

Carotenoids from the Peel of Shatian Pummelo (*Citrus grandis* Osbeck) and Its Antimicrobial Activity

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Abstract: In present study, the ethanol method for carotenoids extraction from peel of Shatian pummelo was introduced. The antimicrobial activity of the carotenoids extract against *Bacillus subtili*, *Staphylococcus aureus*, *Escherichia coli*, *Aspergillus niger*, *Aspergillus flavu*, *Penicillium chrysogenum*, *Rhizopus oryzae* and *Saccharomyce scerevisiae* was also elucidated. Results showed that the optimum extraction conditions was at a temperature of 50°C, a solvent-solid ratio of 10:1 and duration of 40 min. The disc diffusion method and minimum inhibitory concentration (MIC) determination showed that the extract has a wide spectrum of antimicrobial activities against *E. coli*, *S. aureus*, *B. subtilis*, *S. cerevisiae* and *R. oryzae*, with their inhibition zones ranging from 8.97 mm to 19.47 mm and the MIC ranging from 18.75µg/ ml to 140µg/ ml. However, it showed no inhibition effect on *A. niger*, *A. flavus* and *P. chrysogenum*. Our present results suggested that Shatian pummelo carotenoids extract would be a natural alternative for chemicals in food preservation.

Key words: Antimicrobial activities • Carotenoids ethanol • Extraction • Shatian pummelo

INTRODUCTION

Citrus is one of the most important commercial fruit crops grown in all continents of the world. According to the FAO report in 2005, citrus in China covers a cultivating area of 17.1 million ha, with a production of almost 16 million tons [1]. In current citrus industry, citrus fruits are marketed fresh. As a result, approximately 50-60% of the fruit becomes citrus peel waste, a waste product consisting of peels, seeds and membranes left over after consuming [2]. This waste was generally regarded mainly as waste, which consequently generated some environmental problems [3]. To resolve this problem, more and more researchers have aimed at the utilizing of the citrus by-products [4, 5].

Carotenoids are one of the most important by-products in citrus fruits. In the past decades, contents and carotenoids compositions in fruits of different *Citrus* cultivars were extensively studied, showing that the peel of mature fruits is one of the richest and more complex sources of carotenoids [6-8]. More than 115 different carotenoids were identified in the peel and pulp of citrus fruits, with the concentration and composition vary greatly among different citrus cultivars [9]. Due to their complex structure and the wide variety of these compounds present in fruits, there was not a reference

method to analyze them [10]. The common method for carotenoids extraction from citrus fruits was organic solvents including ethyl acetate [11], acetone/methanol [12], methanol [6, 7, 13-16], ethanol/hexane [17], hexane/acetone/ethanol [18,19], petroleum ether/acetone [20], acetone [21,22] etc. However, the ethanol method was not well documented from citrus fruits, although it was successfully performed in other plants [23,24].

Carotenoids were observed to be highly involved in photosynthesis and photoprotection in plants [19]. They were also found in animal tissues where they may act as antioxidants or as immunomodulating, antimutagenic and tumor-preventing agents. The application of carotenoids in medicine and cosmetics were well documented as is their utilization as food additives (colorants and antioxidants) [25]. Recent studies have suggested that the carotenoids have the potential antimicrobial activity [26-30].

The aim of this work was to extract the carotenoids from peel of Shatian pummelo (*Citrus grandis* Osbeck), a popular and large-scale planted elite cultivar in south China, by the ethanol method and to study the effect of the carotenoids extract on the growth of microorganisms commonly associated with both food spoilage and food safety such as *Bacillus subtili*, *Staphylococcus aureus*, *Escherichia coli*, *Aspergillus niger*, *Aspergillus*

flavu, *Penicillium chrysogenum*, *Rhizopus oryzae* and *Saccharomyce scerevisiae*, in an effort to evaluate the economical value of this cultivar.

MATERIALS AND METHODS

Plant Material: Mature fruits of Shatian pummelo (*C. grandis* Osbeck) were collected from orchard of Rong county, Guangxi province, P.R. China.

Extraction and Determination of Carotenoids: Fresh flavedo of Shatian pummelo was freeze-dried and ground into powder. About 5 g powder was extracted with 10 ml 95% ethanol and incubated in 50°C for 60 min until the extraction phase was colorless. The final volume of the carotenoids extract was adjusted to 75 ml by adding 95% ethanol. The absorbance value of the carotenoids extract was determined by UV-vis spectrophotometer (Shimadzu UV 2450, Japan) at 450 nm. The total carotenoids yield (µg/g dried weight) was calculated according to the formula as follows [31].

$$\text{Carotenoids yield (}\mu\text{g/g dried weight)} = \frac{V(A - 0.0051)}{0.175W}$$

Where; A is the absorbance value of diluted extraction at 450 nm, V is the final volume of extract, 0.175 is the extinction coefficient of carotenoids and W (g) is the weight of dried powder.

Experimental Design: Firstly, the single factor experiment was performed with the analysis of the effect of three factors (temperature, solvent-solid ratio and extraction time) on carotenoids yield from peel of Shatian pummelo. Then, the optimization of carotenoids extraction process parameters of extraction temperature, solvent-solid ratio and extraction time (Table 1) was performed using orthogonal design (L_93^3). Every experiment was replicated three times. Conventional statistical methods were used to calculate means and standard deviations.

Microbial Strains: The carotenoids extract was tested towards eight microorganisms: *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Aspergillus niger*, *Aspergillus flavu*, *Penicillium chrysogenum*, *Rhizopus oryzae* and *Saccharomyce scerevisiae*. All these microorganisms were provided by the Department of Biotechnology and Food Engineering, Xiangtan University, Xiangtan, P.R. China.

Antimicrobial Activity Test: The agar disc diffusion method [32] was used as preliminary assay for testing antimicrobial effect of the carotenoids extract. Nutrient agar (NA) and Potato dextrose agar (PDA) sterilized in a flask and cooled to 45-50°C were distributed to sterilized Petri dishes with a diameter of 9 cm. A suspension of the tested bacteria (2.0×10^6 CFU/mL) and fungal (2.0×10^5 spore/mL) was spread on the solid media plates. The filter paper discs (6 mm in diameter) were individually impregnated with 10 µL of the carotenoids extract (140 µg/ml) and then placed onto the agar plates which had previously been inoculated with the tested microorganisms. The plates inoculated with bacteria were incubated at 37°C for 24 h, while the plates inoculated with fungal were incubated at 30°C for 48 h. Ampicillin Na (30µg) and 95 % ethanol served as positive control and negative control, respectively.

The broth microdilution method [32] was used to determine the minimum inhibitory concentration (MIC). The carotenoids extract was first diluted to the highest concentration (140µg/ml) to be tested and then serial twofold dilutions were made in a concentration range from 1.18 to 140 µg/ml with nutrient broth or PDA broth. The MIC was defined as the lowest concentration of the carotenoids extract at which the microorganism did not demonstrate visible growth.

RESULTS

Effect of Temperature on Carotenoids Yield: As revealed by Fig. 1, the carotenoids yield increased with the temperatures increasing from 30 to 50°C, but declined when the temperatures were above 60°C and the yield was lower at 70°C than that at 30°C. No remarkable increase was observed between 50°C and 60°C. Therefore, 50°C was the optimal temperature at which carotenoids was extracted from Shatian pummelo.

Effect of Solvent-solid Ratio on Carotenoids Yield: The effect of solvent-solid ratio on carotenoids yield was shown in Fig. 2. The carotenoids yield increased when the ratio was in 1:1 to 10:1, while it significantly increased when the ratio was higher than 15:1. This suggested that the solvent-solid ratio of 10:1 was the optimal ratio for carotenoids extraction.

Effect of Extraction Time on Carotenoids Yield: Figure 3. showed the effect of different extraction time on carotenoids yield. The carotenoids yield increased

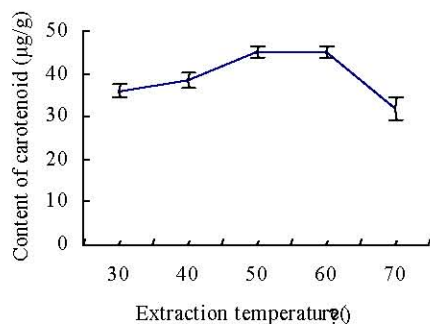


Fig. 1: Effect of temperature on carotenoids yield

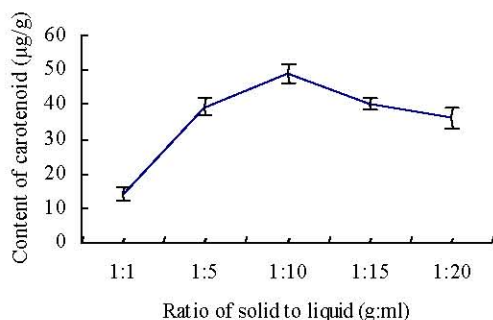


Fig. 2: Effect of solvent-solid ratio on carotenoids yield

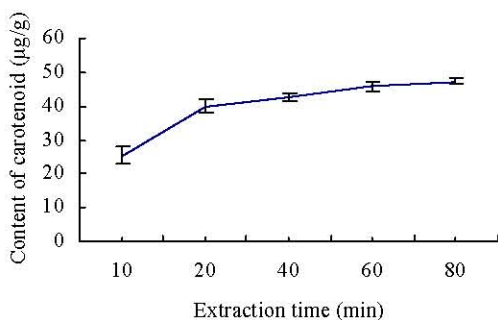


Fig. 3: Effect of extraction time on carotenoids yield

when the duration was extended from 10 to 40 min, but it remained approximately the same when the duration was further extended from 40 to 80 min, implying that duration of 40 min was the optimal duration for carotenoids extraction.

Orthogonal Experiments: The optimum conditions for the extraction were obtained by using orthogonal design (L_93^3) based on single factor experiments. Table 1 listed the coded levels of the orthogonal test factors. Table 2 showed factors at different levels in nine experiments conducted and the statistical analysis. Results showed that the order of the effect of factors on carotenoids extraction was found to be: $B > A > C$. The optimum extraction conditions obtained from the

Table 1: The coded levels and real levels of orthogonal test factors

| Factor/level | 1 | 2 | 3 |
|---------------------------------|----|------|----|
| A: Temperature (°C) | 40 | 50 | 60 |
| B: Solvent-solid ratio (g : ml) | 50 | 10:1 | 40 |
| C: Extraction time (min) | 60 | 15:1 | 60 |

Table 2: The experimental designs and the orthogonal test results

| Run | A | B | C | Contents of carotenoids (µg/g) |
|-------|---------|---------|---------|--------------------------------|
| 1 | 1 | 1 | 1 | 32.314 |
| 2 | 2 | 2 | 2 | 51.600 |
| 3 | 3 | 3 | 3 | 45.686 |
| 4 | 1 | 2 | 3 | 50.400 |
| 5 | 2 | 3 | 1 | 42.343 |
| 6 | 3 | 1 | 2 | 44.314 |
| 7 | 1 | 3 | 2 | 32.743 |
| 8 | 2 | 1 | 3 | 41.486 |
| 9 | 3 | 2 | 1 | 45.343 |
| K_1 | 115.457 | 118.114 | 120.000 | |
| K_2 | 135.429 | 147.343 | 128.657 | |
| K_3 | 135.343 | 120.771 | 137.571 | |
| R | 19.972 | 29.229 | 17.571 | |

statistical analysis were $A_2B_2C_3$. That was to say, the optimum extraction conditions for carotenoids from peel of Shatian pummelo was at a temperature of 50°C, a solvent-solid ratio of 10:1 and duration of 40 min. These conditions were tested later to ascertain the dependability of our results; the recovery of carotenoids was 57.812 µg/g. So the hypothesis of the orthogonal experiment was valid.

Antimicrobial Activity of the Carotenoids Extract:

The in vitro antimicrobial activity of carotenoids extract of Shatian pummelo against the microorganisms employed and its activity potentials were qualitatively and quantitatively assessed by the presence or absence of inhibition zones, zone diameters and MIC values. The data obtained from the disc diffusion method indicated that the carotenoids extract displayed a variable degree of antimicrobial activity on different tested strains (Table 3). The highest antibacterial activity of the carotenoids extract was observed against *E. coli*, with inhibition zone of 19.47 mm, followed by *S. aureus* and *B. subtilis* with their inhibition zones ranging from 18.63 mm on *S. aureus* to 18.57 on *B. subtilis*. The carotenoids extract also exhibited high antifungal activity against *S. cerevisiae*, with the inhibition zone of 13.17 mm, although it seemed resistant to 95% ethanol. The carotenoids extract showed moderate activity to

Table 3: Antimicrobial activity of Shatian pummelo carotenoids extract

| Tested microorganism | Inhibition zone in diameter (mm) | | |
|-----------------------|----------------------------------|----------------------|--------------------|
| | Carotenoids extract (200µL) | Ampicillin Na (30µg) | 95% ethanol (10µL) |
| <i>B. subtilis</i> | 18.57±0.64 | + | 6.20±0.10 |
| <i>S. aureus</i> | 18.63±0.65 | + | 6.30±0.00 |
| <i>E. coli</i> | 19.47±0.81 | + | 6.47±0.21 |
| <i>A. niger</i> | - | - | - |
| <i>A. flavus</i> | - | - | - |
| <i>P. chrysogenum</i> | - | - | - |
| <i>R. oryzae</i> | 8.97±0.25 | 10.57±0.40 | 6.10±0.00 |
| <i>S. cerevisiae</i> | 13.17±0.21 | 20.57±0.40 | - |

Note: “-” Mean no inhibition zone was observed. “+” mean no microorganism was observed

Table 4: The MIC values (µg/ml) of Shatian pummelo carotenoids extract against microorganism tested

| | Tested microorganism | MIC value (µg/ml) |
|----------|----------------------|-------------------|
| Bacteria | <i>B. subtilis</i> | 70.0 |
| | <i>S. aureus</i> | 35.0 |
| | <i>E. coli</i> | 18.75 |
| Fungal | <i>R. oryzae</i> | 140.00 |
| | <i>S. cerevisiae</i> | 140.00 |

R. oryzae, evidenced by the inhibition zone of 8.97 mm. *A. niger*, *A. flavus* and *P. chrysogenum* were considered resistant to the carotenoids extract and even to the reference antibiotic and 95% ethanol, since no obvious inhibition zone was observed.

Results of the MIC were shown in Table 4. The data indicate that the oil exhibited varying levels of antimicrobial activity against the investigated microorganisms. The inhibitory properties of the oil were observed within a range of concentrations from 18.75 to 140.00µg/ml. Maximum activity was observed against *E. coli*, with MIC of 18.75µg/ml. *S. aureus* and *B. subtilis* showed similar susceptibility to the investigated oil, with MIC of 35 and 70 µg/ml. Weak inhibitory effect was also observed against *S. cerevisiae* and *R. oryzae*, with MIC of 140µg/ml. These results were in accordance with those obtained by disc diffusion assay.

DISCUSSION

In present study, the ethanol method was used to extract the carotenoids from peel of the Shatian pummelo. Results showed that the average yield of carotenoids extracts ranged from 30-50 µg/g, which was consistent with reports by other method [11-18, 20-22], suggesting this method could be used to isolate total carotenoids

from peel of Shatian pummelo. However, whether this method was suitable for other citrus cultivar or not need further confirmation, due to the fact that the carotenoids concentrations and compositions varied greatly among citrus cultivars. For example, Satsuma mandarin (*C. unshiu* Marc.) accumulated β-cryptoxanthin predominantly in the flavedo and juice sacs in mature fruits [6,13], while violaxanthin isomers such as zeaxanthin have been determined predominantly in samples of mature sweet orange (*C. sinensis* Osbeck) [6, 7, 11, 14, 16, 17, 21]. The presence of lycopene and β-carotene was usually found in Star Ruby grapefruit and Cara Cara navel orange [18,20,22]. For this reason, when different methods were applied to extract total carotenoids from citrus fruits, the extraction efficiency would be greatly affected by the solvent polarity of the organic solvent.

The carotenoids extract of Shatian pummelo showed significant antibacterial activities against *E. coli*, *S. aureus* and *B. subtilis*. In contrast, weaker antifungal activities against *S. cerevisiae* and *R. oryzae* were observed. It was quite interesting that the carotenoids extract showed no inhibition effects on *A. niger*, *A. flavus* and *P. chrysogenum*. This result was in according with reports by other researchers. The *Vaccinium* fruit pigment was found to have certain antibacterial effects on *E. coli* and the antibacterial effect increased with increasing pigment density [30]. Zhang *et al.* [33] revealed that carotenoids extract from citrus peel had good effect on *S. aureus*, *B. subtilis* and *E. coli* bacterium, but had no effect on *S. cerevisiae*, *A. niger* and *Penicillium*. It deserved attention that the antimicrobial activity of citrus carotenoids extract was highly linked with the extraction method. The ether-soluble pigment from citrus peel showed strong antifungal activity against *Penicillium*, while no inhibitions to *A. niger*, *E. coli* and *S. aureus* [27]. On the contrary, the ethanol-soluble

pigment from citrus peel showed strong antibacterial activity against *E. coli* and *S. aureus*, while no inhibitions to *A. niger*, *Penicillium* and *B. subtilis* [27].

The reason for antimicrobial activity of carotenoids was still kept poorly understood. Cucco *et al.* [29] suggested that β -carotene could lead to the accumulation of lysozyme, an antibacterial immune enzyme that digests bacterial cell walls, therefore generate the antibacterial activity. The phytochemicals present in *Aframomum danielli* spice as alkaloids, carotenoids and polyphenols showed higher antibacterial activities towards *B. subtilis* and *S. aureus*, which provided the possibility of the use of *A. danielli* spice in reducing the incidence of food spoilage and food toxins [34]. As mentioned above, the composition of the citrus carotenoids covered a wide range of compounds such as β -cryptoxanthin, violaxanthin isomers, lycopene, β -carotene etc., it could be inferred that its antimicrobial activities might be highly linked with these compounds. Further confirmations were needed.

CONCLUSIONS

In summary, our present results demonstrated that the ethanol method was suitable for isolating of total carotenoids from peel of Shatian pummelo. In addition, the resulting carotenoids extract showed a wide spectrum of antimicrobial activity against the test microorganisms. These results suggested that Shatian pummelo carotenoids extract would be a natural alternative for chemicals in food preservation.

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