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

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Abstract

In this study, we evaluated anti-hepatitis B virus (HBV) activity of the extracts (dichloromethane, ethyl acetate, n-butanol, acetone, and methanol respectively) of fruit (peels and pulp) of *Citrus madurensis* Lour. (calamondin) using HBV transected cell line MS-G2. The ethyl acetate extracts from the peels strongly reduced the HBsAg expression of HBV in dose 50 µg/mL. According to the previous result, we chose this extract to elute by column chromatography and then separately eluted into 7 fractions. In dose in 50 µg/mL, fractions 2, 3, and 4 had lower HBsAg expression (compare with control). Furthermore, fraction 3 had a strongest inhibitory ability. The flavonoids (hesperidin, diosmin, neohesperidin, nobiletin, tangeretin, and 5-hydroxy-3',4',6,7,8-pentamethoxyflavone) were analyzed each in fractions by high-performance liquid chromatography (HPLC). The results shown fraction 2, 3, 4 were detected containing nobiletin, tangeretin, and 5-hydroxy-3',4',6,7,8-pentamethoxyflavone. The anti-HBV activity of the flavonoids show that nobiletin, tangeretin, and also had a lower HBsAg expression. The IC₅₀ value were nobiletin (33.9 µM), tangeretin (20.7 µM), and 5F (5.12 µM), respectively. Taken together, we suggest that 5F could be used as a standard marker for the anti-HBV effect of calamondin.

Keywords: *Citrus madurensis* Lour.; Citrus flavonoids; Hepatitis B virus; High performance liquid chromatography

Introduction

*Citrus madurensis* Lour. (calamondin), a perennial tree in the family Rutaceae, is a 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 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.) Osbeck) 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 transected 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-*n*-hexane-*n*-hexane-20% ethyl acetate-*n*-hexane-40% ethyl acetate-*n*-hexane-60% ethyl acetate-*n*-hexane-80% ethyl acetate-*n*-hexane-90% ethyl acetate-n-hexane-*n*-hexane, and eluted step wise with *n*-hexane-*n*-hexane-*n*-hexane-20% ethyl acetate-*n*-hexane-40% ethyl acetate-*n*-hexane-60% ethyl acetate-*n*-hexane-80% ethyl acetate-*n*-hexane-90% ethyl acetate-n-hexane to obtain the fractions. 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).

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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 peel 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 1H 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. Cells were seeded in 24-well culture plates at a density of 2×10⁵ cells/mL per well. Cells were properly attached after overnight and changed 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).

To investigate the effect of calamondin on HBsAg expression, five solvents to extract the peel and pulp of calamondin 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 from ethyl acetate extract on HBsAg expression

To investigate the effect of calamondin on HBsAg expression, five solvents to extract the peel and pulp of calamondin 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 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 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 64.3% 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 with different solvents and the tracing of the effective fractions, higher activity expressions were expected. The ethyl acetate peel extracts of calamondin 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 liver 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 from 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 polymethoxylflavones (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 examine 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, and extracts of effective fractions were traced using column chromatography. Although these fractions could effectively reduce the HBsAg expression, they induced cytotoxicity in normal liver cells. Therefore, toxicity in host cells must be considered when using crude extracts to process anti HBV activities.

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, as excellent inhibitory effects on HBsAg, and extracts of effective fractions of calamondin could be considered when using crude extracts to process anti HBV activities. HBsAg Expression (% of control)

**References**


