

ฤทธิ์ยับยั้งจุลชีพของน้ำคั้นผลไม้ตระกูลส้ม

Antimicrobial Activity of Citrus spp. Fruit Juices

นิพนธ์ต้นฉบับ

Original Article

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บทคัดย่อ

วัตถุประสงค์: เพื่อศึกษาและเปรียบเทียบฤทธิ์ยับยั้งจุลชีพของน้ำคั้นจากผลไม้ตระกูลส้มที่นิยมรับประทาน ซึ่งประกอบด้วย มะนาว (*Citrus aurantifolia*) ส้มซ่า (*Citrus aurantium*) มะกรูด (*Citrus hystrix*) ส้มจี๊ด (*Citrus japonica*) ส้มโอ (*Citrus maxima*) ส้มเขียวหวาน (*Citrus reticulata*) และส้มแขก (*Citrus sinensis*) **วิธีการศึกษา:** ทำการทดสอบการยับยั้งเชื้อจุลชีพที่เจริญเติบโตบนอาหารเลี้ยงเชื้อและสมบัติเคมีกายภาพ (ความหนืด ความเป็นกรดต่าง และ ปริมาณของแข็ง) ของน้ำคั้นจากผลไม้ตระกูลส้มดังกล่าว รวมทั้งทำการหาปริมาณสารฟีนอลิกทั้งหมดด้วย **ผลการศึกษา:** น้ำคั้นจากมะนาว มะกรูด และส้มจี๊ด มีฤทธิ์สูงสุดในการยับยั้งเชื้อ *Staphylococcus aureus* ทั้งนี้ น้ำคั้นจากส้มจี๊ดให้ผลที่น่าสนใจในการยับยั้งเชื้อ *Escherichia coli* ได้สูงสุด แต่ไม่พบความสัมพันธ์ระหว่างสมบัติเคมีกายภาพ และฤทธิ์ยับยั้งเชื้อโรค **สรุป:** น้ำคั้นจากผลไม้ตระกูลส้มมีฤทธิ์ต้านเชื้อแบคทีเรียได้ดี อย่างไรก็ตามไม่พบฤทธิ์การยับยั้งเชื้อ *Candida albicans* จากน้ำคั้นของผลไม้ที่ศึกษาเหล่านี้ ข้อมูลจากการวิจัยนี้ให้แนวคิดสำหรับการสกัดสารยับยั้งเชื้อจุลชีพจากน้ำคั้นพืชตระกูลส้มต่อไป

คำสำคัญ: พืชตระกูลส้ม น้ำคั้นผลไม้ ฤทธิ์ต้านแบคทีเรีย และฤทธิ์ต้านเชื้อรา

Abstract

Objective: To determine the antimicrobial activity of some citrus juices including *Citrus aurantifolia*, *Citrus aurantium*, *Citrus hystrix*, *Citrus japonica*, *Citrus maxima*, *Citrus reticulata* and *Citrus sinensis*. **Method:** The tests for antimicrobial activity using agar diffusion method and some physicochemical properties (viscosity, pH and solid content) were determined for those mentioned citrus juices. Total phenolic compound of citrus juice was also analyzed. **Results:** The juice of *C. aurantifolia*, *C. hystrix* and *C. japonica* exhibited the high activity against *Staphylococcus aureus*. Interestingly, *C. japonica* juice presented the greatest activity to inhibit *Escherichia coli* growth. However there was no correlation between the physicochemical properties and the antibacterial activity. **Conclusion:** The *C. japonica* juice contained the most potential for antibacterial activity. However, antifungal activity against *Candida albicans* had not been observed. This information would provide an idea for further study to extract the antimicrobial agents from the citrus juices.

Keywords: Citrus spp., citrus juice, antibacterial and antifungal

Introduction

Microbial infection causes both serious and non-serious health problems. There are many infectious found in a tropical country. Infection with *Staphylococcus aureus*, *Propionibacterium acnes* and *Escherichia coli* are frequently observed.¹ *Candida albicans* also remains the main cause of oral and vaginal fungal infections.² Although there are many effective medicines such as penicillin, cephalosporin and azole but many patients still die with infectious diseases. Bacterial resistant medicine seems to be the worst case.³ Drug hypersensitivity is another limitation to cure a disease and protect people. Therefore, a new antibiotic drug discovery is still needed.

Plants are recognized as potential sources for novel drug discovering. Antimicrobial activity was reported in many plants in *Citrus* spp. For example, *Citrus aurantifolia* L. and *Citrus hystrix* DC. extracts are reported that they inhibit the

growth of microorganism.⁴⁻⁶ *Citrus maxima* (Burm.f.) Merr crude extract and *Citrus reticulata* volatile oil also exhibit the antimicrobial activity.^{7,8} Nevertheless, *Citrus aurantifolia* Swing juice also inhibited *E. coli* growth while *Citrus sinensis* (L.) Osbeck peel essential oil has been reported that it contains the activity against fungi^{9, 10}. Notably, the previous study focused on citrus oil or crude extract, but its juice (the most consumed part) has not been clearly studied for the antimicrobial activity.¹¹⁻¹³

This experiment investigated the antimicrobial activity of seven citrus juices including *C. aurantifolia*, *C. aurantium*, *C. hystrix*, *C. japonica*, *C. maxima*, *C. reticulata*, and *C. sinensis*. The activity against *Staphylococcus aureus* (Gram-positive), *Streptococcus mutan* (Gram-positive), *Propionibacterium acnes* (Gram-negative), *Escherichia coli*

(Gram-negative) and *Candida albicans* (yeast) were tested using agar diffusion method.

Materials and Methods

Materials

A hundred percent of citrus juice was prepared from fresh fruit. The fruit of *C. aurantifolia*, *C. aurantium*, *C. hystrix*, *C. japonica*, *C. maxima*, *C. reticulata* and *C. sinensis* were harvested in Amphur Sampran, Nakhon Pathom Province, Thailand. The fruits were washed with sterile water, cleaned with 70% ethanol and dried. The peel of all test citrus fruits were removed. Then they were cut open with a sterile knife before pressing under laminar air flow cabinet. Sodium carbonate (Ajax Finechem, Seven Hills, Australia), Folin-Ciocalteu reagent (CarLo ErbaReagenti, Milano, Italy) and gallic acid (Fluka Chemie GmbH, Switzerland) were used as received. Ampicillin disc was obtained from Oxoid Company Limited (Basingstoke, Hampshire, England) and clotrimazole (ctz) (batch no. 20001304, Lambrochem, Italy) was kindly supported from T MAN Pharma, Bangkok, Thailand. Microorganism including *S. aureus* (ATCC 6538P), *S. mutans*, *P. acnes* (ATCC 6919), *E. coli* (ATCC 25922), and *C. albicans* (ATCC 17110) were purchased from department of medical sciences (Nonthaburi, Thailand). Brain Heart Infusion (BHI) media, Tryptic Soy agar and Sabouraud dextrose agar (SDA) were acquired from Difco™ (Beckton Dickinson, New Jersey, USA).

Screening for Antimicrobial Activity of Citrus spp. Juice

The antimicrobial activity of *Citrus* spp. juice was assessed using modified agar diffusion method¹⁴. Briefly, *S. aureus*, *S. mutans*, *P. acnes* and *E. coli* were spread on BHI agar plate. *C. albicans* was cultured on SDA. Two hundred microliter of each citrus juice was filled into a sterilized cylinder cup with 0.7 mm diameter. Sterile distilled water and ampicillin (10 µg/disk) were used as negative and positive control, respectively. The positive control for antifungal test was two hundred microliter/cup of 40 µg/mL clotrimazole. The culture plates were further incubated in the microorganism incubator. Diameter of inhibition zone was determined manually after the incubation for 24 hours. The experiment was performed triplicately.

Physicochemical Property Test

The physical properties of the citrus juices were studied. The pH value was measured using a pH meter (Professional Meter PP-15 Sartorius, Goettingen, Germany). The viscosity was determined using brookfield viscometer (Model: DV-I, Brookfield Engineering Laboratories, INC., USA) at room temperature with constant shear rate for 5 min. The solid content in the citrus juices was quantified by a hand-held refractometer (Pocket PAL, Japan). All measurements were triplicated.

Phenolic Compound Assay

Total phenolic compound of citrus juice was analyzed using Follin-Ciocalteu method¹⁵. The absorbance was measured under the UV-Vis spectrophotometer (Perkin Elmer, 1100 series, Agilent) at 760 nm wavelength. Gallic acid was used as a standard phenolic compound.

Investigation of Antibacterial Activity of *C. japonica* Juice

A hundred percent of *C. japonica* juice was diluted as 75%, 50%, 25% and 1% with sterilized water for the test of dose-dependent manner effect. The fresh *C. japonica* juice was also test for heat resistance by boiling at about 100°C and sampling at 5, 15, 30, 45 and 60 minutes in the closed container. The prepared samples were examined the activity against *S. aureus* and *S. mutans* using agar diffusion method as previously described.

Statistical Analysis

Statistical analysis was performed using statistical package for the social sciences software (SPSS version 17). The significant difference was considered when *p-value* less than 0.05.

Results

Antimicrobial Activity

To evaluate the antimicrobial activity of citrus juices, the agar diffusion method was employed in this study. The result revealed that the juice of *C. aurantifolia*, *C. aurantium*, *C. hystrix* and *C. japonica* presented the antibacterial activity against *S. aureus*, *S. mutans*, *P. acnes* and *E. coli*. *C. reticulata* presented its antibacterial activity against *S. aureus*

and *E. coli* while *C. maxima* and *C. sinensis* did not show activity against any bacterium (Table 1). There is no significant difference of antibacterial activity against *S. aureus*, *S. mutans* and *P. acnes* between *C. aurantifolia*, *C. hystrix* and *C. japonica* (p -value > 0.05, one way ANOVA). However, the *C. japonica* juice presented the highest activity against *E. coli* (p -value < 0.05, Post Hoc test). The antifungal activity against *C. albicans* was not found in citrus juices that were investigated in this experiment.

Table 1 Antibacterial activity of citrus juices against *S. aureus*, *E. coli*, *S. mutans*, and *P. acnes*. The activity was compared with ampicillin which was referred as 100% growth inhibition (n = 3). There was no inhibition zone surrounding the negative control disc.

Citrus spp.	Microorganism			
	<i>S. aureus</i>	<i>E. coli</i>	<i>S. mutans</i>	<i>P. acnes</i>
<i>C. aurantifolia</i>	22.7 ± 0.6	24.8 ± 0.3	27.0 ± 1.0	35.0 ± 1.4
<i>C. aurantium</i>	12.3 ± 0.6	15.0 ± 0.0	12.3 ± 1.5	18.7 ± 2.1
<i>C. hystrix</i>	22.8 ± 0.3	25.7 ± 0.3	28.3 ± 1.5	40.7 ± 1.2
<i>C. japonica</i>	23.7 ± 0.6	26.2 ± 0.8	26.7 ± 1.2	40.7 ± 2.3
<i>C. maxima</i>	-	-	-	-
<i>C. reticulata</i>	10.5 ± 3.0	10.2 ± 0.8	-	-
<i>C. sinensis</i>	-	-	-	-
ampicillin disc 10 mcg	46.7 ± 3.5	12.0 ± 0.0	41.2 ± 0.3	46.7 ± 3.5

(-) There was no inhibition zone

Note: There was no inhibition zone for testing with *C. albicans*.

Physicochemical Properties

The physicochemical property study presented that citrus juices were acidic solution (pH 2.31 to 4.03). *C. hystrix* juice had the highest viscosity (2.55 cps) while the lowest was found for that of *C. sinensis* (1.52 cps). The highest brix degree was observed in *C. hystrix* (6.3 degree). All physicochemical data of the juices of *Citrus* spp. are presented in Table 2. There was no correlation between the physicochemical properties and the antibacterial activity (P -value > 0.05, Pearson correlation).

Table 2 Physicochemical properties of the citrus juices.

Citrus spp.	Physicochemical properties		
	pH	Viscosity (cps)	Brix (degree)
<i>C. aurantifolia</i>	2.31 ± 0.03	1.63 ± 0.02	2.9 ± 0.1
<i>C. aurantium</i>	3.72 ± 0.01	1.96 ± 0.02	3.8 ± 0.5
<i>C. hystrix</i>	3.84 ± 0.09	2.55 ± 0.13	6.3 ± 0.4
<i>C. japonica</i>	2.39 ± 0.02	2.36 ± 0.02	3.9 ± 0.1
<i>C. maxima</i>	3.93 ± 0.03	2.35 ± 0.02	4.2 ± 0.1
<i>C. reticulata</i>	3.81 ± 0.00	1.69 ± 0.00	3.8 ± 0.1
<i>C. sinensis</i>	4.03 ± 0.01	1.52 ± 0.01	5.3 ± 0.2
Distilled water	6.51 ± 0.24	1.27 ± 0.03	0.0 ± 0.0

Phenolic Compound

Since phenolic compounds have been reported that they related with an antioxidant and antimicrobial activities, this study therefore quantified these compounds in citrus juices. The data showed that *C. hystrix* juice contained the highest amount of phenolic compounds (58.52 ± 3.47 µg/mL) while *C. japonica* and *C. reticulata* juices contained the lowest amount of the compound (28.29 ± 1.01 and 28.86 ± 0.42 µg/mL, respectively). The record is presented in Table 3. Statistic test revealed that there is no correlation between phenolic amount in citrus juices and its antibacterial activity (P -value > 0.05, Pearson correlation).

Table 3 Total phenolic compounds of the citrus juices.

Citrus spp.	Phenolic compound
<i>C. aurantifolia</i>	30.32 ± 0.77
<i>C. aurantium</i>	42.27 ± 1.15
<i>C. hystrix</i>	58.52 ± 3.47
<i>C. japonica</i>	28.29 ± 1.01
<i>C. reticulata</i>	28.86 ± 0.42
<i>C. sinensis</i>	33.85 ± 0.25

Antibacterial Activity of *C. japonica* Juice

C. japonica juice was further investigated for its activity. The dose-dependent effect was tested. The data revealed that equal or more than 5% of the juice showed the growth inhibition against *S. aureus* while the juice at concentration lower than 50% did not present the activity against *S. mutans*. The activity increased as the concentration was increased (Fig 1A).

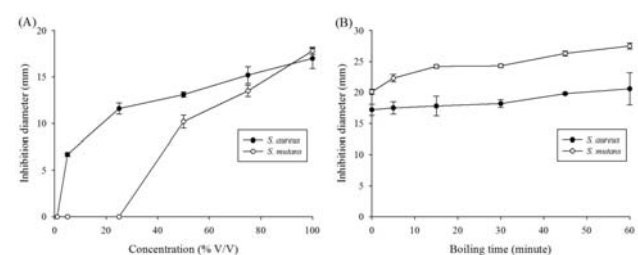


Figure 1 Antibacterial activity of *C. japonica* juice. Graph A presents the dose-response curve. Graph B shows the heat-resistant antibacterial property. The inhibition zone of ampicillin disc (10 µg/disc) for A of *S. aureus* and *S. mutans* was 12.2 ± 0.5 mm and 46.3 ± 0.2 mm, respectively and for B of *S. aureus* and *S. mutans* was 15.1 ± 0.5 mm and 47.0 ± 0.3 mm.

Statistic test indicated the correlation between dose and activity (p -value < 0.05, Pearson correlation). Finally, the heat-resistance test presented that the sample collected at 60 minutes showed the highest activity against *S. mutans* (1.37-fold and 1.14-fold higher than the fresh juice and the sample that was boiled for 15 minutes, respectively, Fig 1B).

Discussion

This study revealed the antibacterial activity of citrus juices. *C. aurantifolia*, *C. japonica* and *C. hystrix* juices were the group that showed the high activity against the tested bacteria. The previous data also reported that *C. aurantifolia* contains the antimicrobial activity¹⁶. The activities of the three citrus juices were not different in gram-positive bacterium, but in the growth of gram-negative bacterium, *C. japonica* juice showed the highest strength. Notably, *E. coli* was highly susceptible to the juice of *C. aurantium*, *C. hystrix* and *C. japonica*. It was just one third of diameter of inhibition zone that ampicillin caused in *E. coli* when compared with its effect in *S. aureus*. This study therefore corresponded with the previous report which indicated that ampicillin exhibited a low strength to destroy *E. coli* and was not practicable in clinical use.¹⁷

For the study of the physicochemical property, there was no correlation between the properties and the antibacterial activity. Viscosity of the juices was quite low which was higher than water, but was lower than milk. These physicochemical properties therefore less affected the antimicrobial results. The brix degree presented that solid component consisted in the juice was less than 10 percent by volume which would be acceptable for manufacture¹⁸. Although the antibacterial activity of citrus juice did not relate with phenolic compound but it might relate with acidity that reported as it consists in the lemon juice as mentioned recently.¹²

Nevertheless, this study also demonstrated that the activity of *C. japonica* juice exhibited the dose-response relationship. The activity slightly increased when it was boiled for longer time. It was possible that the activity was activated by heating, but it has not been clarified. However, its heat-stable property would provide an advantage for utilization.

This finding was interesting since the *C. japonica* juice widely consumed by human beings showed the very great

potential antibacterial activity. It was therefore expected to be a safety agent. However, the study to compare the antibacterial activity between citrus juices and essential oil of citrus peel should be further studied. This information would provide an idea for drug discovery or food preservation.

Conclusion

The *C. japonica* juice exhibited the highest activity against both gram-positive and gram-negative bacterium. It could inhibit both aerobic bacterium and anaerobic bacterium, also, with dose-response dependent manner and heat resistant. However the citrus juice did not exhibit the antifungal activity against *C. albicans*.

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