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Evaluation of chemical compositions and functional properties of bioactive compounds in Pomelo's seeds

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Abstract
Citrus seed is a source of limonoids which brings many benefits for human health. In this study, total limonoids content of extracts from three different pomelo seeds which were grown in Vietnam including Tan Trieu seed, Thanh Tra seed, and Doan Hung seed were investigated. The limonoids were extracted using a group of three organic solvents which were ethyl acetate, acetone and methanol and then limonoids content was determined by colorimetric method using DMAB indicator reagent. The DPPH radical scavenging method was used to determine antioxidant capacity of the extracts. Moreover, anticancer of pomelo seed's extract were examined on cancer cells. The results showed that limonoid content of Tan Trieu pomelo seed was the highest (82.6 ± 0.01 µg/mg RE), followed by that of Thanh Tra pomelo seed (78.8 ± 0.02 µg/mg RE) and Doan Hung pomelo seed (66.3 ± 0.01 µg/mg RE). However, the extract of Doan Hung pomelo seed had the highest antioxidant capacity (29.6 ± 0.01%), followed by that of Thanh Tra and Tan Trieu pomelo seed (21.0 ± 0.01%). Doan Hung pomelo seed was also had significant effect on lung cancer cells with the lowest percentage in cell survival value (CS value) (67.0 ± 2.6%), followed by Thanh Tra pomelo seed (71.9 ± 1.5%) and Tan Trieu pomelo seed (87.8 ± 3.2%). In addition, Doan Hung and Tan Trieu pomelo seeds also had influence in breast cancer cells with CS values of 78.3 ± 2.7% and 79.6 ± 2.5%, respectively, whereas Thanh Tra pomelo seed had no effect on breast cancer cells. As a result, pomelo seed was considered to be a good source of bioactive compounds, antioxidants and anticancer.

Keywords: pomelo seed, limonin, antioxidant, anticancer

1. Introduction
Pomelo (Citrus grandis) is a member of citrus family. It is grown in many eastern countries including China, Japan, India, Vietnam and Thailand. Vietnam also exports pomelo and their products to all over the world such as UK, China, Pakistan, Bangladesh, etc. with good quality and cheap price. However, after processing of pomelo for juice, seeds become a primary byproduct. Therefore, it is necessary to take full of advantages to develop another kind of products from seeds.

In recent years, pomelo has attracted more attention because of their nutritional and antioxidant properties. Moreover, human beings are currently facing with many diseases, especially cancer diseases. Evidence from the World Health Organization, states that 65% people on around the world tends to use traditional and herbal medicines to cure. This trend has dramatically increased in the last 2 decades. According to GLOBOCAN 2012, there were 8.2 million deaths in 2012 and this number is increasing quickly. The most common diagnosed cancers were lung (1.82 million cases), breast (1.67 million cases) and colorectal (1.6 million cases). The most common cancers which caused death on human being were lung cancer (1.6 million deaths), liver cancer (745,000 deaths) ad stomach cancer (723,000 deaths). Recently, many studies have been found that limonoids can affect to the endothelial tissues involving in initial stages of cancer.

Limonoids are a factor causing bitter taste in citrus fruit. There are many limonoids compounds isolated from plant kingdom. Limonoids are highly oxygenated modified triterpenes derives from a precursor with 4,4,8-trimethyl-17-furanylsteroid skeleton biosynthesized from acetate-mevalonate pathway in citrus.[1] The chemical composition of limonoids is $C_{26}H_3O_8$ with molecular weight is 470. All naturally occurring citrus limonoids contain a furan ring attached to the D-ring, at C-17, as well as oxygen containing functional groups at C-3, C-4, C-7, C-16 and C-17 [2]. Previous studies reported that limonoids were highly oxygenated. Moreover, they also indicated that limonoids have been able to antimicrobial as well as antibacterial, antifungal, antimalarial, anticancer, antiviral and others. Limonoids are phytochemicals, abundant in citrus fruit and other plants. Citrus fruits contain about 36 aglycones and 17 limonoid glucosides, be taken from citrus processing and us seeds as byproducts. Especially limonin glucosides were presented abundant compounds in citrus. Limonoids are important quality constituents of citrus fruit. In fact, aglycones are considered as pest deterrents in plants. They are popular in young leaves and fruit when these tissue need to be protected from pathogen attack. The seed residues are the primary by-product fraction. Furthermore, citrus seeds have interesting antioxidant activities. Their extraction could be useful to prevent oxidation in fruit juices and essential oils. They are a good source of unsaturated fatty acids and can be used to recover limonoids. Several studies have been realized on antioxidant activity in food system of citrus fruit, peel and seed either directly or extract form. Citrus species have a lot of biological effective compound that be able to attack radical free and work as natural antioxidant such as flavanoids, limonoids. They play a vital role in reducing the risk of many diseases such as cancers and cardiovascular diseases.

However, to the best of our knowledge, there is no report on

the effect of limonoids which are extracted from pomelo which is grown in Vietnam can affect to cancer diseases. Therefore, the objectives of my research are to optimize the method for extracting flavonoids and limonoids from pomelo peels and seeds. Then, antioxidant and anticancer activities are investigated to prove the ability of bioactive compounds in pomelo residues.

2. Materials and Methods

2.1 Materials

Pomelo's seeds from Tan Trieu, Doan Hung and Thanh Tra were collected before removing the shell. Then it was dried to remove water and ground to powder.

2.2 Extraction methods

Soxlet system was used for limonin and nomilin extraction.[3] In which, 5 gram of sample was treated with ethyl acetate for 4 hours, then continuing to treat with acetone for 1 hour before treating with methanol for 1 hour. The solutes then mixed together and were evaporated by rotating evaporation. After evaporating, 20 ml of methanol/water (1:1) were used to dissolve before centrifuging to collect solid residue.

2.3 Measurement of total limonoid equivalents using DMAB reagent

The quantitation of total limonoids was employed by colorimetric method.[4] Sample residue was reconstituted with 10 ml of acetonitrile. The collected samples after reconstitution were stored in amber bottle. DMAB indicator reagent was prepared as followed: Perchloric acid was diluted with water to 70%. Then 24 ml of them was combined with 30 ml of acetic acid (glacial form) to prepare stock solution. The DMAB reagent was freshly prepared by dissolving 0.56g of DMAB in 15ml of stock solution. Then 330 µl of DMAB indicator and 330 µl of stock solution added into 220µl of sample and then incubated at room temperature for 30 min. After that, the mixture was measured at 503nm in the UV-Vis spectrophotometer. The standard curve was repaired in acetonitrile (0.1 mg/ml) and followed the same measurement process. The result expressed as µg/g.

2.4 HPLC apparatus

An aliquot of 20 µl of reconstituted sample in acetonitrile was injected to HPLC system. The mobile phase composed of 3mM phosphoric acid (A) and acetonitrile (B). The gradient elution was run at 85% of solvent A at 0 min, reduced to 77% A at 5 min, 74% A at 25 min, further reduction to 60%A at 30 min and completed at 54%A at the end of 45 min. The column was equilibrated for 5 min with 85% A and 15% B before next run [1]. The elution was determined at 210nm in ambient temperature.

Standardize limonoids compound: Limonin mixed with acetonitrile to get the standard at 100µg/ml.

2.5 Antioxidant capacity by DPPH radical scavenging assay

The antioxidant activity of peels and seeds of citrus fruits was determined according to the method of Hung and Morita (2008) [5]. Final concentration of DPPH solution used was 0.075 mM. DPPH solution (3.9 ml) was mixed with sample solution (0.1 ml). The mixture was kept in dark at ambient temperature. Absorbance was read at 515 nm for 30 minutes. Blank was made by mixing 3.9 ml of DPPH solution and 0.1 ml of methanol and measured absorbance at t=0. The scavenging of DPPH was calculated according to the following equation (Liyana-Pathirana & Shahidi, 2007).

$$\%\text{DPPH scavenging} = \{(Abs(t=0) - Abs(t=30))/Abs(t=0)\} \times 100$$

Abs(t=0) = absorbance of DPPH radical + methanol at t= 0 min;

Abs(t=30) = Absorbance of DPPH radical+ phenolic extracts at t= 30 min

2.6 Anticancer activity by Cytotoxicity Assay

Colorimetric cytotoxicity assay was carried out to investigate the effects of limonoid compounds on the survival of cancer cells including Hep-G2 (Human hepatocellular carcinoma); NCI H460 (Human lung adenocarcinoma) and MCF-7 (Human breast adenocarcinoma) by using SRB (Sulforhodamine B), a basic colorimetric method to investigate the cytotoxicity of compounds.[6] These cancer cells were maintained in E'MEM (Dulbecco's Modified Eagle Medium) supplemented with L- glutamine, Sodium pyruvate, NaHCO3, PSF (Penicillin- Streptomycin sulfate - Fungizone); NAA (Non-Essential Amino Acids); 10% BCS (Bovine Calf Serum) was added before keeping at 37ºC in 5% CO_2 incubator. A 96-well plate was used to grow cells at 104 cells/well for Hep-G2 and MCF-7, 7.5±103 cells/well for NCI-H460 in growth medium. After 24 hour of growing, these cells were incubated in samples with different concentration in 48 hours. Then the total protein was maintained by using Trichloroacetic acid (Sigma) 50% and dyed by SRB 0.2%.The standard was measured by Ellipticine, Vinblastine or Taxol dissolving DMSO. The result was read by ELISA at 495-515nm.

$$\%\ \text{Cell survival} = (Abs_{sample} - Abs_{control})/ (Abs_{DMSO} - Abs_{control})$$

3. Results and Discussion

3.1 Determination of limonoids in pomelo's seeds.

Total limonoid contents of the pomelo's seeds are shown in Table 1. Tan Trieu pomelo seeds showed the highest total limonoids content (82.6 µg/mg RE), whereas Doan Hung pomelo seeds had the lowest value (66.3 ug/mg RE). Among limonoid aglycones, limonin was the most representative compound in juices, peel, and seed whereas nomilin was the most representative compound in pulps and seeds. These quantitative data are good agreement with those published previously for citrus fruit, in which limonoid glucosides were described as more abundant in juices and pulps because they are water-soluble, while water-insoluble limonoid aglycones are mostly present in seeds and peels [7, 8].

Table 1: Total limonoid contents of pomelo's seeds

Sample	Limonoids concentration (µg/mg RE)
Tan Trieu pomelo seeds	82.6 ± 0.01^a
Thanh Tra pomelo seeds	78.8 ± 0.02^b
Doan Hung pomelo seeds	66.3 ± 0.01^c

*Values with different letters with in a column and a row are significantly different ($p<0.05$) *RE: Residue

3.2 HPLC analysis of limonin in pomelo's seeds

The chromatograph of limolin compound in the pomelo seeds saperated by the HPLC with a C_{18} reversed phase column of 5 µm particle size (25 mm × 0.5 mm) was presented in Figure 1. Amounts of limonin found in three kinds of pomelos are given in Table 2. Tan Trieu pomelo seeds had the highest amount of limonin (52.1 µg/g), followed by Doan Hung pomelo seeds (40.6 µg/g) and Thanh Tra pomelo seeds (32.7 µg/g). Thus, limonin compound occupied around 78% of total limonoids in Doan Hung seeds, 51% of total limonoids in Thanh Tra seeds and 39% of total limonoids in Tan Trieu seeds. The different amounts of total limonoids and limonin might affect antioxidant capacity and bioactivities of the extracts. Limonin concentration of these kinds of pomelo was consistent to previous study reported [1].

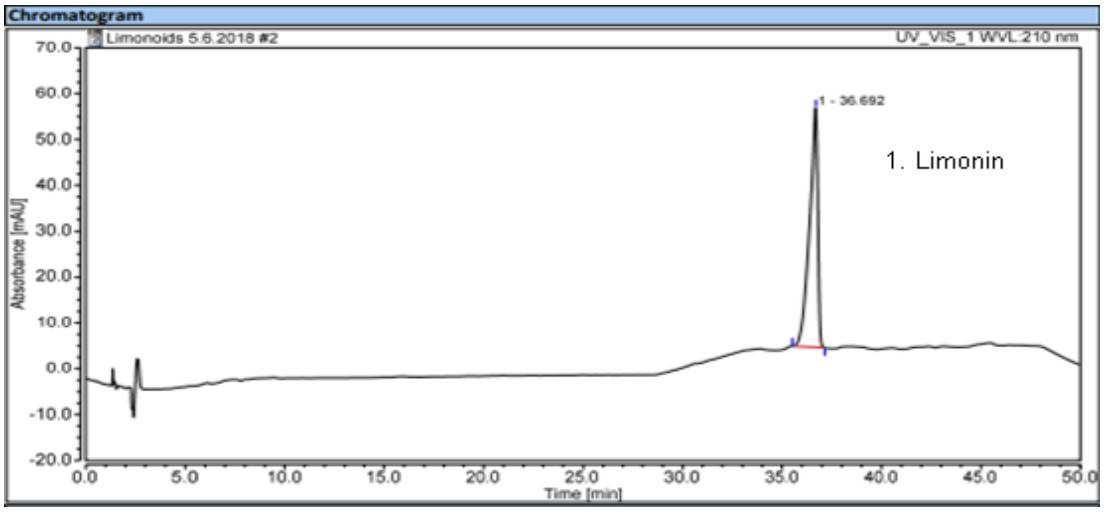

Fig 1: Chromatograph of limonin by HPLC measurement

Table 2: The HPLC method for limonin analysis in pomelo seeds

Sample	Limonin concentration (µg/g)
Tan Trieu pomelo seeds	52.1 ± 0.58
Thanh Tra pomelo seeds	32.7 ± 0.32
Doan Hung pomelo seeds	40.6 ± 0.54

Values are means of triplicate measurements. Values with different letters with in a column and a row are significantly different ($p<0.05$)

3.3 Antioxidant capacities of limonoids extracting from pomelo's seeds

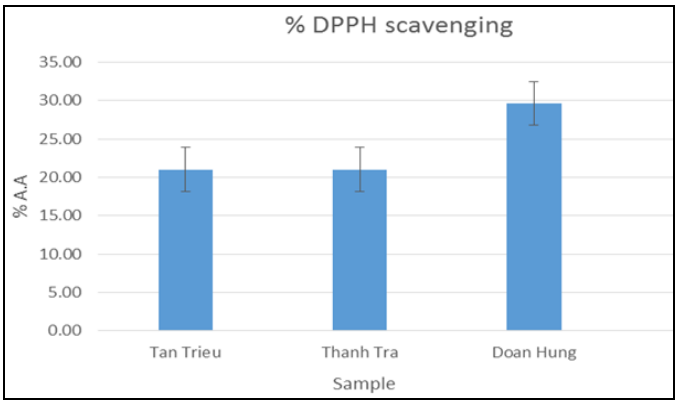

Fig 2: Antioxidant activity of Pomelo's seed extracts measured by DPPH

Antioxidant capacity of the extracts from pomelo's seeds is shown in Figure 2. Doan Hung pomelo seeds had the highest antioxidant activity (29.6 ± 0.01%), followed by Thanh Tra (21.0 ± 0.01%) and Tan Trieu pomelo seeds (21.0 ± 0.01%). However, the antioxidant activities of Thanh Tra and Tan Trieu pomelo seeds were not significantly different. Limonoids are highly oxygenated triterpenoids with fewer hydroxyl groups than flavonoids. Therefore, these structural compounds probably contributed to the weak antioxidant activity of limonoid compounds. Previous study demonstrated that limonin had relative stronger antioxidant activity than others, especially in LDL oxidation assay system.[9] Therefore, with higher limonin content, Doan Hung pomelo seeds had the higher antioxidant activity compared to the Tan Trieu and Thanh Tra pomelo seeds.

3.4 Anticancer actitity of seed's extracts

The viability of MCF-7, LU-1, Hep- G2 cells in the presence of limonoids at 100 µg/ml and 48 hours of incubation is given in Table 3 as percentage of Cell Survival Value. All three samples did not affect to the Hep-G2 (Human hepatocellular carcinoma) which had high CS value. Only 1.27%, 1.55% and 0.41% cancer cells were killed by using Doan Hung, Tan Trieu or Thanh Tra pomelo seed extract, respectively. To determine whether other effects might be found in other cancer cell lines, the limonoids were tested against two other cells, LU-1 and MCF-7. The most potent inhibitor of Doan Hung pomelo seed extract was found for LU-1 with the lowest percentage in CS value (67.0 ± 2.6 %), followed by Thanh Tra (71.9 ± 1.5%) and Tan Trieu (87.8 ± 3.2%). Moreover, both Doan Hung and Tan Trieu pomelo seed extracts had mild activities on breast cancer cells (21.7% and 20.3% death cells, respectively). The results also indicated that Thanh Tra pomelo seed extract did not affect to MCF-7 (Human breast adenocarcinoma). Tian *et al.* [10] also found the mild activities of citrus limonoids on human cancer cells similar to the results of this study.

Table 3: Anticancer activity of pomelo seed's extracts.

Sample	Initial concentration (µg/ml)	CS value (%)		
		Hep-G2	LU-1	MCF-7
DMSO	-	100	100	100
Control (+)	5	4.3 ± 0.4	1.1 ± 0.4	2.5 ± 0.1
Doan Hung seeds	100	98.7 ± 1,2[a]	67.0 ± 2.6[b]	78.3 ± 2.7[e]
Tan Trieu seeds	100	98.4 ± 0.5[a]	87.8 ± 3.2[c]	79.6 ± 2.5[e]
Thanh Tra seeds	100	99.6 ± 0.3[a]	71.9 ± 1.5[d]	98.4 ± 1.6[f]

Values with different letters with in a column and a row are significantly different ($p<0.05$)

4. Conclusions

The limonoid compounds, antioxidant and anticancer capacity of the extrats from pomelo seeds were investigated in the present study. The results indicated that all samples possessed antioxidant, in which DoanHung seeds had the highest antioxidant capacity because of higher limonin compounds than other samples. In addition, anticancer capacity of limonoids extracted from pomelo seeds was only found high ability against LU-1 and MCF-7.

5. Acknowledgement

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The authors have declared no conflict of interest.

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