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[Purpose] We aimed to investigate the systemic pharmacological analysis of gardenia fructus (GF) and the proof of concepts. We examined the antioxidant and anti-inflammatory effects in high-fat (HF) diet mice.

[Methods] The active compounds of GF and the target genes were identified using the Traditional Chinese Medicine Database and Analysis Platform (oral bioavailability ≥ 30%, Caco-2 permeability ≥ -0.4, and drug-likeness ≥ 0.18). The rats were divided into four groups: untreated group, HF group, HF and metformin (17 mg/kg) treated group, and HF and treated with GF (28 mg/kg) for 8 weeks group. Hepatic lesion changes and markers were analyzed using hematoxylin and eosin staining and immunohistochemistry assay.

[Results] In the systemic analysis, we identified 14 active compounds including A, B, and C. From these 14 compounds, 242 biological target genes were identified. The top 10 Gene Ontology were analyzed using GO-biological process analysis: removal of superoxide radicals, regulation of endothelial cell apoptotic process, and cellular response to lipopolysaccharide. GF extracts in high-fat diet-induced non-alcoholic fatty liver disease (NAFLD) mice models significantly regulated hepatic lesion markers, such as mTOR, 8-Hydroxy-2'-deoxyguanosine as well as oxidative stress activities, TGF-β, and phosphorylation of ERK1/2.

[Conclusion] These results suggest that GF, as an exercise supplement, can alleviate NAFLD disease or fatty liver inflammation. Further studies are required to verify the synergistic effect of GF treatment combined with exercise, which is known to alleviate NAFLD and fatty liver inflammation.

[Keywords] gardenia fructus, non-alcoholic fatty liver disease, anti-oxidant, 8-Hydroxy-2'-deoxyguanosine, anti-inflammatory

Pharmacological systemic analysis of gardenia fructus against non-alcoholic fatty liver disease and validation of animal models

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INTRODUCTION

Owing to westernized eating habits and sedentary lifestyles, people have a high prevalence of obesity and various metabolic diseases in modern times[1,2]. Fat accumulation due to excessive nutritional intake suggests a causal relationship with the increase in lesions in fatty liver disease[3]. Non-alcoholic fatty liver disease (NAFLD) is classified as a fatal disease because it is difficult to diagnose, and it increases histological lesions, similar to alcoholic steatohepatitis, even without alcohol consumption[4]. However, there is currently no standardized treatment for NAFLD; therefore, research on treatment is urgently needed.

The liver is involved in the process of metabolism, including metabolizing energy required by the tissue and disposing off the waste products from the tissue[5]. Although the liver participates in the body's metabolism by synthesizing fatty acids in the blood into triglycerides, excessive activation of adipogenesis in liver leads to disease.[6]. Mitogen-activated protein kinase (MAPK) changes in TGF-β and its sub-pathways appear to be a contributing factor in the increase of non-alcoholic steatohepatitis[7]. In addition, an increase in inflammation markedly indicates changes in oxidative stress and SOD1 expression.

Several studies have suggested exercise as a possible alternative for the treatment of liver disorders and metabolic diseases[8]. In addition, various studies suggested exercise combined with treatment using natural products[9]. Some researchers have reported the development of a sports drink containing a physiologically active substance that can increase the effectiveness of exercise performance. For example, gardenia fructus (GF) is widely cultivated in Asia, and has been reported to contain various physiologically active substances. GF has been reported to have several biological effects, including anti-neurotoxicity, anti-inflammatory, and anti-oxidant effects[10,11]. Some studies have also shown that GF is effective in suppressing liver diseases. However, its pharmacological action and molecular mechanisms have not yet been elucidated. Therefore, in this study, bioactive substances with excellent oral availability, intestinal absorption, and pharmacokinetics in the body were selected from Gardenia extracts and systematically analyzed for their hepatoprotective or

fatty acid inhibitory action to provide basic data.

METHODS

Active compound analysis of gardenia fructus (GF)

To identify the active compounds in GF, we used the Traditional Chinese Medicine System Pharmacology Database and Analysis Platform (TCMSP, https://old.tcmsp-e.com/index.php). Analysis of the GF active compounds was performed as previously reported[12]. Briefly, the drug ability of the bioactive substances was analyzed based on the pharmacokinetic properties (absorption, distribution, metabolism, and excretion) of the drug, including oral bioavailability (OB), Caco-2 permeability (Caco-2), intestinal epithelial permeability, and drug-likeness (DL). As recommended by TCMSP, the main active compounds with OB ≥ 30%, Caco-2 ≥ -0.4, and DL ≥ 0.18, were selected as candidates for further analysis.

Target network analysis of GF bioactive substances

The genes encoding the key active ingredients in GF were obtained from TCMSP. The names of proteins encoded by these genes were obtained using the Uniprot database. To analyze the mechanisms of GF active proteins, target networks were constructed using Cytoscape software 3.7.2. The networks were constructed as previously reported[13]. The selected candidate ingredients and targets were input into the software, and the gene ontology (GO) network was analyzed. The relationships between various active GF ingredients and target genes as well as the biological metabolic processes related to exercise metabolism were selected, and a process-target network (PT network) was subsequently constructed.

Extraction of GF

Extracts from GF were obtained from the Seoul Yangyeongsi herb market (Seoul, Korea), and 100 g of GF was boiled in 1 L of distilled water at 100 °C for 3 h. This decoction was reduced to 50 mL using a rotary evaporator, and the supernatant was lyophilized at −60 °C (yield, 24%). In this study, GF extract (28 mg/kg) was orally provided to the study animals as per the recommended daily intake (4 g of decoction/70 kg body weight) for adults considered in Korean medicine. Previous studies have reported that non-toxic concentrations of GF administered to animals were less than 100 mg/kg.

Animal care and animal study

We used 6-week-old male C57BL/6 mice, which were obtained from Orient bio (Gyeonggi-do, Korea) and acclimatized for 2 weeks. All experiments and animal care were performed in accordance with the institutional guidelines (smecae-2017-08-01). Experimental animals were kept in standard cages in a breeding room maintained at a constant temperature of 25 ± 2 °C, a humidity of 55 ± 5%, and 12-hour light/dark cycles. Mice were divided into four groups (n = 40): untreated group (UN; normal condition); high-fat diet group (HF; negative control); high-fat diet and treated with metformin (ME; 17 mg/kg/ 100uL/ daily) group (HF + Me; positive control); and high-fat diet and treated with GF (28 mg/kg/ 100uL/ daily) group (HF + GF; test group). Although metformin is not a direct inhibitor of NAFLD, we used it as a reference drug because it is effective in suppressing insulin resistance and is clinically adopted in non-alcoholic fatty liver[14]. The UN group was treated with 100 μL of normal saline and a normal diet. The mice of the HF, HF + Me, and HF + GF groups were fed a high fat diet for 8 weeks. The low-fat diet provided 10% of total kcal as fat (D12450B, Korea Research Institute of Bioscience and Biotechnology, Seoul, Korea) for the UN group, and the high-fat diet provided 60% of total kcal as fat (D12492) for the HF, HF+Me, and HF+GF groups.

Immunochemistry

Immunochemistry was performed as previously described[11]. The mice were sacrificed and liver tissues were fixed in 4% formalin. The tissues were embedded in paraffin and serially sectioned into 5-μm slices. The prepared sections were cleared with xylene and hydrated using an ethanol gradient (70%, 80%, and 90%). The sections were incubated with anti-8-hydroxy-2'-deoxyguanosine (8-OHdG), anti-TGF-β, anti-phosphorylation of ERK (P-ERK), and anti-mTOR primary antibodies, overnight at 4 °C. The sections were incubated with biotinylated secondary antibodies. The images were captured using a microscope (Nikon, Tokyo, Japan) and analyzed using the ImageJ software.

Statistical analysis

Results are expressed as means ± standard deviation. Multiple comparisons were performed using one-way analysis of variance, followed by Tukey's post-hoc test (GraphPad Prism ver. 4.00, GraphPad, CA, USA). Statistical significance was set at p-values < 0.05.

RESULTS

Identification of active compounds

The TCMSP database was used to identify the active compounds of GF. GF contains 113 active compounds. Among them, there were 14 active compounds that satisfied the criteria of OB ≥ 30%, caco-2 ≥ -0.4, and DL ≥ 0.18: (4aS,6aR,6aS,6bR,8aR,10R,12aR,14bS)-10-hydroxy-2,2,6a,6b,9,9,12a-heptamethyl-1,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydropicene-4a-carboxylic acid, 3-methylkempferol, 5-hydroxy-7-methoxy-2-(3,4,5-trimethoxy-phenyl)chromone, ammidin, beta-sitosterol, crocetin, ethyl oleate, isoimperatorin, kaempferol, mandenol, quercetin, stigmasterol, sudan III, and supraene (Table 1). The target genes associated with each active compound are listed in Table 2.

GO network analysis of GF

To analyze the mechanisms of GF based on active ingredients and networks, GO networks were created using the

Table 1. Active compound of GF.

Active compound name	OB (%)	Caco-2	DL
(4aS,6aR,6aS,6bR,8aR,10R,12aR,14bS)-10-hydroxy-2,2,6a,6b,9,9,12a-heptamethyl-1,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydropicene-4a-carboxylic acid	32.03	0.61	0.76
3-Methylkempferol	60.16	0.37	0.26
5-hydroxy-7-methoxy-2-(3,4,5-trimethoxyphenyl)chromone	51.96	0.88	0.41
Ammidin	34.55	1.13	0.22
beta-sitosterol	36.91	1.32	0.75
crocetin	35.3	0.54	0.26
Ethyl oleate (NF)	32.4	1.4	0.19
isoimperatorin	45.46	0.97	0.23
kaempferol	41.88	0.26	0.24
Mandenol	42	1.46	0.19
quercetin	46.43	0.05	0.28
Stigmasterol	43.83	1.44	0.76
Sudan III	84.07	0.42	0.59
Supraene	33.55	2.08	0.42

Table 2. List of proteins associated with GF active compounds.

Active compound	Related protein
(4aS,6aR,6aS,6bR,8aR,10R,12aR,14bS)-10-hydroxy-2,2,6a,6b,9,9,12a-heptamethyl-1,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydropicene-4a-carboxylic acid	-
3-Methylkempferol	AR, CDC2, DPP4, GSK3B, HSPB1, MAPK14, NOS2, PIK3CG, PRKACA, PTGS1, PTGS2,
5-hydroxy-7-methoxy-2-(3,4,5-trimethoxyphenyl)chromone	ADRB2, AR, BACE1, CACNA2D1, CaM, CHEK1, DPP4, ESR1, ESR2, F10, F2, GSK3B, HSPB1, KCNH2, KCNMA1, MAPK14, NCOA1, NCOA2, NOS2, NOS3, PPARG, PRSS1, PTGS1, PTGS2, SCN5A, TOP2A
Ammidin	-
beta-sitosterol	ADRA1A, ADRA1B, ADRB2, BAX, BCL2, CASP3, CASP8, CASP9, CHRM1, CHRM2, CHRM3, CHRM4, CHRNA2, CHRNA7, CYP450, DRD1, GABRA1, GABRA2, GABRA3, GABRA5, HSPB1, HTR2A JUN, KCNH2, MAP2, NCOA2, OPRM1, PDE3A, PGR, PIK3CG, PON1, PRKACA, PRKCA, PTGS1, PTGS2, SCN5A, SLC6A4, TGFB1
crocetin	ADRA1A, ADRA1B, CHRM1, CHRM2, CHRM3, CYP450, GABRA1, GABRA2, GABRA3, GABRA5, IGHG1, NCOA2, PTGS2, VCAM1
Ethyl oleate (NF)	NCOA2
isoimperatorin	-
kaempferol	ACHE, ADRA1B, AhR, AHSA1, AKR1C3, AKT1, ALOX5, AR, BAX, BCL2, CaM, CASP3, CDC2, CHRM1, CHRM2, YP1A1, CYP1A2, CYP1B1, CYP3A4, DIO1, DPP4, F2 , F7, GABRA1, GABRA2, GSTM1, GSTM2, GSTP1, HAS2, HMOX1, Hsp90, ICAM1, IKBKB, INSR, JUN, MAPK8, MMP1, NCOA2, NOS2, NOS3, NR1I2, NR1I3, PGR, PIK3CG, PPARG, PPARG, PPP3CA, PRKACA, PRSS1, PRXC1A, PSMD3, PTGS1, PTGS2, RELA, SELE, SLC2A4, SLC6A2, SLPI, STAT1, TNF, TOP2A, VCAM1, XDH, NCOA2, PTGS1, PTGS2
Mandenol	NCOA2, PTGS1, PTGS2
quercetin	PARP1, ABCG2, ACACA, ACHE, ACP3 , ADRB2, AhR, AHSA1 AKR1B1, AKT1 , ALOX5 , AR, BAX, BCL2, BCL2L1, BIRC5 CASP3, CASP8, CASP9, CAV1, CCL2 , CCNB1, CCND1, CD40LG CDC2 , CDKN1A , CDKN2A, CHEK2, CLDN4, COL1A1, COL3A1, CRP CTSD, CXCL10, CXCL11, CXCL2 , CYP1A1, CYP1A2, CYP1B1, CYP3A4 DCAF5 , DIA4, DIO1, DPP4, DUOX2, E2F1, E2F2 , EGF EGFR , EIF6, ELK2, ERBB2, ERBB3, F10 , F2 , F3 F7, FOS, GABRA1, GJA1, GSTM1, GSTM2, GSTP1, gyrB, HAS2 HERC5, HIF1A, HK2, HMOX1, HSF1, Hsp90, HSPA5, HSPB1, ICAM1 IFNG, IGF2_C, IGFBP3, IKBKA, IL10, IL1A , IL1B, IL2, IL6, IL8 INSR, IRF1 , JUN, KCNH2, MAOB, MAPK1, MGAM, MMP1 MMP2 , MMP3, MMP9, MPO, Myc, NCF1, NCOA2, NFE2L2 NFKBIA, NKX3-1, NOS3, NOS3, NPEPPS, NR1I2, NR1I3 , ODC1 PCOLCE, PIK3CG, PLAT, PLAU, PON1, POR , PPARA, PPARD PPARG, PPARG, PRKACA, PRKCA, PRKCB, PRSS1, PRXC1A, PSMD3

| Active compound	Related protein
quercetin	PTEN , PTGER3 , PTGS1 , PTGS2, RAF1, RASA1, Rassf1, RB1, RELA, RUNX1T1 RUNX2, RXRA, SCN5A , SELE, SERPINE1, SLC2A4, SOD1, SPP1 STAT1, SULT1E1, TGFB1, THBD, TNF, TOP1, TOP2A, TOP2A , TP53, VCAM1 VEGFA, XDH
Stigmasterol	ADH1C, ADRA1A, ADRA1B, ADRA2A, ADRB1, ADRB2, AKR1B1, CHRM1, CHRM2 CHRM3, CHRNA7, CTRB1, GABRA1, GABRA3, HTR2A, IGHG1, LTA4H MAOA , MAOB, NCOA1 , NCOA2, NR3C2, PGR, PLAU, PRKACA PTGS1, PTGS2, RXRA, SCN5A , SLC6A2, SLC6A3
Sudan III	CCNA2, CDC2, DPP4, ESR1 ESR2, F2, F7, GSK3B, MAPK10 MAPK14, PIM1, PRKACA , PTGS2
Supraene	
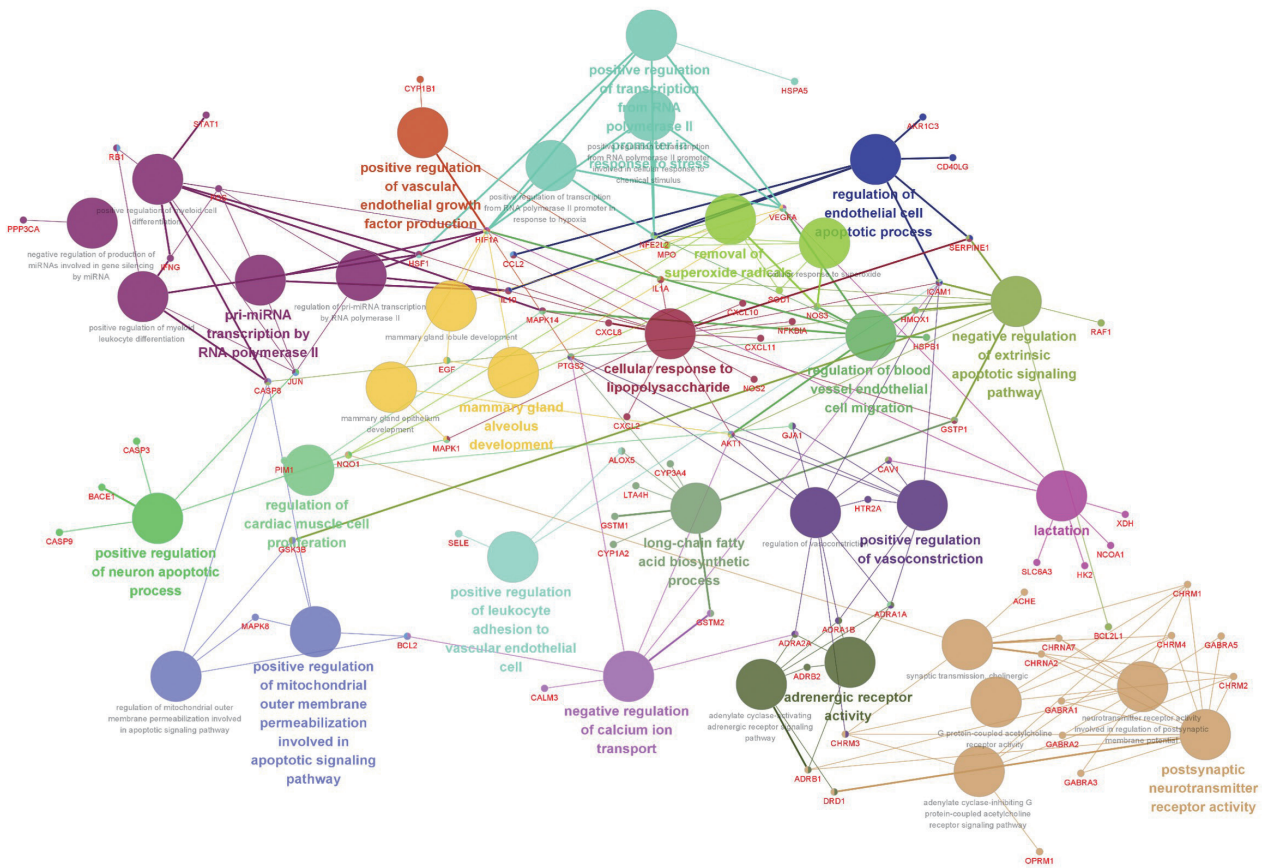
Figure 1. Biological processes of gene ontology by gardenia fructus (GF). The large circles idcicate the gene ontology. The red letters indicate the GO-associated gene.

Cytoscape visualization software. The 242 selected genes were analyzed using GO biological processes. As shown in Figure 1, 242 genes were associated with 21 GOs: positive regulation of vascular endothelial growth factor production, positive regulation of transcription from RNA polymerase II promoter in response to stress, removal of superoxide radicals, regulation of endothelial cell apoptotic process, negative regulation of extrinsic apoptotic signaling pathway, regulation of blood vessel endothelial cell migration, cellular response to lipopolysaccharide, long-chain fatty acid

biosynthetic process, positive regulation of vasoconstriction, lactation, negative regulation of calcium ion transport, adrenergic receptor activity, postsynaptic neurotransmitter receptor activity, positive regulation of vascular endothelial growth factor production, pri-miRNA transcription by RNA polymerase II, mammary gland alveolus development, regulation of cardiac muscle cell proliferation, positive regulation of neuronal apoptotic process, positive regulation of mitochondrial outer membrane permeabilization involved in apoptotic signaling pathway, positive regulation of leuko-
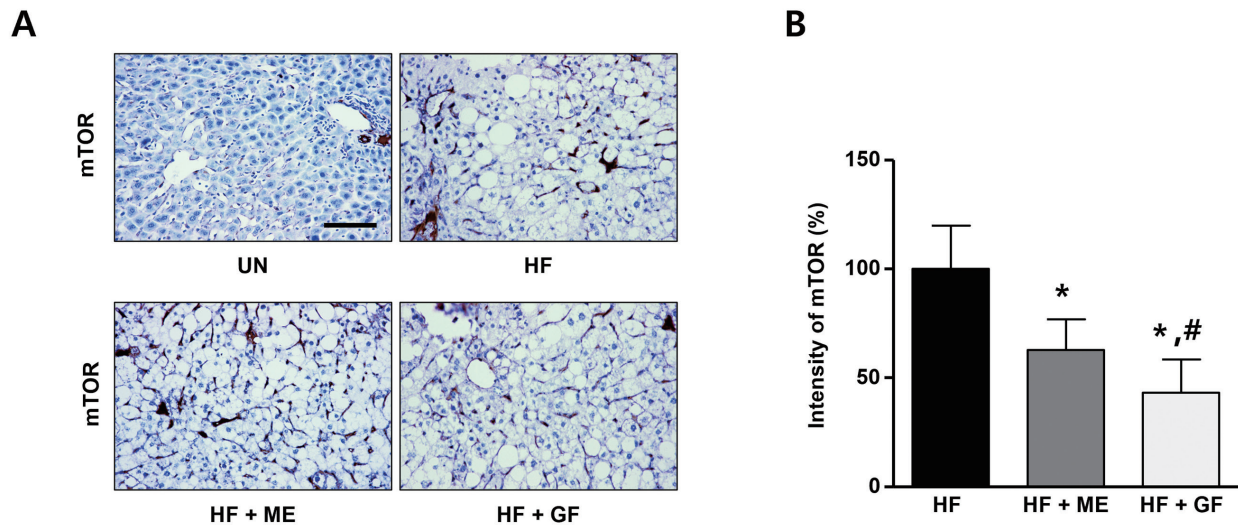
PAN
Physical Activity and Nutrition

A

B

Figure 2. The lipogenesis effect of gardenia fructus (GF) in high fat diet mice. The mice were sacrificed, and liver tissues were isolated. The liver tissues were section in 5-μm- thick. The sections were incubated with anti-mTOR for 16 h. (A) The representative images indicated the protein expression of mTOR. (B) The graphs indicate the intensity of mTOR. The HF is expressed as 100 %. Data are presented as mean ± standard deviation. *P<0.05 verse HF, # P<0.05 verse HF +ME. HF: high-fat diet; ME: metformin; GF: gardenia fructus.

cyte adhesion to vascular endothelial cells, and negative regulation of calcium ion transport. The removal of superoxide radicals was related to six proteins: MPO, NFE2L2, NOS3, NQO1, SOD1 and TNF.

Effects of GF on reducing lipid accumulating in high-fat diet mice

To investigate whether GF can regulate the high-fat-induced lipogenesis in the liver specimen of mice, we performed the immunohistochemistry with specific anti-body as anti-mTOR. As shown in Figure 2A, expression of mTOR (HF expression given as 100%) in HF showed a significant increase compared with the UN, whereas HF + ME was 37.1 ± 6.0% and HF + GF was 56.7 ± 4.7% compared to HF, respectively.

Anti-NAFLD effect of GF on high-fat diet mice

To investigate the anti-NAFLD effects of GF on high-fat diet-fed mice, 8-OHdG, TGF-β, and p-ERK expressions were observed. To confirm the level of reactive oxygen stress, the tissues were incubated with anti-8-OHdG. The treatment with ME or GF reduced the high fat diet induced 8-OHdG to 15.2 ± 3.2 % and 36.1 ± 4.2 %, respectively. Also, the treatment with ME or GR reduced the high fat diet induced P-ERK to 39.7 ±1.7 % and 70.2 ± 5.2 %, respectively. TGF-β induced the hepatic stellate cells. The high fat diet and treatment with ME or GF reduced the expression of TGF-β to 50.3 ± 14.7 % and 71.8 ± 14.5 %, respectively (Figurer 3 A and B).

DISCUSSION

Obesity and metabolic diseases are associated with the most serious problems in modern society. In this study, we suggest a methodology that combines exercise and sports supplements to find a treatment method for non-alcoholic fatty liver. In particular, this study is an attempt in convergence science to explore the candidates for sports drinks to improve exercise effects by combining them with Korean medicine. These core points suggest that indiscriminate ingestion of natural products can harm health, but pharmacological interpretation and approaches can be responsible for human health. This study attempted to study GF, which is mainly used in oriental medicine, and represents an attempt to validate substances through systemic analysis using in vivo experiments. GF is effective for liver disease, but it is still unclear whether it is possible through pharmacological interpretation. Therefore, in this study, information collection and GO analysis were performed using TCMSP for systemic analysis to identify the pharmacological effects of GF. The purpose of this study was to confirm that pharmacological studies of GF can affect NAFLD and to conduct a study to verify the effect of high-fat diet-induced liver disease in vivo.

Excessive fat accumulation in the liver causes problems in the regulation of fat metabolism and leads to inflammation[15,16]. In addition, the pathological signal cascade can induce inflammatory factors and apoptosis of damaged cells[14]. The increasing inflammatory signaling system is closely related to the signal transduction system associated with cardiovascular diseases such as abnormal vasocontraction, endothelial cell loss, and proliferation of vascular smooth muscle cells[17,18,19,20]. In summary, if fat accumulation leads to oxidative stress and inflammatory signaling, it is associated with cardiovascular diseases. In this study, we first analyzed the pharmacological effect through the pharmacological interpretation of gardenia. As shown in the Table 1 and Figure
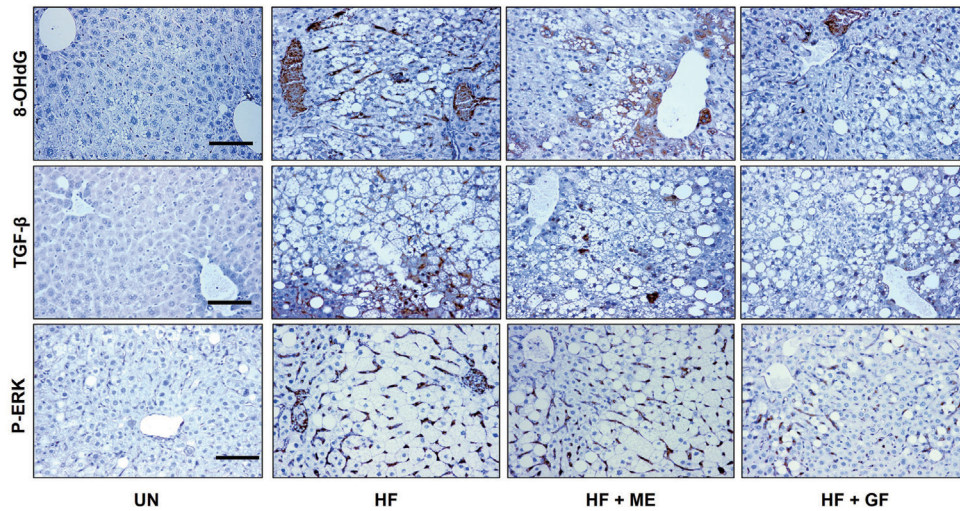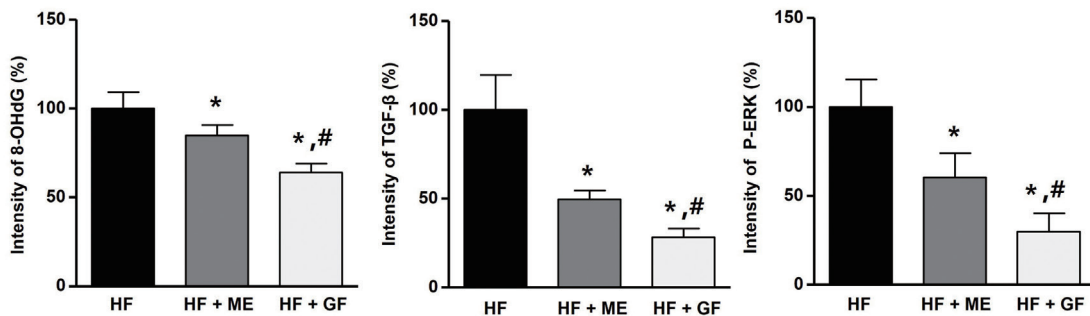
Figure 3. The histological analysis of inflammatory related factors in high fat diet mice. (A) Representative images showing the protein expression of 8-OHdG, TGF-β and p-ERK. (B) The graphs indicate the intensity of 8-OHdG, TGF-β and p-ERK, respectively. The HF is expressed as 100 %. Data are presented as mean ± standard deviation. *P <0.05 verse HF, # P <0.05 verse HF +ME. HF: high-fat diet; ME: metformin; GF: gardenia fructus.

1, GF is predicted to regulate liver disease through biological effects, such as antioxidant, anti-inflammatory effects, and anti-lipogenesis. In particular, it also has an effect on the factors that decrease cardiovascular disease. Therefore, we speculated that GF could control non-alcoholic fatty liver. Next, we attempted to verify the concept interpreted through a systemic analysis of animal experiments.

8-OHdG is a well-known marker that reflects the antioxidant activity in liver lesions[21]. TGF-β expression and phosphorylation of ERK have been reported to be very important key mechanisma in the liver fibrosis process[22,23]. In our study, the antioxidant factor 8-OHdG was significantly reduced. As shown in Figure 3, the reduction of p-ERK was reported to control non-alcoholic fatty liver. These results suggest that non-alcoholic fatty liver can be controlled. Next, we verified TGF-beta signaling, which also showed a significant decrease. These results verified the pharmacological efficacy of gardenia.

We obtained proof of concept of gardenia as an NAFLD treatment option through systemic pharmacological analysis and animal experiments. Taken together, we speculated

that gardenia may be a candidate for sports supplements to promote exercise performance and improve athletic performance. Additional research is needed to examine the potential of gardenia as a candidate for sports supplements. In addition, it can be a candidate for controlling non-alcoholic fatty liver. However, further research is required to confirm whether there is a synergistic effect with exercise.

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