Anti-hepatitis B Virus Activities of *Geranium carolinianum* L. Extracts and Identification of the Active Components

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Received October 8, 2007; accepted January 12, 2008; published online January 24, 2008

The ethanol extract of *Geranium carolinianum* L., a domestic plant grown in China, was subjected to sequential extractions with different organic solvents. The extracts were assayed for anti-hepatitis B virus (HBV) activities. The ethyl acetate fraction was found to contain the highest level of anti-HBV activity. In order to identify the active ingredients, the ethyl acetate fraction was further fractionated by column chromatography. Seven compounds were identified including ellagic acid, geraniin, quercitrin, hyperin, hirsutrin, quercetin, and kaempferol, whose structures were determined by NMR. The presence of the anti-HBV compounds geraniin, ellagic acid and hyperin in *G. carolinianum* L. may account for the effectiveness of this folk medicine in the treatment of HBV infections. Geraniin inhibited hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) secretion by more than 85.8% and 63.7%, respectively, at the non-cytotoxic concentration of 200 μg/ml. The inhibitions of HBsAg and HBeAg secretion by geraniin were higher than the inhibition by the positive control Lamivudine, 33.5% and 32.2% respectively, at the same concentration. Since HBeAg is involved in immune tolerance during HBV infection, the newly identified anti-HBV compound geraniin might be a candidate agent to overcome the immune tolerance in HBV-infected individuals. This is the first report of the anti-HBV effects of geraniin and hyperin, the active substances derived from *G. carolinianum* L.

**Key words** *Geranium carolinianum* L.; hepatitis B virus; geraniin; ellagic acid; hyperin; anti-hepatitis B virus activity

Viral infections are important health problems all over the world, both in the developed and developing countries, due to their morbidity and mortality. Among the many viruses, hepatitis B virus (HBV) has chronically infected over 300 million individuals. In addition to causing both acute and chronic liver disease, HBV infection is epidemiologically linked to the formation of primary hepatocellular carcinoma (HCC). Several treatments with nucleoside analogues have been developed for individuals with chronic HBV infections. However, the treatments have moderate to serious side effects and are associated with a rising rate of resistance due to the emergence of mutant HBV. Moreover, they are effective for only a small percentage of the HBV-infected population. It has been postulated that, in persistently infected individuals, the HBV-specific immune response is too weak to eliminate HBV from all infected hepatocytes, but sufficiently strong to continuously destroy HBV-infected hepatocytes to induce chronic inflammatory liver disease. Thus, in the treatment of chronic hepatitis B infection, an effective therapeutic needs not only to induce sustained disease remission, but also to prevent serious complications like liver failure and/or HCC. The development of new antiviral drugs to eradicate HBV in chronic carriers is still urgently needed.

Natural compounds, because of their structural diversity, are a good source for identifying anti-HBV agents with novel structures and mechanisms of action. The screening of plant compounds for potential antiviral agents has led to the discovery of potent inhibitors of viral growth. Moreover, the use of the ethnomedicinal approach increases the probability to identify new bioactive plant compounds. To discover agents for the development of new treatments, it is critical to isolate and identify the active components in the plant extracts.

*Geranium carolinianum* L. (Geraniaceae) is a widely used herb in China. Aqueous extracts from the aerial parts of the plant have diuretic and hemostatic properties. The plant has been used to treat several human diseases including diarrhea and rheumatic arthritis. Extracts of *G. carolinianum* L. have also been shown to have inhibitory effects towards several types of viruses. Indeed, the aqueous extract has been shown to improve the clinical symptoms of HBV infections in patients. Therefore, it has been postulated that *G. carolinianum* L. might possess pharmacological properties that inhibit the replication of HBV and the expression of viral antigens. However, its direct anti-viral activity against HBV has not been demonstrated. In the current study, we report an anti-HBV activity in the ethyl acetate extract prepared from *G. carolinianum* L. We also showed the presence of large quantities of polyphenol compounds in the extract, which may contribute to the virus inhibitory effect of *G. carolinianum* L. We further identified the compounds in the extract and tested their anti-HBV activities. This is the first report of the anti-HBV activities of geraniin and hyperin, the active substances isolated from *G. carolinianum* L. These compounds warrant further investigation as potential new anti-HBV agents.

**MATERIALS AND METHODS**

**Plant Materials** Aerial parts of *G. carolinianum* L. were collected from Xianfan District, Hubei Province, P. R. China. The collected samples were kindly identified by Prof. Jixian Guo in the Department of Pharmacognosy, School of Pharmacy, Fudan University, Shanghai, China. Voucher specimens were deposited in the School of Pharmacy, Fudan University.

**Chemicals** Ethanol, methanol, ethyl acetate, petroleum ether, chloroform, n-butanol, NaNO₂, AlCl₃ were obtained from Sinopharm Chemical Reagent Co., Ltd. Rutin was purchased from National Institute for the Control of Pharmaceutical and Biological Products in China. Lamivudine ([(-)-β-L-2',3'-dideoxy-3-thiacytidine] (3TC) was from Glaxo-
SmithKline, Inc. Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, U.S.A.).

**Extract Preparation** The dried and powdered aerial parts of *G. carolinianum* L. (5 kg) were refluxed for 2 h with 95% ethanol (401, ×3). The extract was filtered and concentrated under reduced pressure. The ethanol extract concentrate (570 g) was dissolved in water (5 l) and successively extracted with petroleum ether (5 l, ×4), chloroform (5 l, ×3), ethyl acetate (5 l, ×3), and n-butanol (41, ×3). The solvent in each extract was evaporated under reduced pressure. The ethyl acetate extract of *G. carolinianum* L. (GCEE) was evaporated in *vacuo* giving a yellow powder (87 g).

**Determination of the Total Flavonoid Content** The total flavonoid content in the ethyl acetate extract of *G. carolinianum* L. was determined using the modified method of Zhuang et al. A 0.5 ml aliquot of properly diluted sample solution was mixed with 2 ml of distilled water and subsequently with 0.15 ml of 5% NaNO2 solution. After 6 min, 0.15 ml of 10% AlCl3 solution was added and the mixture was allowed to stand for 6 min, followed by the addition of 2.0 ml 4% NaOH. Distilled water was added immediately to a final volume of 5.0 ml. The solution was then thoroughly mixed and allowed to stand for another 15 min. Absorbance of the solution at 510 nm (UNICO WFZ UV 2000 spectrophotometer, China) was determined against water. Rutin was used as the standard compound for the quantification of total flavonoid content. All values were expressed as mg of Rutin eq. per gram of GCEE. Data were reported as means±S.D. from three replicates.

**Separation and Identification of the Anti-HBV Compounds** The ethyl acetate extract (30 g) of *G. carolinianum* L. was first fractionated with silica gel column chromatography (mesh 200—300, 500g, 8.0×30.0 cm, Qingdao Marine Chemical Factory, China) into 5 fractions with a gradient mobile phase of chloroform and ethanol. Repeated Sephadex LH-20 column chromatography (25g, 2.0×69 cm, Sigma, U.S.A.) of fraction 1, eluted with chloroform/ethanol (4 : 6–8 : 2, v/v), yielded compounds 1 (105 mg) and 2 (35 mg) (Fig. 1). Flash column chromatography (silica, mesh 230—400, 8.0×30.0 cm, Merck) of fraction 2 eluted with chloroform/ethanol (95 : 5) afforded compounds 3 (14 mg), 4 (122 mg), and 5 (32 mg). Fraction 3 was subjected to a Sephadex LH-20 column chromatography (25g, 2.0×69 cm, Sigma, U.S.A.) with methanol as the mobile phase to give compounds 6 (35 mg) and 7 (24 mg). Their structures were elucidated on the basis of spectroscopic studies of UV, 1H- and 13C-NMR (Bruker DRX-400 spectrometers, U.S.A.) by comparing with that of the authentic samples. Compound 1 was faint yellow crystals (pyridine) with a mp of more than 300 °C. The UV λmax (MeOH) was 256 and 369 nm. ESI-MS showed a molecular ion of m/z 301. [α]D20 +78° (c = 0.5, MeOH) (JASCO P-1020). Compound 1 was identified as ellagic acid. Its 1H-NMR (DMSO-d6) and 13C-NMR (DMSO-d6) data were in agreement with the literature values. The ethyl acetate extract of the aerial parts of the plant was most effective among all extracts to inhibit the secretion of HBsAg and HBeAg from HepG2 2.2.15 cells as shown in Table 1. The ethyl acetate was also the least toxic to cells (data not shown).

**Cell Viability Assay** The cytotoxicities of different extracts and isolated compounds were analyzed by the MTT assay as described. Each experiment was performed in triplicate.

**Measurement of HBsAg and HBeAg** After incubation with various concentrations of different extracts and isolated compounds at 37 °C for 6, 9, and 12 d, the culture medium was harvested. The concentration of HBsAg or HBeAg was measured by an enzyme-linked immunosorbent assay (ELISA) kit (Keuh Hua Inc., China) following the manufacturer’s protocol. Each experiment was performed in triplicate. The inhibition effect was calculated as follows: 

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\text{inhibition ratio} = \frac{[\text{OD(control)} - \text{OD(sample)}]}{\text{OD(control)}} \times 100\%
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RESULTS

**Anti-HBV Activity of Each Solvent Extract of *G. carolinianum* L.** Five different extracts (ethanol, petroleum ether, chloroform, ethyl acetate, n-butanol) prepared from the aerial parts of *G. carolinianum* L. were tested for their inhibitory effect on the secretion of HBsAg and HBeAg from HepG2 2.2.15 cells. The antigens in the culture supernatants were quantified using specific ELISA kits. The anti-HBV activity of each extract was shown by its inhibition of the antigen secretion by HepG2 2.2.15 cells after treatment with the corresponding extract.
Anti-HBV Effect

Large quantities of flavonoids and tannins were found in the ethyl acetate extract. We investigated the effect of the flavonoid concentration on the secretion of HBsAg and HBeAg. As shown in Fig. 1, there was a positive correlation between flavonoid content in the sample and the inhibition of the secretion of HBsAg and HBeAg from HepG2 2.2.15 cells. Ethyl acetate extract inhibited the secretion of HBsAg and HBeAg in a concentration dependent manner.

Isolation and Identification of the Compounds in the Ethyl Acetate Extract

In order to identify the active compounds in the ethyl acetate extract, the ethyl acetate extract was separated into 5 fractions and 3 of the 5 fractions were chromatographed. Seven compounds were identified including two tannins and five flavonoids: ellagic acid (1), geraniin (2), quercitrin (3), hyperin (4), hirsutrin (5), quercetin (6), and kaempferol (7) (Fig. 2). The chemical structures of these compounds were elucidated by comparing their Rf values of TLC and spectral data (UV, 1H- and 13C-NMR) with that of the authentic samples.

The HBV Inhibitory Effects of the Identified Compounds

All the isolated compounds were evaluated for their anti-HBV activity. Ellagic acid, geraniin and hyperin showed promising activity against HBV (Table 2). In HepG2 2.2.15 cells, ellagic acid, geraniin or hyperin showed no inhibitory effect on cell proliferation up to 200, 200 and 50 µg/ml, respectively, as assessed by the MTT assay. Treatment of HepG2 2.2.15 cells with ellagic acid, geraniin or hyperin at various concentrations for 9 d resulted in a significant reduction of HBsAg and HBeAg secretion in a dose-dependent manner (Fig. 3). Quercitin and quercetin showed no inhibitory effect on HBeAg secretion, but hyperin showed significant inhibitory effect on HBsAg and HBeAg secretion.

Among the seven compounds, geraniin exhibited the highest inhibitory effect on HBV antigen secretion. Treatment of HepG2 2.2.15 cells with geraniin at various concentrations for 9 d resulted in significant reduction of HBsAg and HBeAg secretion in a concentration-dependent manner. Geraniin inhibited HBsAg secretion by 87% and HBeAg secretion by 63.7% at non-cytotoxic concentrations of 200 µg/ml.
which was higher than that of the positive control Lamivudine, 33.53% and 32.2%, respectively at 200 μg/ml.

DISCUSSION

Flavonoids and tannins are a group of polyphenolic compounds broadly distributed as secondary metabolites in plants and used in pharmaceutical, cosmetic and food industries. Recent interest in these natural products is attributed to their reported biological activities. Flavonoids and tannins are a group of polyphenolic compounds broadly distributed as secondary metabolites in plants and used in pharmaceutical, cosmetic and food industries. Recent interest in these natural products is attributed to their reported biological activities.24) By sequential organic solvent extractions of the ethanol extract of *G. carolinianum* L., a domestic plant grown in China, and studying the activities of the extracts by a bioassay, we found a strong anti-HBV activity in the ethyl acetate fraction. Large amounts of polyphenol compounds such as flavonoids and tannins were found in this fraction. It is well-known that the polyphenols bind to proteins to form unstable complexes.25) Therefore, it is possible that enveloped viruses such as HBV may be affected by polyphenols, since this class of naturally occurring substances might interact with the glycoproteins of the viral envelope.

It has been suggested that the extracts of *G. carolinianum* L. may contain virus inhibitors. However, the active substances responsible for the antiviral effect have not been identified. In order to identify the active ingredients responsible for the specific antiviral activity,26) we fractionated the ethyl acetate extract of *G. carolinianum* L. using column chromatography and identified the following compounds: ellagic acid (1), geraniin (2), quercitrin (3), hyperin (4), hirsutrin (5), quercetin (6), and kaempferol (7). Among them, ellagic acid has been shown previously to effectively block HBeAg secretion in HepG2 2.2.15 cells.5) We evaluated all seven compounds for their anti-HBV activities. Quercetin and kaempferol did not show activities against HBeAg secretion, which might be due to the absence of the saccharide group in their structures. The anti-HBV activities of quercitrin and hirsutrin were lower than that of hyperin, likely due to the differences of saccharide groups. Geraniin showed specific antiviral activity by inhibiting HBsAg and HBeAg secretion in HBV-infected cells. The inhibition ratios of the section of HBsAg and HBeAg by geraniin were 85.78 and 63.7% respectively, comparing with 33.53 and 32.2% respectively, by Lamivudine. The higher anti-HBV activity of geraniin might be attributed to its multi-hydroxy groups. Galactose receptor is known to be present in HepG2 2.2.15 cell line and geraniin contains one galactose group. Studies of the other possible mechanisms of the activities of geraniin to decrease HBeAg secretion, such as the possible destabilization of the secreted HBeAg, are in progress.

HBeAg is involved in the inhibition of core antigen during viral assembly,26) and the involvement of HBeAg in host immune tolerance has also been suggested.27) Since HBeAg is involved in immune tolerance of HBV-infected individuals, our finding suggests that host immune tolerance induced by HBeAg during HBV infection might be overcome by geraniin. The potential therapeutic value of this compound should be explored. Compared with Lamivudine, ellagic acid and hyperin also have higher activities in reducing HBsAg and HBeAg secretion (Table 2). The presence of the anti-HBV compounds geraniin, ellagic acid, hyperin, and others may explain the effectiveness of this plant in folk medicine.22) And tannins such as geraniin and ellagic acid might play an important role against HBV.

From the structure–activity relationship point of view, it is of great interest to observe that a galactose group at C-3 of quercetin enhances the anti-HBV potency, but not other sugars. Our current data suggest that geraniin, ellagic acid and heparin could be used as lead compounds for the development of novel therapeutics for the treatment of HBV. Further structural modifications might be needed to increase their efficacy and to decrease their cytotoxicity.

In conclusion, we have identified seven known compounds in *Geranium carolinianum* L., and some of which showed potent anti-HBV activity. Compounds geraniin, ellagic acid and hyperin were found to be the most active molecules among all the isolated compounds. Further chemical transformation of hyperin, ellagic acid and geraniin is in progress.

**Fig. 3. Inhibitory Effects of Geraniin (A), Ellagic Acid (B), Hyperin (C) on the Secretion of HBsAg and HBeAg from HepG2 2.2.15 Cells**

The HepG2 2.2.15 cells were cultured in the presence of geraniin, ellagic acid and hyperin at various concentrations or with Lamivudine (3TC) at 200 μg/ml. HBsAg and HBeAg in the supernatants were quantified using specific ELISA kits. The experiments were performed in triplicates and the data were presented as mean±S.D. **p<0.01,** *p<0.05 as compared with the control group.
to improve their biological profiles. Studies on the mechanisms of their virus inhibition activities are also being pursued.

Acknowledgements We thank Science and Technology Commission of Shanghai Municipality for financial support given (06DZ22906).

REFERENCES