The Flavonoids in *Citrus madurensis* Lour and their Anti-Hepatitis B Virus Activity

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**Introduction**

*Citrus madurensis* Lour. (calamondin), a perennial tree in the family Rutaceae, is an important citrus tree in Taiwan where its juice and fruit are popular among consumers. However, juice production often results in a considerable amount of waste, such as peels, seeds and pulp; fortunately, the waste are rich in flavonoids, carotenoids, polyphenols, and limonoids compounds which all have excellent bioactivities [1-10] and the development of these compounds could create additional value.

The human hepatitis B virus (HBV) is a global disease that is especially prevalent in Asia and Africa. Infections could induce acute hepatitis that could cause the infected into chronic hepatitis carriers. Approximately one-quarter to one-third of those infected will develop hepatitis that could cause the infected into chronic hepatitis carriers. Early liver cirrhosis and are at elevated risk of hepatocellular carcinoma [11,12]. Current treatments include Interferon-α, Lamivudine, Adefovir, Entecavir, and Telbivudine, but these drugs can only reduce the activity or temporally inhibit the reproduction of HBV and cannot eradicate HBV from the human body. Therefore, in recent years, developing antivirus drugs from natural products has become an important research topic.

Research has revealed that flavonoids in citrus demonstrated antiviral activities [13-17], but little research has been conducted on the anti-HBV properties of citrus. So far the only citrus anti-HBV research consisted of isolated imperatorin purified from pomelo (*C. grandis* L.) peels, in which findings displayed that imperatorin possessed excellent inhibition effect on the surface antigen and e-antigen of HBV [18]. The purpose of this study is to investigate the anti-HBV activity of calamondin and citrus flavonoids. We used HBV transacted cell line MS-G2 to assess that anti-HBV ability.

**Materials and Methods**

**Plant material**

Calamondin fruit were bought at random on 9. Oct. 2009 at Jiou Township, Pingtung County, Taiwan, and verified by Professor Chung-Ruey Yen. Voucher specimens (Specimen No. CM2009001) were maintained in the laboratory of the corresponding author in the Department of Plant Industry, NPUST.

**Crude extracts preparation from calamondin**

The peel and pulp were separate from of fruit, that were freeze-dried and powdered, then the dried material was extracted by ultrasonic extraction for 30 min. The extraction was carried out using organic solvents (dichloromethane, ethyl acetate, n-butanol, acetone, and methanol respectively). The filtrate was concentrated by evaporation under reduced pressure (ca. 42°C) and further freeze-dried. The dried extract powder was used in anti-HBV assays.

The better anti-HBV activities were evaluated from the different organic solvent extracts described above, and the one with the lowest HBsAg expression was chosen for column chromatography to clarify the bioactive fractions. 200 grams of dried sample powder were extracted with ethyl acetate (2L×3) then filtered. The filtrate was concentrated and freeze-dried to yield the ethyl acetate extract (13.8 g).

The ethyl acetate extract was chromatographed by a silica gel column (3.5 I.d.×30 cm) and eluted step wise with n-hexane×80% n-hexane-20% ethyl acetate×60% n-hexane-40% ethyl acetate×40% n-hexane-60% ethyl acetate×20% n-hexane-80% ethyl acetate×ethyl acetate×methanol.

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Citrus flavanoids of calamondin

Pure compounds of hesperidin, diosmin, neohesperidin, and synephrine were acquired from Sigma Chemical Co.; tangeretin (1), nobiletin (2), and 5-hydroxy-3',4',6,7,8-pentamethoxyflavone (3) were purified from fruit peels of calamondin.

Dry fruit peels were extracted with methanol. After evaporation of the solvent, the residue was partitioned between water and ethyl acetate. The ethyl acetate extract was chromatographed on a silica gel column and eluted with gradients of n-hexane: ethyl acetate to yield compound 1–3. The physical and 'H NMR spectroscopic data of 1–3 were in agreement with the reported data for tangeretin, nobiletin, and 5-hydroxy-3',4',6,7,8-pentamethoxyflavone [19-21]. Using osthole as a positive controls that origin, isolation and structure identification to refer publication [22].

Cell culture and measurement of HBsAg

The human HBV transected cell line MS-G2 was cultured in Dulbecco’s modified Eagle medium (DMEM) containing 10% (v/v) FBS (fetal bovine serum), 2.5 μg/mL amphotericin B, 1% penicillin streptomycin solution, and 0.1 μM MEM (modified eagle medium) non-essential amino acid solution at 37°C under 5% CO₂. Cell were seeded in 24-well culture plates at a density of 2×10⁵ cells/mL per well. Cells were properly attached after overnight and the test samples, which were all dissolved in DMSO. The concentration of DMSO in the culture medium was kept below 2.5 μL/mL to ensure it did not affect cell growth. The culture media were collected on Day 3 for the measurement of hepatitis B surface antigen (HBsAg). The HBsAg expression in the culture medium was measured using ELISA kits (General Biologicals, Co., Hsinchu, Taiwan) following the Manufacturer’s recommendations. Each experiment was performed in triplicate. The expression was calculated as follows:

Expression (%)=[OD₄₅₀ (sample) /OD₄₅₀ (control)] ×100%

Cell viability assay

Cytotoxicity was assessed by the MTT assay as previously described [23]. Cells were cultured for 48h with tested compounds. The cells with media alone were used as a negative control. After 48 hours, MTT (1 mg/mL) reagent was added to each well. After 4 hours at 37°C, the medium was then replaced by 400 μL of DMSO and mixed thoroughly to dissolve the formazan crystals. Absorbance was measured at 540 nm. Cell viability was determined as a percentage of the negative control.

Cell viability(%)=[OD₄₅₀ (sample) /OD₄₅₀ (control)] ×100%

Cell viability above 80% can be regarded as indicating nontoxicity, and the pharmacy and dose can then be used for investigating HBsAg expression in advance.

HPLC analysis

High Pressure Liquid Chromatography (HPLC) was conducted with a Hitachi system equipped with a degasser DG-2410, pump L-7100, UV/Vis detector L-7420, and auto-sampler L-7200. Peak areas were calculated with D-7000 HSM software.

The samples were eluted using an Inertsil ODS-2 column (4.6 mm i.d.×250 mm) at 40°C. Elution was carried out on a gradient. Mobile phases were 10% (solvent A) and 70% (solvent B) acetonitrile (85% H₃PO₄ was added to adjust the pH value to 2.8). The gradient program was started with 100 : 0 (A : B), and A was gradually decreased to 90% after 10 min, 84% after 55 min, 51% after 65 min where it settled for 31 min, and then to 30% at 105 min and to 0% at 110 min. Re-equilibration of the column was achieved with a linear gradient to 100% A (initial condition) in 5 min, followed by 3 min of isocratic elution before the next injection. The flow rate was 1.0 mL/min, injected volume was 20 μL, and the components were detected at 220 nm.

Statistical analysis

All the experiments were carried out in triplicate and each experiment was repeated three times. The data were analyzed by SAS (Statistical Analysis System). One-way ANOVA with Duncan’s multiple range test was used to determine the significance at p<0.05.

Results

Effect of solvent extracts on HBsAg expression

To investigate the effect of calamondin on HBsAg expression, five solvents to extract the peel and pulp of calamond in respectively. The ethyl acetate and acetone extracts from the peels reduced the HBsAg expression of HBV by 41.6% and 71.4%, respectively, in doses 50 μg/mL (Figure 1). The ethyl acetate extract exhibited a high inhibitory effect. In pulp, the n-butanol extracts light reduced HBsAg expression to 78.7%, four other solvents extract had no inhibitory effect on the HBsAg of HBV (HBsAg expression were higher than 80%). The solvent control had no inhibition effect on the HBsAg of HBV.

Effect of different fractions of ethyl acetate extract on HBsAg expression

According to the previous result, the extracts of ethyl acetate from the peels had the highest inhibitory effect on HBsAg expression. We chose this extract to elute by column chromatography and then separately eluted into 7 fractions. The dose at 50 μg/mL, fractions 2, 3, and 4 had lower HBsAg expression (Figure 2). Furthermore, fraction 3 had a strongest inhibitory effect (HBsAg expression 40.4%) then fraction 2 and 4 (HBsAg expression 70.6% and 52.5%, respectively).
Effect of flavonoids of calamondin HBsAg expression

The effect on the HBsAg expression of HBV of citrus flavonoids was investigated with results shown in Figure 3. The results shown nobiletin, tangeretin, and 5F had lower HBsAg expression than other compounds. 100 and 50 µM doses of nobiletin lowered the HBsAg expression to 19.3% and 35.1%, respectively, and had a very high inhibitory effect. Meanwhile, a 25 µM dose of nobiletin could still lower the HBsAg expression to 56.0% and had a medium inhibitory effect. 100, 50, and 25 µM doses of tangeretin greatly lowered the HBsAg expression to 33.1%, 38.4%, and 41.5%, respectively, and had a very high inhibitory effect. A 6.25 µM dose of 5F lowered the HBsAg expression to 42.1% and had a very strong inhibitory effect, while a 3.13 µM dose of 5F still lowered the HBsAg expression to 64.33% and had a medium inhibitory effect. The IC₅₀ of nobiletin, tangeretin, and 5F were calculated. The IC₅₀ of 5F was 5.12 µM and 5F had lowest inhibiting concentration followed by tangeretin (20.7 µM) and nobiletin (33.9 µM). The positive control (osthole) have medium inhibition of HBsAg expression with 48.7% expression at 80 µM.

Quantitative analysis and fingerprint variation of different fractions

Ethyl acetate extracts of peels of calamondin were divided into 7 fractions by column chromatography elution. The fingerprint variation of each fraction was investigated using HPLC and the marker components were quantitatively analyzed, with results shown in Table 1 and Figure 4. In fraction 1, none of the 6 marker components were detected. In fraction 2, only tangeretin and 5F were detected at 47.5 and 26.0 mg/g D.W., respectively. In fraction 3, only nobiletin and tangeretin were detected at 45.0 and 291.2 mg/g D.W., respectively. In fractions 4, 5 and 6, only nobiletin was detected at 420.9, 2.2, and 2.2 mg/g D.W., respectively. In fraction 7, synephrine (without chromatogram), hesperidin, diosmin, and neohesperidin were detected at 12.8, 16.4, 3.9, and 16.7 mg/g D.W., respectively.

Discussion

Organic solvents with different polarities could induce different expressions of activity in their extracts [24-26]. Calamondin methanol extracts had no significant inhibitory effect on the HBV surface antigen or e-antigen in the pre-test of this study. Therefore, dichloromethane, n-butanol, ethyl acetate, acetone, and methanol were chosen for the extraction of calamondin in accordance with their polarities. Results exhibited a 50 µg/mL dose of ethyl acetate extracts of calamondin peel significantly lowered the HBsAg expression of HBV. Meanwhile, MTT assay of the ethyl acetate peel extract displayed no toxicity to normal cells. Hence, organic solvents extracted in different polarities apparently can enhance expression activity and massively elute effective fractions. According to effective extraction methods previously assessed with different solvents and the tracing of the effective fractions, higher activity expressions were expected. The ethyl acetate peel extracts of calamondin were analyzed using column chromatography and were divided into several fractions. Each fraction was processed using the HBV activity assays. Results showed fractions 2, 3, and 4 significantly lowered the HBsAg expressions of HBV without cytotoxicity with doses of 100, 50, and 50 µg/mL, respectively, but these dosages induced cytotoxicity on normal liver cells. It may be that the components which exhibited anti-HBV effects as well as induced cytotoxicity on normal cells were condensed in the column chromatography process; thus, enhancing their anti-virus activities and rendering them toxic to normal cells.

Imperatorin, a constituent of the fruit peel of shaddock (C. maxima (Burm. f.) Merr. form. Buntan (Hay.) Hort.) and its biotransformation from fermentation using Aspergillus flavus displayed excellent anti HBV surface antigens [17]. The three polymethoxyflavones (PMFs) investigated here (nobiletin, tangeretin, and 5F) also had excellent inhibitory effects on the HBV surface antigen, and were higher more effective than imperatorin [17]. Although a 100 µM dose of 5F had lower inhibitory effect on the HBV surface antigen when compared to nobiletin or tangeretin, there was still a high inhibitory effect when the dose was reduced to 6.25 µM.

PMFs are a kind of flavonoid, a specific component of citrus, and they display anti-inflammatory, anti-mutation, and anti-tumor activities [27-30]. This study showed that nobiletin, tangeretin, and 5F all have excellent HBV surface antigen inhibitory effects. Real-time PCR tests will be needed to confirm that the effective compounds that were filtered out in this study could reduce HBV DNA. Further
investigation could examined the influence of intracellular molecules on HBV inhibition mechanism.

**Conclusion**

In this study, ethyl acetate peel extracts of calamondin have excellent inhibitory effects on HBsAg. Moreover, after processing anti hepatitis B virus activities using all the marker components in this study, three polymethoxyflavones, namely nobiletin, tangeretin, and 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone, were found to significantly inhibit the hepatitis B virus surface antigen, which has potential antiviral activity. The results of this study could provide a reference for the development of hepatitis B treatments and diversify the use of citrus.

**References**
