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Isolation of quinic acid derivatives and flavonoids from the aerial parts of *Lactuca indica* L. and their hepatoprotective activity in vitro

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Abstract—In our continuing study of biologically active compounds from Korean medicinal plants, we investigated the hepatoprotective constituents of the aerial parts of *Lactuca indica* L. (Compositae), since the methanolic extract of *L. indica* has hepatoprotective activity against hepatitis B virus (HBV) production. The bioactivity-guided separation of the methanolic extract of the aerial parts of *L. indica* resulted in the isolation of seven quinic acid derivatives (**1**, **3**–**4**, **6**, and **10**–**12**), along with five flavonoids (**2**, **5**, and **7**–**9**). All the isolated compounds were evaluated for hepatoprotective activity by the HBV assay in vitro. In the human HBV-transfected liver cell line HepG2.2.15, all the compounds except **2** and **5** effectively reduced HBV DNA level in the release of mature HBV particles from HepG2.2.15 cultivation. Of the ten active compounds, treatment with **1**, **3**, and **12** led to significant reduction in the extracellular HBV DNA level, suggesting that they could be potent phytochemical agents against hepatitis B virus.

Hepatitis B virus (HBV) infection in humans is still a major medical problem worldwide. Although the rate of new infections has decreased due to vaccination with recombinant subunit vaccines and several new antiviral targets, an estimated 500 million affected individuals need treatment.[1,2] Early vaccination against chronic infection of HBV has been effective in the reduction of new infections. Chronic viral hepatitis infections can progress to cirrhosis, which may ultimately lead to hepatic failure or the development of hepatocellular carcinoma.[3] During chronic infection, treatment with nucleoside analogs and/or interferon-α may decrease the virus titer or prevent the development of chronic viral liver disease. However, there are problems such as the development of drug resistant HBV mutants after prolonged treatment and adverse side-effects.[4] Thus, better therapeutic regimens are still needed for afflicted individuals. The use of alternative therapies to complement existing antivirals for chronic viral infections is generally recognized.[5,6] The investigation of medicinal plant extracts that exhibit antiviral properties may lead to the development of compounds to complement conventional therapies.[7,8]

In our continuing study of biologically active compounds from Korean medicinal plants, we investigated the hepatoprotective constituents of the aerial parts of *Lactuca indica* L. (Compositae), since the methanolic extract of *L. indica* was found to be hepatoprotective against HBV production (Fig. 2). *L. indica* is widely distributed throughout Korea. This indigenous herb is an edible wild vegetable that is traditionally used as a folk medicine for the treatment of inflammation, bacterial infection, and intestinal disorders.[9] Various biological activities have been reported, including antimutagenic activity against indirect-acting mutagens (AFB_1 and B(a)P)[10] and stimulation of differentiation of the mouse melanoma cell line, B16 2F2.[11] Although there have been several studies of the constituents and biological activities of this plant, the hepatoprotective constituents have seldom been investigated. A bioassay-guided column chromatographic separation of the methanolic

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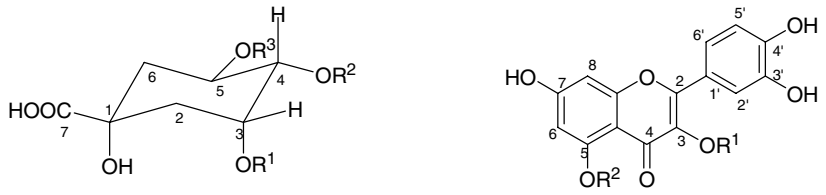
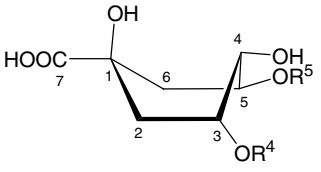
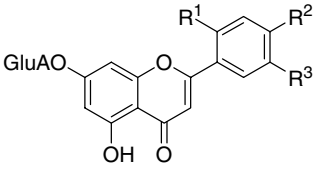


1 R^1 = caffeoyl R^2 = caffeoyl R^3 = H
4 R^1 = caffeoyl R^2 = H R^3 = caffeoyl
6 R^1 = H R^2 = caffeoyl R^3 = caffeoyl
10 R^1 = H R^2 = H R^3 = caffeoyl
11 R^1 = caffeoyl R^2 = H R^3 = H
12 R^1 = H R^2 = H R^3 = (*E*)-*p*-coumaroyl

2 R^1 = Rut R^2 = H
5 R^1 = H R^2 = Glu
9 R^1 = Glu R^2 = H



3 R^4 = caffeoyl R^5 = caffeoyl



7 R^1 = H R^2 = OH R^3=OH
8 R^1 = OH R^2 = H R^3=H

Rut = rutinose, Glu = glucose, GluA = glucuronic acid

Figure 1. The structures of the isolated compounds **1**–**12** from *L. indica*.

extract of this plant led to the isolation of seven quinic acid derivatives (**1**, **3**–**4**, **6**, and **10**–**12**), together with five flavonoids (**2**, **5**, and **7**–**9**) (Fig. 1). The hepatoprotective activity of all the isolated compounds was assessed using an in vitro HBV assay.

The aerial parts of *L. indica* were collected at Suwon city, Korea, in May, 2005. A voucher specimen (SKKU 2005-5) of the plants was deposited at the herbarium of the College of Pharmacy at Sungkyunkwan University (Korea). The aerial parts of *L. indica* (5.0 kg) were extracted three times with 80% MeOH at room temperature for 72 h. The concentrated methanolic extracts (200 g) were suspended in distilled water (800 mL) and successively partitioned with *n*-hexane, $CHCl_3$, and *n*-BuOH yielding 20 g, 12 g, and 45 g, respectively. Since the *n*-BuOH-soluble fraction showed hepatoprotective activity against HBV production (Fig. 2), this active fraction (45 g) was subjected to silica gel column chromatography using a stepwise gradient solvent system of $CHCl_3$:MeOH:H_2O (from 30:10:1 to 13:7:1) to give six fractions (B1–B6). Fr. B4 (7.0 g) was purified by preparative reversed-phase HPLC, using a solvent system of 30% MeOH over 30 min at a flow rate of 2.0 mL/min (Econosil® RP-18 10 μm column; 250 × 22 mm; 10 μm particle size; Shodex refractive index detector) to yield compounds **1** (8 mg), **2** (12 mg) and **3**, (20 mg). Fr. B5 (5.0 g) was also separated by repeated RP-C_{18} silica gel column chromatography and reversed-phase MPLC on LiChroprep® RP-18 column (25 × 310 mm; 40–63 μm particle size; 5 ml/min; eluted with 30% MeOH), and, finally, purified by preparative reversed-phase HPLC, using a solvent system of 30% MeOH over 30 min as described above, to afford compounds **4** (10 mg), **5** (31 mg), **6** (10 mg), **11** (7 mg), and **12** (12 mg), respectively. Fr. B3 (7.5 g) was further purified by preparative reversed-phase HPLC, using a solvent system of 45% MeOH over 30 min as described above, to yield compound **9** (32 mg). Fr. B6 (3.0 g) was chromatographed over a RP-C_{18} silica gel column chromatography and a Sephadex LH-20 column chromatography, using a solvent system of 80% MeOH, and purified by preparative reversed-phase HPLC, using a solvent system of 25% MeOH over 30 min as described above, to yield compound **10** (55 mg), and then purified by preparative HPLC, using a solvent system of EtOAc–MeOH–H_2O (15:7:1) over 30 min at a flow rate of 2.0 mL/min (Apollo Silica 5 μm column; 250 × 22 mm; 5 μm particle size; Shodex refractive index detector) to yield compounds **7** (8 mg) and **8** (15 mg). The isolated

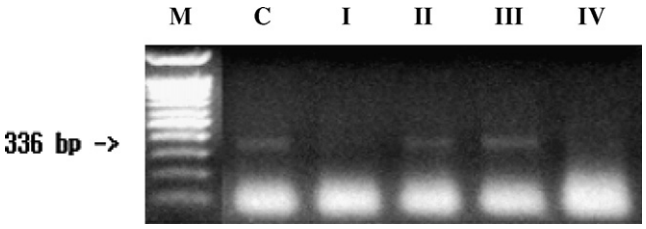


Figure 2. Amplification of extracellular HBV DNA fragment by PCR. HepG2.2.15 cells were treated with MeOH extract (**I**), hexane fraction (**II**), chloroform fraction (**III**), and butanol fraction (**IV**) or were not treated (**C**). After 4 days cultivation of the cells, 10 μl medium for each sample was used to amplify the 336-bp length of the HBsAg region of HBV DNA.

compounds were identified as 3,4-di-*O*-caffeoylquinic acid (**1**),[12] quercetin 3-*O*-α-L-rhamnopyranosyl(1 → 6)-β-D-glucopyranoside (**2**),[13] 3,5-di-*O*-caffeoyl-*muco*-quinic acid (**3**),[14] 3,5-di-*O*-caffeoylquinic acid (**4**),[15] quercetin 5-*O*-β-D-glucopyranoside (**5**),[16] 4,5-di-*O*-caffeoylquinic acid (**6**),[15] luteolin 7-*O*-β-D-glucuronide (**7**),[17] 5,2′-dihydroxy-7-*O*-β-D-glucuronylflavone (**8**),[18] quercetin 3-*O*-β-D-glucopyranoside (**9**),[19] 5-*O*-caffeoylquinic acid (**10**),[20] 3-*O*-caffeoylquinic acid (**11**),[21] and 5-*O*-(*E*)-*p*-coumaroylquinic acid (**12**)[22] by comparison of their physical and ^{1}H, ^{13}C NMR, and MS data reported in the literature.

The hepatoprotective activity of the isolated compounds was investigated using the HBV assay in vitro. The HepG2.2.15 cell line constitutively expresses HBV via an integrated HBV genome and is used extensively for drug evaluation.[23] Cultures of HepG2.2.15 cells grown in DMEM were supplemented with whole crude-extracts and/or purified compounds from plant extracts. On days 4 or 8 of treatment the presence of released HBV particles in the culture media (10 μL) was determined by PCR techniques and compared to untreated controls, positive control with 1-deoxynojirimycin,[24] and plant extract or purified compounds. Intracellular HBV-specific RNAs were extracted from the harvested HepG2.2.15 cells and could be also determined by RT-PCR techniques.[25]

To monitor the inhibition of HBV secretion from HepG2.2.15 cells, the target region of the HBV surface antigen was amplified in a PCR or RT-PCR. The following set of primers was employed to amplify the HBsAg sequences: forward primer, 5′-TGC CTC ATC TTC TTG TTG GTT CT-3′; backward primer, 5′- CCC CAA TAC CAC ATC ATC CAT ATA-3′ amplifying a 336-nt length of DNA products. The amplified DNA fragments at the HBsAg sequences were then revealed by conventional agarose gel electrophoresis. In order to identify intracellular HBV-specific RNA expression in the HepG2.2.15 cells during treatment, the same target region of HBV surface antigen was amplified in a RT-PCR with the primer sets used above. Since *n*-BuOH-soluble fraction of the methanolic extract showed the most effective antiviral activity compared to other fractions (Fig. 2), this fraction was purified by column chromatography resulting in the isolation of twelve compounds. The effect of these twelve isolated compounds on HepG2.2.15 cells was assessed in order to explore their antiviral properties (Fig. 3). After each compound was added to the culture media and incubated for 4 days, 10 μL medium was used for HBV DNA amplification by PCR. The release of virus particles or the expression of HBV-specific RNAs was positively inhibited by most of the tested compounds at micromolar concentrations after 4 days of treatment. No amplified DNA or a very weak DNA band at the position of 336 bp after agarose gel electrophoresis indicated a positive result, that is, inhibition of virus production. No inhibitory effect on virus production or viral gene expression resulted in a unique DNA band at the 336 bp position of the agarose gel. The lack of

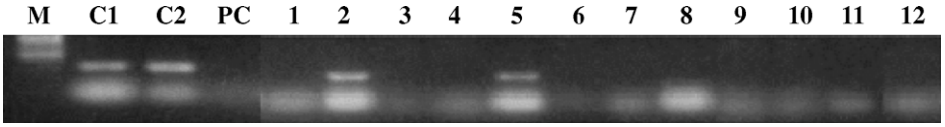


Figure 3. Twelve compounds isolated from the active *n*-BuOH fraction were assayed for amplification of 336-bp length of HBsAg DNA fragment. About 0.39 mM of each compound was added and tested by PCR. **M** lane: 1 kb size marker; **C1** and **C2** lanes: untreated mock and butanol treated mock as control, respectively. PC lane is treated with 1-deoxynojirimycin as a positive control for HBV inhibition.

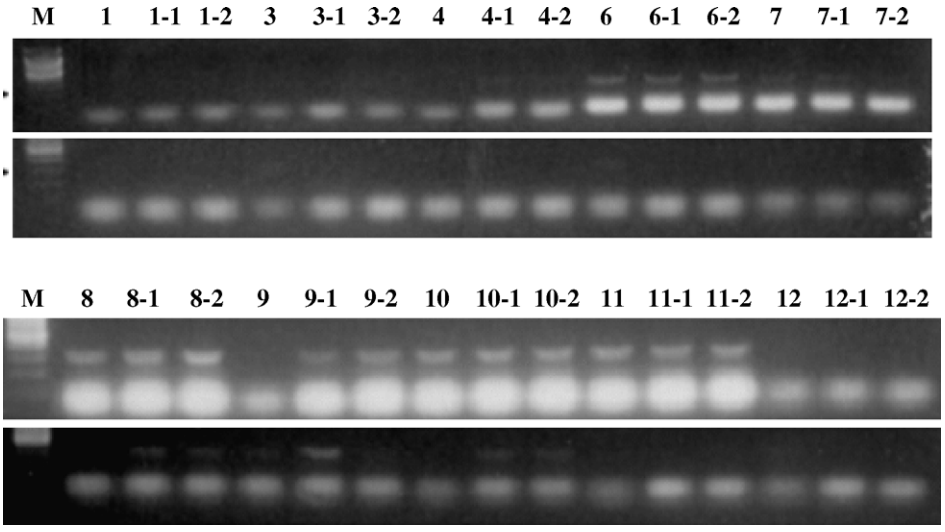


Figure 4. Serially diluted molecules of each selected active constituent (except compounds **2** and **5**) were confirmed by HBsAg PCR amplification after 4 days treatment (upper half gel) and 8 days treatment (lower half gel). Each component was serially diluted 10 times.

amplification of viral DNA meant that a specific inhibitory molecule had interrupted the processes of viral DNA replication or transcription. From analysis of these PCR products on the agarose gel electrophoresis, we found 10 compounds (**1**, **3**–**4**, and **6**–**12**) of the 12 tested that had stronger antiviral activity than that of the control molecule of 1-deoxynojirimycin,[24] as shown in Figure 3. Of the compounds tested, the quinic acid derivatives (**1**, **3**–**4**, **6** and **10**–**12**) and three flavonoids (**7**–**8** and **9**) showed significant hepatoprotective activity against hepatitis B virus replication, while two flavonoids (**2** and **5**) had little hepatoprotective activity.

We further assayed the ten active compounds with stronger antiviral activity on the inhibition of HBV viral particle production (**1**, **3**–**4**, and **6**–**12**) using serially diluted concentrations in order to quantitate their hepatoprotective activity (Fig. 4). We found that 3,4-di-*O*-caffeoylquinic acid (**1**), 3,5-di-*O*-caffeoyl-*muco*-quinic acid (**3**), and 5-*O*-(*E*)-*p*-coumaroylquinic acid (**12**) were the most effective antiviral compounds as assayed by the inhibition of HBV viral particle production in HepG2.2.15 cells, as shown in Figure 4. The quinic acid derivatives and flavonoids isolated from this plant may inhibit the HBV DNA replication, so that the formation of pol-primer complex was inhibited by their binding to the pol protein or to the chaperoning proteins such as HSP90. On the epsilon structure, the pol-primer was not properly formed in order to synthesize the negative strand of HBV DNA, resulting in the absence of released virion into the HepG2.2.15 medium. It was confirmed that the formation of pol-primer was inhibited in the HBV priming reaction by using a functional pol protein expressed in the recombinant baculovirus expression system.[8] Some compounds may inhibit the priming reaction by inhibition of pol-binding to epsilon and/or chaperone protein such as hsp90, resulting in the absence of HBV DNA replication. The isolated quinic acids and flavonoids inhibited the release of HBV viral virion particles in the treatment of HepG2.2.15 cells. This inhibition of release of HBV virion from HepG2.2.15 cells may be directly related to the formation of pol-primer complex. We need to further investigate how they interfere with viral multiplication steps to explore whether they inhibit the activities of polymerizing enzyme or any other cellular factors involved in the replication mechanism.

It was recently reported that a mixture of medicinal plant extracts showed antiviral potency against HBV, woodchuck hepatitis virus (WHV), and bovine viral diarrhea virus (BVDV).[8] It was also reported that iminosugar derivatives could prevent the replication of HBV, WHBV, and BVDV.[26] The mechanism of the hepatoprotective effect of the active compounds needs to be elucidated by investigating their effect on the HBV polymerase priming reaction, HBV viral DNA replication, and at the transcriptional and translational levels. It is known that HBV DNA replication is initiated on the epsilon-structure of the pregenomic RNA molecules by pol-priming reaction.[27] The pol-primer complex may be composed of pol protein and some cellular factors such as hsp90, which is known to be the chaperone of the priming reaction of HBV pol proteins.[28] Thus, compounds **1**, **3**, and **12** are promising candidates for chemotherapeutic agents, especially against HBV, and might be involved in the formation or reaction of the pol-primer complex. If this is the case, the pol-primer complex formation would be negatively inhibited so a protein-primed primer would not be formed. This would prevent viral DNA production by HBV pol proteins. We need to determine which steps are specifically inhibited during viral multiplication by these compounds. Although the mechanism of hepatoprotective action of the active compounds has not been directly elucidated yet, our results suggest that active quinic acid derivatives might be associated with the HBV inhibition caused by plant, which contains abundant quinic acid derivatives.

Future experiments will be done to see if the quinic acid derivatives (**1**, **3**, and **12**) inhibit the viral release through HepG2.2.15 cell membrane or the HBV DNA replication, and their gene expression in the cytoplasm of HepG2.2.15. These would be assayed by isolation of cellular RNAs and analysis of their PCRs.

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