Anti-hepatitis C virus compounds obtained from Glycyrrhiza uralensis and other Glycyrrhiza species

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学位論文の内容要旨

Anti-hepatitis C virus compounds obtained from *Glycyrrhiza uralensis* and other *Glycyrrhiza* species

ウラルカンゾウ及び他のカンゾウ属植物から由来の抗Ｃ型肝炎ウイルス化合物

神戸大学大学院医学研究科医科学専攻

微生物学

（ 指導教員： 堀田　博 教授 ）

Myrna Adianti

BACKGROUND: Hepatitis C virus (HCV) is among the major causative agents of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. A variety of standard treatment regimens has been adopted with considerable success. However, there still remain clinically important unsolved issues, such as the emergence of drug-resistant virus and the cost of the drugs. Therefore, development of complementary and/or alternative drugs for treatment of HCV infection is still much needed.

Glycyrrhiza uralensis and *G. Glabra* have been commonly used in both traditional and modern medicine. Glycyrrhizic acid, also known as glycyrrhizin, considered as the principal component in *Glycyrrhiza* spp. Antiviral activities of glycyrrhizin and other compounds isolated from *Glycyrrhiza* species against a variety of viruses, including human immunodeficiency virus, herpes simplex virus, influenza virus, SARS coronavirus, hepatitis viruses and enteroviruses, have been reported. For hepatitis viruses, glycyrrhizin has been used for the treatment of liver diseases, including chronic hepatitis B or C. Glycyrrhizin lowered serum alanine aminotransferease levels in HCV-infected patients, but there was no significant reduction in HCV RNA level. Recently, anti-HCV activity of glycyrrhizin *in vitro* was reported. However, clear evidence for it still appears to be lacking. In this study, we examined extracts of *G. uralensis* roots and their components for anti-HCV activities using the HCV cell culture system.

MATERIALS AND METHODS: The sub-fractionation and purification of *G. uralensis* root extract were conducted with high-performance liquid chromatography (HPLC). Other bioactive constituent of *Glycyrrhiza* species were purchased commercially. The anti-HCV activities of samples were examined with cell culture system. Huh7.5 cells infected with HCV genotype 2a (J6/JFH1-P47) were treated with sample extracts or compounds. The infectious virus production titers were determined with immunofluorescence assay. To

further confirm anti-HCV activities of the samples, we also observed the HCV protein expression level with immunoblotting and HCV RNA replication with real-time quantitative RT-PCR.

RESULTS: First, we examined a crude methanol extract of *G. uralensis* roots for anti-HCV activities. The IC_{50} and CC_{50} values of the crude methanol extract were 20.0 and 300 μg/ml, respectively, with the selectivity indexes (SI: CC_{50}/IC_{50}) being 15. From the further partitioned of the methanol extract, we found that the anti-HCV activities were concentrated into a chloroform partition, with IC_{50} and CC_{50} were 8.0 and 93 μg/ml, respectively, with the SI being 11.6. Next, we sub-fractionated the chloroform partition by using recycling HPLC and examined the sub-fractions for anti-HCV activities. The result revealed that significant anti-HCV activities were observed with fractions 6 to 10, with IC_{50} ranging between 2.9 and 4.9 μg/ml. We further purify a major component(s) in the fractions 6 to 10 by using recycling HPLC and glycycoumarin, glycyrin, glycyrol and liquiritigenin were identified by NMR spectrum analysis.

Bioactive constituents of *Glycyrrhiza* species can be classified into triterpenoids (glycyrrhizic acid, etc.), coumarins (glycycoumarin, glycyrin, glycyrol, etc.), flavones (liquiritin, liquiritigenin, etc.), chalcones (isoliquiritigenin, licochalcon A, etc.), isoflavans (glabridin, etc), stilbenoids and other miscellaneous compounds. For this experiment we purchased glycyrrhizic acid, glycyrrhizic acid monoammonium salt *n*-Hydrate, glycyrrhetinic acid, glycyrrhetic acid 3-O-glucuronide, liquiritin, liquiritigenin, licochalcone A, isoliquiritigenin, glabridin, licorice-saponins G2 and H2.

Anti-HCV activities of *G. uralensis*-derived purified compounds and related chemical compounds obtained from other *Glycyrrhiza* species examined. The results revealed that glycyrrhizin and glycyrrhizic acid monoammonium, possessed only marginal levels of anti-HCV activities, with IC_{50} values being 180 and 320 μg/ml, respectively. Compare with previously reported experiments, some reported that glycyrrhizin did inhibit HCV infection in Huh7 cells, but there also some controversial observations reported in clinical settings were glycyrrhizin did not show any significant effect on HCV RNA levels while it lowered alanine aminotransferase levels in HCV-infected patients. This might be explained by a membrane-stabilizing effect of glycyrrhizin and the clear evidence for the possibility of glycyrrhizin as an antiviral was still lacking.

Liquiritin and glycyrrhetinic acid, showed more significant anti-HCV activity than that of glycyrrhizin, with IC_{50} being 75 and 40 μg/ml. However, this results still significantly weaker than the coumarins, such as glycycoumarin, glycyrin and glycyrol, obtained from the *G. uralensis* extract, with IC_{50} being 4.6 to 8.8 μg/ml. Liquiritigenin, a flavanone obtained from *G. uralensis* extract also shown to possess potent anti-HCV activity, although still slightly weaker compared to the coumarins.

Glycyrrhetic acid 3-O-glucuronide, licorice-saponins G2 and H2 did not show significant anti-HCV activities. Isoliquiritigen, licochalcone A and glabridin exhibited potent anti-HCV activities with IC_{50} being 3.7, 2.5 and 6.2 μg/ml, respectively. But licochalcone A and glabridin were reported as a species-specific component of *G. inflata* and *G. glabra*, respectively, and reported to be missing in *G. uralensis*.

To determine whether the anti-HCV effects of the compounds of *Glycyrrhiza* species are exerted on the entry or post-entry stage, we performed time-of-addition experiments. The results obtained showed that all the *Glycyrrhiza* species-derived compounds and the chloroform partition of the *G. uralensis* extract exerted their antiviral effects after the virus inoculation. These results suggested that all the anti-HCV compounds of *Glycyrrhiza* species tested in this study, i.e., glycycoumarin, glycyrin, glycyrol, liquiritigenin, isoliquiritigenin, licochalcone A and glabridin as well as the chloroform extract, act primarily at the post-entry

step. Consistent with our observations, other researchers also reported that two flavonoids, isoliquiritigenin and glycycoumarin, inhibited replication of an HCV subgenomic RNA replicon *in vitro*, with IC_{50} being 6.2 and 15.5μg/ml, respectively.

To further confirm that *G. uralensis*-derived compounds exert their anti-HCV activities at the post-entry step, Huh7.5 cells were inoculated with HCV for 2 hours, followed by treatment with each of compounds for 1 to 2 days. The results obtained clearly demonstrated that glycycoumarin, glycyrin and glycyrol suppressed HCV RNA replication, resulting in the inhibition of HCV protein synthesis as demonstrated by real-time quantitative RT-PCR and immunoblotting analyses. We also confirmed that production of HCV infectious particles was inhibited by glycycoumarin, glycyrin and glycyrol at 1 and 2 days post-infection.

To the best of our knowledge, there is no report so far regarding anti-HCV activities of glycyrin and glycyrol. It was reported that glycyrin possess peroxisome proliferator-activated receptor-γ ligand binding activity and showed antibacterial activity. While glycyrol reported to exert an anti-inflammatory effects. However, the possible antimicrobial activity of glycyrol has not been reported. Further study is needed to elucidate the detailed mechanism of anti-HCV activities of glycyrin and glycyrol, and also possible antiviral activities against other viruses than HCV.

CONCLUSION: From our present results, we could suggest that glycycoumarin, glycyrin, glycyrol and liquiritigenin isolated from *G. uralensis* as well as isoliquiritigenin, licochalcone A and glabridin would be good candidates for seed compounds to develop antivirals against HCV.

神戸大学大学院医学(系)研究科（博士課程）

論文審査の結果の要旨			
受付番号	甲 第 2420 号	氏 名	Myrna Adianti
論文題目 Title of Dissertation	Anti-hepatitis C virus compounds obtained from Glycyrrhiza uralensis and other Glycyrrhiza species ウラルカンゾウ及び他のカンゾウ属植物から由来の抗C型肝炎ウイルス化合物		
審査委員 Examiner	主 査 Chief Examiner 森 康子 副 査 Vice-examiner 川端 眞人 副 査 Vice-examiner 林 祥剛		

（要旨は1，０００字～2，０００字程度）

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