氏名（本籍）	司（し）　南（なん）（中国）
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学位論文題目	Improvement of Renal Function by Acteoside and Its Reactive Metabolites from Total Glycoside of the Leaves of *Rehmannia glutinosa* Libosch.
論文審査委員	（主査）教授　佐藤　隆 教授　高木　教夫 教授　袴田　秀樹 教授　杉浦　宗敏

論文内容の要旨

INTRODUCTION

The leaves of *Rehmannia glutinosa* Libosch. (Dihuangye)'s Total Glycoside (DTG) capsule, which is made by phenylethanol glycoside group in the Dihuangye, has been developed by our team as a new Class II Chinese drug for the treatment of renal disease. Since DTG capsule has a favorable reputation to improve kidney function and protecting kidney with a high degree of safety, the main active ingredients of DTG capsule as traditional Chinese medicine (TCM) preparations are not well understood, which may seriously affect the clinical usage and global promotion. On the other hand, acteoside (ACT, $C_{29}H_{36}O_{15}$) (**Figure 1**) is known as a verbascoside and widely exists in various TCM, especially in the Dihuangye. It is worth mentioning that ACT is the main component in DTG accounting for about 35%, and that in DTG capsule about 12% (capsule preparation made from DTG as raw material with an appropriate amount of starch added). It has been reported that metabolites of ACT are detectable in diabetic nephropathy (DN) animals treated with DTG. Therefore, ACT is suggested to be an active candidate of DTG and DTG capsule for treating kidney diseases. However, it is not known whether acteoside and/or its metabolites improve renal dysfunction and exert renoprotective activity.

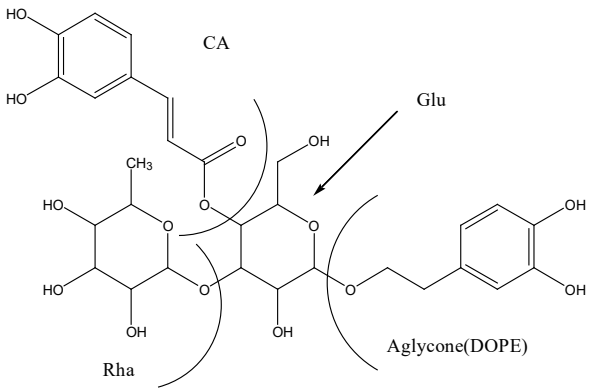

Figure 1 The structure of ACT

In the present study, to identify the major active component of DTG capsules for the treatment of renal failure as ACT and analyze its medicinal effects, I have characterized *in-vivo* metabolites of ACT

in animal models of renal failure, and investigated the effects of these components on renal function *in vivo* and *in vitro*. In CHAPTER 1, I have characterized ACT metabolites and therapeutic effects of ACT and its metabolites in a rat model of chronic glomerulonephritis (CGN). In CHAPTER 2, I have demonstrated the improvement effects of ACT and its metabolites on another renal damage model; diabetic nephropathy (DN) in mouse. Furthermore, in CHAPTER 3, I have demonstrated the pharmacological actions of ACT and its metabolites on cellular functions in three types of renal glomerulus-derived cells under inflammatory and high-glucose conditions *in vitro*.

CHAPTER 1 Identification of ACT and its metabolites and their renal protective effects in a rat model of chronic glomerulonephritis (CGN)

First, metabolites of ACT were analyzed by ultra-high performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry (UHPLC-Q/TOF MS) using plasma, urine, and feces from normal and a CGN model of CGN rats treated orally with ACT. A total of 49 metabolites of ACT was identified, of which 21 metabolites were detectable in CGN. In addition, this analysis indicated that ACT was rapidly absorbed into the blood and transferred to the kidney, while the metabolites were discharged via urine. These results suggested that the main metabolic organ of ACT may be the kidneys.

Next, I analyzed the dynamics of biomarkers related to renal failure in the ACT-administrated CGN rats. ACT administration was found to decrease; i) the serum level of creatinine, ii) urinary protein, iii) the plasma level of CD18, iv) the platelet aggregation, and v) the expression of TGF-β and fibronectin (**Figure 2**). However, there was no change in the serum level of endothelin and the plasma level of CD3, CD4, CD8, IgG, IgM, IgA in CGN rats. Furthermore, the level of TNF-α and IL-6 was decreased in serum and splenocytes in CGN rats. Similar ACT-mediated suppression of both cytokines was reproduced in lipopolysaccharide-stimulated macrophage RAW264.7 cells.

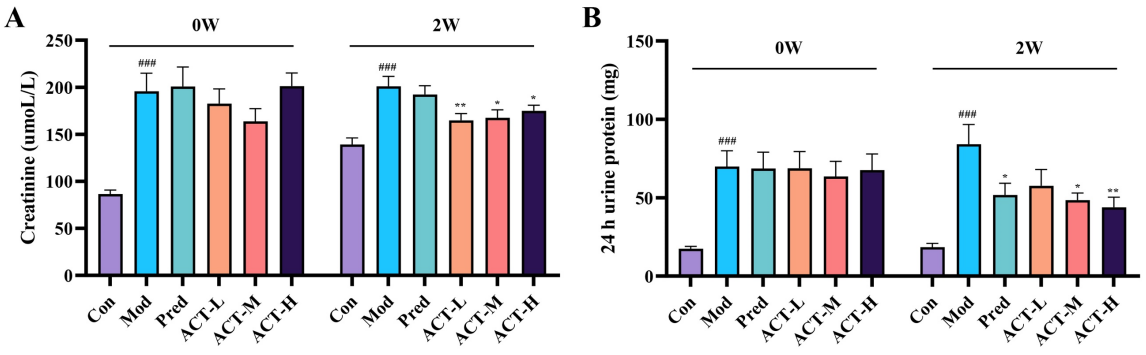

Figure 2 The level of creatinine and urinary protein in the ACT-administrated CGN rats

CHAPTER 2 Characterization of renal protective actions of ACT in *db/db* mice with diabetic nephropathy (DN) and their network pharmacology analysis

As the Chapter 1 showed that ACT improved renal functions in CGN rats, the pharmacological

mechanisms of the ACT actions were examined in another renal disease model DN. Administration of ACT was found to i) decrease the body weight gain values of mice without the alteration of water consumption and feed intake (**Figure 3**); ii) restore the morphology of glomeruli and glomerular capillary lumen and basement membrane, and iii) inhibit the proliferation of glomerular thylakoid region and renal tubulointerstitial tissues and collagen deposition in glomeruli and tubular interstitium in DN rats. A principal component analysis (PCA) using serum, urine, and kidney showed that pathways associated with the metabolism of amino acids were markedly changed by ACT treatment. In addition, a network pharmacology analysis using Pharmmapper and Swiss Target Prediction databases indicated that a total of 293 targets for ACT and 1748 targets for DN were extracted, with 122 targets overlapping between them. Furthermore, the "component-target-pathway" network revealed that targets in signaling transduction pathways such as AKT2, AKT1, MAPK1, HRAS, MAPK10, EGFR, MAPK8, MAPK14, IGF1R, and SRC were associated with the pharmacological actions of ACT in DN. Moreover, the verification test by targeted metabolomics was performed in kidney samples. The urea cycle and metabolisms of arginine, proline, glutamate, aspartate, asparagine, tyrosine, tryptophan, pyrimidine, phosphatidylinositol phosphate, purine, lysine, glycine, serine, alanine, and threonine, which closely influence endocrine and immune system, was suggested to be a high relevant pathway along with the signaling transduction pathways in the renal protective actions of ACT. These results suggested that the actions of ACT are associated with the regulation of signal transduction, the metabolism of carbohydrate, lipid, and amino acid, and mainly the endocrine and immune systems.

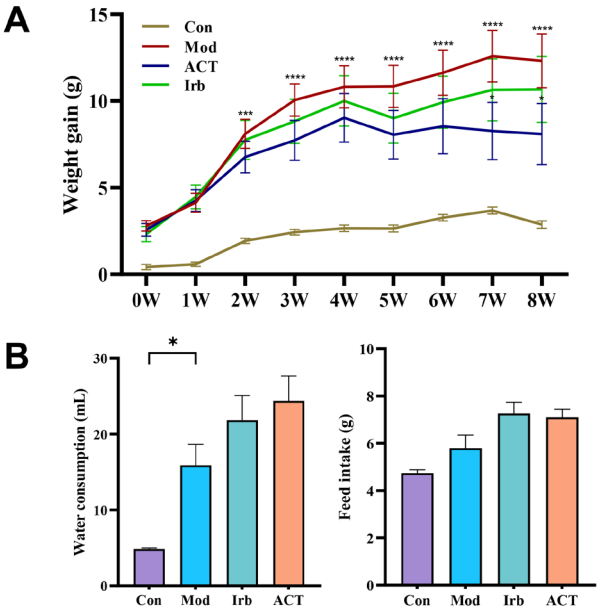

Figure 3 Body weight gain, water consumption, and feed intake in DN rats treated with ACT

CHAPTER 3 Molecular mechanisms of renal protective actions of ACT in rat glomerular cells *in vitro*

To elucidate the molecular mechanisms of the therapeutic actions of ACT for kidney disease, I examined the effects of ACT on the expression of kidney disease-associated factors; matrix metalloproteinase (MMP), connective tissue growth factor (CTGF), TGF-β, angiotensin II (AngII), and nephrin in rat mesangial cells, glomerular endothelial cells, and podocytes under inflammation and high-glucose (HG) damage conditions. When those cells were treated with IL-1β, LPS, and HG, the cell

viability was slightly decreased. Both ACT and DTG dose-dependently augmented the cell viability in

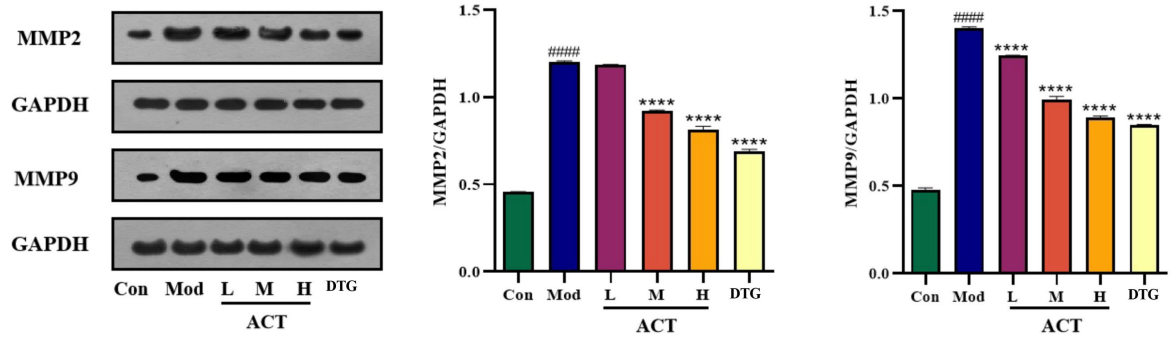

Figure 4 The suppression of MMP-2 and MMP-9 production by ACT and DTG in IL-1β-stimulated rat mesangial cells

the IL-1β-, LPS-, and HG-treated cells. The IL-1β-induced production of MMPs-2 and -9 was suppressed by ACT and DTG in three cell types (**Figure 4**). In addition, the LPS-augmented production of CTGF and TGF-β was decreased by ACT and DTG in mesangial cells. Both ACT and DTG was found to suppress the HG-induced production of AngII in glomerular endothelial cells. Furthermore, the HG-reduced level of nephrin was augmented by ACT and its metabolite, DOPAC, but not DOPE in glomerular podocytes.

CONCLUSION

The present study demonstrated that ACT improved the symptoms of nephritis by the inhibition of inflammatory responses, the regulation of immune function, and the improvement of tissue hemodynamics and hemorheology in CGN rats. In addition, ACT exhibited similar protective activities due to the reduction of not only histopathological but also functional damage to the kidney in DN. Furthermore, the network pharmacology analysis suggests that the actions of ACT are associated with the regulation of signal transduction, the metabolism of carbohydrate, lipid, and amino acid, and mainly the endocrine and immune systems. Moreover, as molecular mechanisms for the improvement and protective actions of ACT to renal functions in CGN and DN, ACT is likely to re-activate the viability of glomerular component cells and suppress the expression of nephritis-inducing factors related closely to the occurrence of renal dysfunction. Thus, these results strongly suggest that ACT is a crucial active component in DTG for the treatment of kidney disease. Given that ATC metabolites existed in the blood and kidneys, ACT is likely to not only improve systemically metabolic regulation, but also restore glomerular component cell functions in renal failure patients. Thus, this study is believed to contribute to the development of new therapeutic and preventive agents for kidney disease.

PUBLICATIONS

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2. Gao W, Gao S, Zhang Y, Wang M, Liu Y, Li T, Gao C, Zhou Y, Bian B, Wang H, Wei X, Sato T, Si N*, *et al*. 他 2 名, *J Ethnopharmacol*, 318, 117073 (2024). *First Corresponding Author
3. Liu Y, Gao W, Wang M, Bian B, Zhou Y, Wei X, Si N*, *et al*. 他 3 名, *Integr Med Nephrol Androl*, 11, e23-00021 (2024). *First Corresponding Author

【論文審査の結果の要旨】

中国における腎機能不全による透析患者数は年々増加しており、またその患者年齢は低下傾向にある。このような背景から腎臓病治療における新薬開発が望まれている。申請者のグループは、腎臓病治療における併用薬として地黄葉（レフマニア・グルティノーサ・リボッシュ）の総グリコシド（DTG）カプセルを上市した。この薬物を投与した膜性腎症、糖尿病性腎症およびその他の腎症患者において、主に蛋白尿のコントロール、血漿アルブミンの増加、血中脂質の減少など補助的治療効果が認められている。DTG カプセルによる腎機能調節は、その主成分である acteoside (ACT)に起因すると推察されているが、ACT による腎機能改善・保護作用については十分に理解されていない。本研究では、ACT の腎機能調節作用を明らかにすることを目的に、2 種類の腎不全動物モデルにおいて ACT の体内代謝物を同定し、ACT およびその代謝物による腎機能改善・保護効果の分子機構を in vivo および in vitro において検討した。

第 1 章では、慢性糸球体腎炎ラットにおいて、質量分析法 (UHPLC-Q/TOF MS) を駆使して DTG カプセルの主成分として ACT を確認するとともに、49 種類の ACT の代謝産物を同定し、その内の 21 種類が慢性糸球体腎炎において検出された。また、投与された ACT は素早く腎臓に移行して、その代謝産物が尿中に排泄されることを見出した。さらに、ACT は慢性糸球体腎炎における抗炎症作用や脾臓由来リンパ球の活性化および腎臓の線維化を抑制することを発見した。

第 2 章では、糖尿病性腎症マウスにおいて ACT がマウスの体重増加を抑制、糸球体、糸球体毛細血管および基底膜の形態を改善することを明らかにした。また、主成分分析 (principal component analysis)からアミノ酸代謝に関連する経路が ACT 投与によって顕著に変化すること、ネットワーク薬理学解析から ACT の作用と糖尿病性腎症において 122 の標的が重複していることを見出した。さらに、ターゲット分析から内分泌および免疫系への関与が推測されるいくつかの細胞内情報伝達経路とアミノ酸代謝が ACT の腎保護作用に関わる可能性を見出した。

第 3 章では、ACT による腎機能改善効果の分子機構を明らかにするために、腎臓病を想定した炎症刺激または高グルコース条件下での糸球体由来メサンギウム細胞、内皮細胞および足細胞（ポドサイト）において、ACT は 3 種の細胞の生存率を増加させることを明らかにした。また、ACT は腎線維化に密接に関わるマトリックスメタロプロテアーゼ(MMP)-2 および MMP-9 と結合組織成長因子やトランスフォーミング増殖因子 β の産生を抑制した。さらに、ポドサイトにおいて糸球体機能調節に関わるネフリンの産生が ACT のみならずその生体内代謝物である DOPAC により増加することを発見した。

本研究より、中国における腎臓病治療において ACT は DTG カプセルの主要な薬効成分であり、迅速に腎臓に移行して治療効果を発揮することが示唆される。また、慢性糸球体腎炎および糖尿病性腎症において ACT は内分泌および免疫系に関わる細胞内情報伝達経路を介したアミノ酸代謝の改善と糸球体構成細胞の機能回復に起因して、腎機能改善・保護効果を発揮するものと示唆される。本研究成果は、ACT が優先的に腎臓に移行して腎機能を調節する新たな腎臓病治療薬として有用であることを強く示唆するとともに、新たな腎機能改善薬の開発にも貢献するものである。

以上、本申請論文は、腎臓病治療薬の開発基礎研究において新規性、進歩性および応用性もあり、博士（薬学）の学位論文として十分な価値があるものと判断する。