Bulletin of Health,
Science and Technology
BHST
ISSN 0858-7531
Volume 13, Number 1, 2015 : 43-49

CHEMICAL CONSTITUENTS AND BIOLOGICAL ACTIVITIES OF *FORTUNELLA JAPONICA* ESSENTIAL OILS

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Abstract: Essential oils of *Fortunella japonica* (*syn. Citrus japonica*) were obtained by hydrodistillation on its leaves and peels. The essential oil compounds were identified by gas chromatography-mass spectrometry (GC-MS). Major constituents of leaf oil were β-pinene (47.44%), d-limonene (10.24%), and linalool (9.79%), meanwhile d-limonene (87.07%) was the most abundant constituent in peel essential oil. The antioxidant activities of these oils were evaluated by using stable 1,1-diphenyl 2-picrylhydrazyl (DPPH) free radical scavenging method. Antioxidant activities were exhibited as percentage of DPPH radical inhibition and IC_{50} values (μg/ml) were compared with synthetic vitamin E and butylated hydroxyltoluene (BHT). Leaf and peel essential oils showed antioxidant potential with the IC_{50} of 53.11 and 102.11 μg/ml, respectively. Both of these oils also demonstrated antibacterial activity against *Bacillus subtilis* ATCC 6633 *and Staphylococcus aureus* ATCC 25923, but no activity against *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853.

Keywords: *Fortunella japonica*, essential oil, antioxidant, antimicrobial

บทคัดย่อ : น้ำมันหอมระเหยของส้มจี๊ดเตรียมได้จากส่วนใบและเปลือกผลโดยวิธีการกลั่นด้วยน้ำ จำแนกองค์ประกอบทางเคมีของน้ำมันหอมระเหยด้วยวิธี gas chromatography-mass spectrometry (GC-MS) องค์ประกอบหลักที่พบในน้ำมันจากส่วนใบได้แก่ β-pinene (47.44%), d-limonene (10.24%) และ linalool (9.79%) ในขณะที่น้ำมันจากส่วนเปลือกผลมี d-limonene (87.07%) เป็นองค์ประกอบเคมีเพียงชนิดเดียวที่มีปริมาณมาก ทดสอบฤทธิ์ต้านออกซิเดชันของน้ำมันหอมระเหยโดยใช้วิธี1,1-diphenyl 2-picrylhydrazyl (DPPH) free radical scavenging ฤทธิ์ต้านออกซิเดชันแสดงด้วยการยับยั้ง DPPH radical และเปรียบเทียบค่า IC_{50} μg/ml กับวิตามินอีสังเคราะห์และ butylated hydoxyltoluene (BHT) น้ำมันจากส่วนใบและเปลือกผลแสดงฤทธิ์ต้านออกซิเดชันด้วยค่า IC_{50} เท่ากับ 53.11 และ 102.11 μg/ml ตามลำดับ น้ำมันทั้งสองชนิดยังแสดงฤทธิ์ต้านแบคทีเรียชนิด *Bacillus subtilis* ATCC 6633 และ *Staphylococcus aureus* ATCC 25923 แต่ไม่มีฤทธิ์ต้านแบคทีเรียชนิด *Escherichia coli* ATCC 25922 และ *Pseudomonas aeruginosa* ATCC 27853

INTRODUCTION

Fortunella japonica (Thumb.) Swingle (syn. *Citrus japonica*) belonged to family Rutaceae. It has many commonly names eg. Kumquat, Round kumquat, or Marumi kumquat. It is native of China, grown throughout subtropical regions of the world. Its fruit is acidic taste like lemon or lime that contains vitamin C up to 0.24 mg/ml (Choopra *et al.*, 1986). In India, it is grown as ornamentals and for the fruit that is rich in pectin and excellent for marmalades and jellies (Talapatra *et al.*, 1974). *F. japonica* has been accepted for its medicinal properties such as antiphologistic, antivinous, carminative, deodorant, and expectorant properties (Quijano and Pino, 2009).

d-Limonene (87-97%) was the most abundant of this fruit peel essential oil, along with germacrene D and linalool (Koyasako and Bernhard, 1983, Choi, 2005 and Quijano and Pino, 2009). Its leaf essential oil in Nepal composed of linalool (35.1 %), eugenol (14.8 %), geraniol (12.7 %), and geranial (7.9 %) (Satyal *et al.*, 2012).

Almost citrus essential oils are interesting since their absorptive aroma, good for insect repellant. They are available to the food industry, and also generally accepted as safe (Fisher and Phillip, 2008). They have antioxidant and antimicrobial activity (Subba *et al.*, 1967, Viuda-Martos *et al.*, 2008, Wang *et al.*, 2012, Janoti *et al.*, 2014). They have been found to be inhibitory against a range of both Gram-positive and Gram-negative bacteria. To our knowledge, there are no reports regarding to antioxidant and antimicrobial activities of leaf and peel essential oils of *Fortunalla japonica* until now. Therefore, the objectives of this study were to evaluate chemical constituents of leaf and peel essential oils of *F. japonica*, along with determine their antioxidant and antibacterial activities.

MATERIALS AND METHODS

Fresh leaves and peels of *Fortunella japonica* were collected in May-June 2014 from plant garden of Faculty of Pharmacy, Rangsit University, Pathum Thani, Thailand. The plant materials were authenticated by comparison with herbal specimen collected in Department of Pharmacognosy, Faculty of Pharmacy, Rangsit University. Fresh leaves and peel were hydrodistillated by Cleventure-type apparatus, collected and stored at 4°C until being analyzed for chemical constituents by gas chromatography-mass spectrometry (GC-MS).

GC-MS Analysis

Agilent Technologies GC7890A, 5975C Inert XL EI/CI MSD with Triple-Axis detector was used for gas chromatography-mass spectrometry analysis. The column was DB-5MS (27.75 m. X 0.25 mm. i.d.; 0.25 μM film thickness); oven temperature programming was 60-240 °C at 3 °C/min; injector and detector temperature were 180 and 290 °C, respectively; sample volume injected was 1 μl; split ratio was 100:1; and the carrier gas was He (1 ml/min).

Compound Identification

Compounds were identified by comparing the Kovats gas chromatographic retention indices of the peaks on the HP-5MS column with literature values, computer matching using the Masslynx database, and comparison of the fragmentation patterns of the mass spectra with those reported in the literature (Adam, 1995 and Davies, 1990).

DPPH radical scavenging activity

The antioxidant activity of the essential oils was evaluated on the basis of the scavenging activity of stable 1, 1- diphenyl 2-picrylhydrazyl (DPPH) free radical according to the method described by Brand-Williams *et al.* with slight modifications (Brand-Williams *et*

al., 1995). 100 Microliter of 0.1 mM DPPH solution in methanol was mixed with 100 µl of essential oil solution of varying concentrations (10-1000 µg/ml). Corresponding blank sample were prepared and various concentrations of synthetic α-tocopherol (vitamin E) and butylated hydroxyltoluene (BHT) (1-100 µg/ml) were applied as reference standard. Mixer of 100 µl methanol and 100 µl DPPH solution was used as control. These samples were incubated for 30 minutes at room temperature, avoid from light, and absorbance was measured at 517nm. The reaction was carried out in triplicate.

Microorganism

Bacillus subtilis ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 were obtained from Sino-Thai Traditional Medicine Research Center [Co-operation between Rangsit University-Harbin Institute of Technology- Heilongjiang University] Faculty of Pharmacy, Rangsit University. These bacteria were cultured on Mueller Hinton agar (MHA).

Antibacterial screening test

The antibacterial activity was determined as in traditional antibiotic susceptibility testing using the disc diffusion method (Bauer *et al.*, 1966). Sterile 6 mm paper disc were impregnated with 15 µl undiluted essential oil and deposited on the agar surface.

Determination of MIC and MBC

The antibacterial effects of the essential oil were evaluated by analyzing the minimum inhibitory concentration (MIC) using broth dilution method according to the modified protocol described by Gutierrez *et al.* (2008). Briefly, the extracts were dissolved in 5% DMSO, then diluted in media and tested over a range of concentrations from 0.3125-10,000 µg/ml against overnight broth cultures of bacterial grown to population $5x10^5$ CFU/ml in MHA. Gentamicin was used as positive control. The MIC was defined as the lowest concentration of the essential oil that completely inhibited the growth of particular microorganisms. The minimum bactericidal concentration (MBC) was established by the minimum concentration showing lack of growth upon reinoculation of bacterial from compound-treated plates to MHA agar plates and incubated at 37°C for 18-24 hours.

RESULTS AND DISCUSSION

Chemical constituents of essential oils

The essential oils obtained by hydrodistillation on *Fortunella japonoca* fresh leaves and peels yielded 0.53 and 0.90%, respectively. They were exhibited clear, pale yellow, and slightly bitter with the characteristic odor. Essential oil compositions of *F. japonica* leaf and peel were listed in Table 1.

Seventeen compounds in *F. japonica* leaf oil were analysed as 13 monoterpenes and 3 oxygenated monoterpenes, and 1 aldehyde (n-decanal). β-Pinene (47.44%) was the major component, followed by d-limonene (10.24%), linalool (9.79%), *trans*-ocimene (7.56%), and α-pinene (7.41%). On the comparison of present results with those reported from sample of other countries, it is quite evident that the concentrations of linalool was lower, some compounds such as eugenol, geraniol, nerol, geranial, germacrene D had not been found in this sample, meanwhile β-pinene, d-limonene, *trans*-ocimene and α-pinene had not been found in previous report (Satyal *et al.*, 2012). The observed differences in the compositions of leaf essential oil of *F. japonica* may be due to different environmental and genetic factors, different chemotypes and nutritional status of the plants.

Peel oil of *F. japonica* performed 19 compounds, which were identified as 10 monoterpenes, 5 oxygenated monoterpenes, and 4 sesquiterpenes. d-Limonene (87.07%) was the major constituent, followed by linalool (1.44%), myrcene (1.32%), and geranyl acetate (1.12%). d-Limonene, linalool and germacrene were found in peel oil like previous reports (Choi, 2005 and Quijano and Pino, 2009).

DPPH radical scavenging activity

DPPH radical scavenging activity is one of the most widely used method for screening the antioxidant activity of essential oil. Table 2 shows antioxidant activity of leaf and peel oils of *F. japonica* assessed using the DPPH radical scavenging. Figure 1 exhibits the comparative data of DPPH radical scavenging activity as determined by the IC_{50} value of each essential oil compared with synthetic vitamin E and BHT. The leaf essential oil exhibited stronger antioxidant activity than those of synthetic vitamin E, with the IC_{50} of 53.11 μg/ml, meanwhile peel essential oil showed the weakest antioxidant activity with the IC_{50} of 102.11 μg/ml.

Several citrus oils had been reported to have strong radical scavenging activity. Choi *et al.* found that the radical scavenging activity of 34 kinds of *Citrus* essential oils on DPPH ranged from 17.7% to 64%. These activities were found to be higher when the oils contained geraniol, terpinolene and γ-terpinene (Choi *et al.*, 2000). Some authentic flavor constituents such as γ-terpinene, terpinolene, geraniol, β-pinene and myrcene have high antioxidant activities, even at low concentrations (Seok *et al.*, 2008, Malhotra *et al.*, 2009). This result that leaf essential oil of *F. japonica* had the highest scavenging activity, may be due to the presence of terpinolene and γ-terpinene much more higher than that of peel essential oil. However, the bioactivity of the essential oils generally results from a complex interaction between its constituents, which produce both synergistic and antagonistic responses.

Antibacterial screening test

Leaf and peel essential oils of *F. japonica* showed the promising inhibition zone against *Bacillus subtilis* and *Staphylococcus aureus*, but there was no antibacterial activity against *Pseudomonas aeruginosa* and *Escherichia coli* (Table 3). This might be according to described by Burt (2004) that test gram-positive bacteria were more sensitive to essential oil than test gram-negative bacteria due to their impermeable outer membrane barrier that surround test gram-negative bacteria.

Antibacterial activity of these oils against *B. subtilis* and *S. aureus* were following examined by broth dilution method. Even though peel oil seem exhibited antibacterial activity stronger than that of leaf oil with the wilder inhibitory clear zone diameter, but only leaf essential oil demonstrated antibacterial activity against *B. subtilis* and *S. aureus* with the MIC of 1.25 and 1.25 mg/ml, respectively (Table 4).

The limitation of oil activity could be clarified by the low water solubility of the oil and its compounds which limits their diffusion through the agar medium in the disc-diffusion method. More water soluble compounds diffuse into the agar, but the hydrocarbon components either remain on the surface of the medium or evaporate (Griffin *et al.* 2000). That could be the reason for the better results obtained by the microdilution method.

Table 1. Chemical constituents of leaf and peel essential oils of *F. japonica*

Compound	Kovat's Index	% Area	
		Leaf oil	Peel oil
Monoterpenes			
α-thujene	931	0.91	-
α-pinene	931	7.41	0.83
camphene	953	3.59	-
β-pinene	980	47.44	-
myrcene	991	3.16	1.32
α-phellandrene	1005	0.95	-
δ-3-carene	1011	0.77	t
α -terpinene	1018	1.82	t
p-cymeme	1026	-	t
β-phellandrene	1031	-	0.19
d-limonene	1031	10.24	87.07
cis-ocimene	1040	0.91	-
trans-ocimene	1050	7.56	0.08
γ-terpinene	1062	2.56	0.03
terpinolene	1088	1.5	0.04
Oxygenated monoterpenes			
linalool	1098	9.79	1.44
4-terpineol	1177	0.57	0.27
α-terpineol	1189	0.44	0.13
geraniol	1255	-	0.02
geranyl acetate	1383	-	1.12
Sesquiterpenes			
germacrene D	1480	-	0.81
β-elemene	1391	-	0.04
α-copaene	1376	-	0.02
α-humulene	1454	-	0.06
Others			
n-decanal	1204	0.4	-

t = trace

Table 2. DPPH scavenging activities of leaf and peel essential oils of *F. japonica*

Concentration of essential oil (μg/ml)	% DPPH scavenging activities*	
	Leaf oil	Peel oil
125	56.36 ± 0.97	51.19 ± 0.90
100	52.45 ± 0.44	50.03 ± 0.94
75	52.01 ± 0.93	-
62.5	50.85 ± 0.67	47.72 ± 0.71
50	49.73 ± 0.67	-
31.25	47.99 ± 0.90	44.80 ± 0.85

*mean ± SD. The tests were done in triplicate

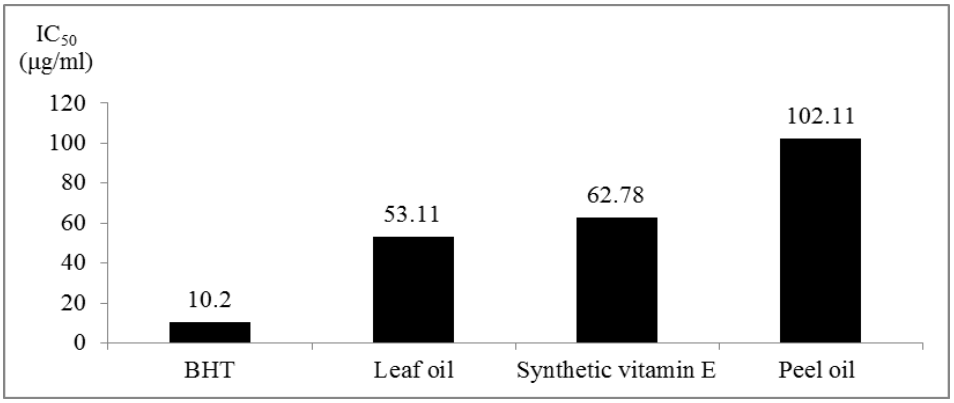

Figure 1. IC_{50} value of leaf and peel essential oils of *F. japonica* and synthetic antioxidants

Table 3. Antibacterial activities of *F. japonica* essential oils by disc diffusion method

Microorganism	Inhibition zone (mm)*	
	Leaf oil	Peel oil
Bacillus subtilis ATCC 6633	15.33 ± 0.58	26.67 ± 1.52
Staphylococcus aureus ATCC 25923	19.37 ± 3.06	34.67 ± 1.15
Pseudomonas aeruginosa ATCC 27853	NA	NA
Escherichia coli ATCC 25922	NA	NA

*mean ± SD, NA = no activity, disc diameter = 6 mm. The tests were done in triplicate

Table 4. Antibacterial activity of *F. japonica* leaf essential oil by broth dilution method

Microorganism	Leaf oil		Peel oil		Gentamicin	
	MIC* (mg/ml)	MBC* (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (µg/ml)	MBC (µg/ml)
B. subtilis ATCC 6633	1.25	>5	>5	>5	<5	<5
S. aureus ATCC 25923	1.25	>5	>5	>5	<5	<5

*MIC = minimum inhibitory concentration, MBC = minimum bactericidal concentration. The tests were done in triplicate

CONCLUSION

Leaf and peel essential oils of *Fortunella japonica* were obtained by hydrodistillation. The smell of these oils was different. The chemical compositions of *Fortunella japonica* leaf and peel essential oil were listed together with their relative percentages. Monoterpenes and oxygenated monoterpenes were the most abundant groups found in these oils, from which β-pinene (47.44%), d-limonene (10.24%), linalool (9.79%), trans-ocimene (7.56%), and α-pinene (7.41%) were the most abundant compounds in leaf essential oil, whereas d-limonene (87.07%) was the only most abundant in peel oil.

Antioxidant activity was evaluated by DPPH radical scavenging method. Both leaf and peel oil of *F. japonica* exhibited antioxidant activity with the IC_{50} of 53.11 and 102.11 µg/ml, respectively. Antibacterial activity was screened by agar disc diffusion method along with broth dilution method. These oils showed antibacterial activity against *B. subtilis* ATCC 6633 and *S. aureus* ATCC 25923, but inactive against *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922. By broth dilution method, it was found that only leaf essential oil demonstrated antibacterial activity against *B. subtilis* ATCC 6633 and *S. aureus* ATCC 25923 with the MIC of 1.25 and 1.25 mg/ml, respectively compare to the reference standard

antibacterial drug gentamicin (<5 µg/ml). These components might contribute to the biological activities of the leaf essential oil of *F. japonica*. Here is the first report on antioxidant and antibacterial activities of *F. japonica* essential oils. However, the further study will be performed with the pure compounds for distinct conclusion of bioactive components contributing these biological activities of this essential oil.

ACKNOWLEDGEMENT

The author would like to acknowledge Department of Pharmacognosy, Faculty of Pharmacy, and Sino-Thai Traditional Medicine Research Center [Co-operation between Rangsit University-Harbin Institute of Technology- Heilongjiang University], Rangsit University for instrumental supports.

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