moro' and 'Sanguinello' essential oils were significantly active against *L. monocytogenes*, while 'Valencia' hexanic extract was active against all the tested microorganisms. According to our study, Moro blood orange has proven to have significant antimicrobial properties. This oil was very effective against both *L. monocytogenes* and *E. coli*. The highest antimicrobial efficacy against *L. monocytogenes* was found in *Star Ruby* essential oil.

Espina et al. (12) investigated the chemical composition of the three commercial citrus essential oils (orange [*Citrus sinensis*], lemon [*Citrus lemon*] and mandarin [*Citrus reticulata*]) and examined their antimicrobial properties. Total of 65 compounds as major volatile ingredients were identified by GS-MS analysis. According to the results obtained by using the agar disc diffusion technique on *E. faecium, S. aureus* (ATCC 6538), *P. aeruginosa* (ATCC 10145), *Salmonella enterica* subsp. enterica ser. Enteritidis (ATCC 49214), *Escherichia coli* O157:H7 and *Listeria monocytogenes* bacteria, mandarin essential oil was identified as the best growth inhibitor. They did not observe that essential oils of oranges and lemons have a potent inhibitory effect. In contrast, it was observed in our study that both orange essential oils had an inhibitory effect on the bacteria. In addition, this effect was stronger than the mandarin essential oil. Al-Howiriny (2) analyzed the volatile components by GC-MS and investigated the antimicrobial effects of these oils after it obtained essential oils of *Salvia lanigera* (Labiatae) by steam distillation. In total 42 components representing 98.8% of the oil were identified. The β-pinene (6.5%), 1,8-cineole (36.2%), α-pinene (10.7%), terpene-4-ol (7.5%), limonene (5.6%) and bornyl acetate (4.5%) were reported as major components. Based on the results of antimicrobial studies, it was reported that the plant essential oil showed inhibitory effect against *Mycobacterium smegmatis, Proteus mirabilis*, *Bacillu subtilis*, *Staphylococcus epidermidis*, *Candida vaginalis* and *Candida albicans* microorganisms, but it was determined that *Escherichia coli* and *Pseudomonas aeruginosa* were resistant to this essential oil. In contrast to this study, all citrus fruit oils except clementine mandarin were found to have inhibitory activity against *E. coli* in our study. Vasek et al. (31) examined the effectiveness of grapefruit (*Citrus paradisi*) essential oil to inhibit the growth of wild food-borne spoilage and pathogenic bacterial strains. They have observed that essential oil of grapefruit had antibacterial activities on 100.00% of Gram (-) and 40.74% of Gram (+). In addition, all strains of *E.coli* were sensitive to this essential oil. This result was somewhat consistent with our study. Essential oil of Star Ruby grapefruit showed inhibitory effect against *E.coli*, but this oil had a higher antimicrobial effect against *L. monocytogenes*.

In the study conducted, it was observed that the amount of d-limonene was directly proportional to the antimicrobial effect. The oils of Moro blood oranges which have higher antimicrobial activity than the others, also had the highest amount of d-limonene. It was as effective as cefazolin on some microorganisms. Cefazolin is a first-generation antibiotic that exhibits bactericidal action by inhibiting bacterial cell wall synthesis. It has been found that most of the citrus essential oils used here have antimicrobial activity on microorganisms. In this effect, we think that d-limonen is an active duty. In addition, we anticipate that these substances may be used as an alternative to chemical preservatives.

IV. Conclusions

In this study, essential oils from different varieties of citrus species were extracted and the main components contained in these substances were identified using GC-MS. Antimicrobial activities of essential oils were performed against various microorganisms by agar disc diffusion method. All eight essential oils had inhibitory effects on the six bacteria tested, and Moro blood orange essential oils were most effective against *E.coli* and *S. typhimurium*.

The major constituents of the essential oils from some citrus varieties were α-pinene, sabinene, β-pinen, β-myrcene, d-limonene, linalool, m-cymene and 4-terpineol. Also, d-limonene appeared to be the major compound of the essential oils obtained by hydrodistillation.

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