Network Pharmacology-based to Investigate Pharmacological Mechanisms of Active Ingredients in Gegen Qinlian Pill to Ameliorate Irinotecan-induced Diarrhea

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Research

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Abstract

Background: The application of natural extracts or compounds derived from Chinese medicine is deemed to a promising strategy to prevent irinotecan (CPT-11)-induced gut toxicity. Based on the protective activity of Gegen Qinlian decoction in our previous study, we speculated that Gegen Qinlian Pill (GQP) would exhibit similar therapeutic effects. However, the effective material basis and the potential mechanisms underlying the effect of GQP in the treatment of diarrhea induced by CPT-11 have not been fully elucidated. Herein, we primarily investigated amelioration effect of GQP on the CPT-11 induced gut toxicity, and further explore its anti-diarrhea mechanism.

Methods: Firstly, the protective effect of GQP towards alleviating diarrhea in mice following CPT-11 administration was investigated. Furthermore, the effective ingredients of GQP in serum sample of mice by HPLC-Q-TOF-MS analysis was obtained. Next, based on these active components, an interaction network linking “compound-target-pathway” was established. Finally, a predicted mechanism within vivo validation of GQP based on Gene Ontology (GO) functional and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were obtained.

Results: A total of 19 chemical compounds derived from the GQP were identified in its serum sample. Then, an interaction network linking “compound-target-pathway” was established to illuminate the interaction between these components absorbed into serum and their targets for diarrhea. GQP exerted a curative effect on diarrhea and diarrhea-related diseases by targeting different targets, regulating inflammation, oxidative stress, and proliferation processes.

Conclusion: Taken together, this study provides a novel and scientific strategy to discover the potential effective constituents of herbs or herbal formula, and elucidated basic pharmacological effects and underlying mechanisms of GQP in the treatment of CPT-11 induced gut toxicity.

Background

Irinotecan (CPT-11), a camptothecin-derived cytotoxic anticancer agent through DNA topoisomerase I inhibition, has been approved to treat various cancers, especially as the clinical first-line drug for colorectal cancer. Unfortunately, the administration of CPT-11 commonly results in severe side-effects including myelosuppression, neutropenia, and gut toxicity particularly delayed-onset diarrhea[1, 2]. Nowadays, there are few of acknowledged guidelines regarding CPT-11-induced diarrhea. In view of the high failure rate of generally recommended octreotide or loperamide treatment in clinics, to investigate effective herbal formula or extracts to alleviate the side effects associated with CPT-11 treatment is growing attractive [3]. Previously, either HuangQin Decoction [4] or its extract named PHY906 [5] has been proved to significantly ameliorate the intestinal toxicity of CPT-11, as well as some other traditional medicines, like Shengjiang Xiexin Decoction [6], Banxia Xiexin Decoction [7].

Gegen Qinlian Decoction [8, 9], a classic TCM prescription firstly recorded in the Treatise on Exogenous Febrile Disease (202 BC-220 AD), is recommended as one of the most efficient formulas for “the intestinal

tract with dampness-heat with the symptom of diarrhea". It is composed of *Pueraria lacei* Craib (Puerariae Lobatae Radix, Gegen), *Scutellaria baicalensis* Georgi (Scutellariae Radix, Huangqin), *Coptis chinensis* Franch. (Coptidis Rhizoma, Huanglian), and *Glycyrrhiza uralensis* Fisch. (Glycyrrhizae Radix et Rhizoma, Gancao). In our previous study, a proof-of-concept evaluation was performed and manifested that the extract of Gegen Qinlian Decoction could activate CPT-11-induced diarrhea in rats mediated by oxidative stress and β -glucuronidase inhibition [10]. Therefore, we speculated that a Chinese patent medicine Gegen Qinlian Pill (GQP) for the treatment of diarrhea [11], originated from Gegen Qinlian Decoction, could possess a similar therapeutic effect. Since the effective components of GQP and the related mechanisms to alleviate gut toxicity induced by CPT-11 still need to investigate in-depth. It is important to find novel strategies to identify the targets and mechanisms in GQP.

How to distinguish the components related to the efficacy and further control quality of herbs effectively now becomes not only one of the hottest issues but also a considerably difficult point in TCM research[12]. Traditionally, isolation and purification of chemical constituents mediated by solvent extraction, column chromatography, and/or combined with pharmacodynamic tests are time-consuming and inefficient. To date, based on serum pharmacochrometry, research on the components in the serum could provide an effective methodology to identify active constituents in vivo from Traditional Chinese medicine (TCM) [13, 14]. Additionally, network pharmacology has been widely used to explore the molecular mechanisms of TCM, based on system biology and multidirectional pharmacology [15, 16]. However, the majority of studies of network pharmacology used compounds from databases to explore the involved targets and pathways [4, 17].

Herein, an integrative approach combining serum pharmacochrometry with network pharmacology was utilized to clarify the potential active components and potential mechanisms of GQP. Based on HPLC-Q-TOF-MS analysis, the absorbed chemicals after the oral administration of GQP were comprehensively identified. And then, these absorbed components in vivo were selected to perform network pharmacology analysis and constructed "ingredients-targets-pathways network" for GQP. According to their internal relation, the active mechanisms were screened and predicted. Therefore, the current study aimed to ameliorate irinotecan-induced gut toxicity and the underlying mechanisms of GQP associated with the identified potential target genes[18]. The detailed workflow is shown in Fig. 1.

Materials And Methods

Chemicals and reagents

Irinotecan (CPT-11, purity $\geq 99\%$) was purchased from Melonepharma Co., Ltd. (Dalian, China). Gegen Qinlian pills were purchase from Guangxi Huahong Pharmaceutical Co., Ltd (Guangxi, China). Standard substances, puerarin, berberine, baicalin and liquiritin, alkaloid, Baicalein, palmatine (purity $\geq 98\%$), were purchased from Weikeqi Biological Technology Co., Ltd (Chengdu, China). loperamide (LPM, purity $\geq 98\%$) was purchased from Xian Janssen Pharmaceutical Co., Ltd. HPLC-grade methanol, acetonitrile, and formic acid were purchased from Thermo Fisher Scientific (Thermo scientific, USA).

Kunming mice (18–25 g, SPF grade, Certificate No. SCXK (Chuan) 2004-15) were provided from Dashuo experimental animals Co., Ltd. (Chengdu, China) and maintained under specific pathogen-free conditions. Animals were kept under a 12 h light/dark cycle at the animal care facility, acclimatized for at least 7 days prior to the experiments, and given a fresh diet with free access to water. All in vivo experiments were carried out under the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Chengdu University of Traditional Chinese Medicine.

Mice diarrhea model induced by CPT-11 administration

The mouse diarrhea model was established based on a previous study [8]. Specifically, Kunming mice were administered 55 mg/kg of CPT-11 per day via intraperitoneal injection for 4 consecutive days. Mice displaying diarrhea symptoms were randomly assigned into four groups with 12 mice in each group. For all groups except the control group that received no treatment, 0.4 mg/kg of loperamide, 3.7 g/kg, and 1.85 g/kg of GQP were administered to mice with CPT-11 pretreatment once per day, named LPM, GQP/H, and GQP/L, respectively. Additionally, mice without CPT-11 administration were employed as a blank control group and were administered saline in the same way as the experimental groups. All agents were administered for another 5 consecutive days. The body weight, stool consistency, food intake, and water intake were recorded daily. On day 10, all mice were sacrificed by euthanasia with isoflurane.

Evaluation of gut toxicity in mice induced by CPT-11 administration

Disease activity index (DAI)

The DAI score was employed to comprehensively evaluate the daily assessment data for body weight, stool consistency, and colonic gross bleeding range based on previous descriptions [34]. As seen in Eq. (1) DAI scores were calculated according to the amount of weight loss, blood in stool, and stool consistency, with DAI scores falling in a range of 0–4 in each index. S1, S2, and S3 represent the bodyweight loss score, feces status score, and bloody stools score, respectively. In addition, mortality was also recorded every day in a double-blind manner. $DAI = (S1 + S2 + S3)/3$.

Histopathological feature

After mice were sacrificed, the colon tissues were rapidly isolated from the proximal rectum, freed of adherent tissues, and rinsed with phosphate buffer saline. Colon tissues in each group were photographed and then measured for length. Subsequently, colon biopsy specimens of 1 cm-length were fixed with 4% paraformaldehyde for 24 h, embedded in paraffin, and sectioned into 4- μ m-thick slices. Slices were hydrated and stained with hematoxylin and eosin (H&E) followed by microscopic observations.

Biochemical indexes measurement

After colon tissues homogenized in 3 × cold PBS buffer (pH 7.4), the tissue homogenate was centrifuged at 3000 rpm at 4 °C for 20 min. The supernatant was collected and levels of pro-inflammatory cytokines were determined, including Interleukin-1 beta (IL-1 β), and tumor necrosis factor-alpha (TNF- α) by enzyme-linked immunosorbent assay (ELISA) kit (ExCellBiology, China). Furthermore, some indexes related to oxidative stress including myeloperoxidase (MPO), glutathione peroxidase (GSH-PX) in the colon supernatant were measured with immunohistochemistry colorimetric method. The assays were performed according to the manufacturer's guidelines.

HPLC-Q-TOF-MS analysis method and Chromatographic Conditions

All analyses were carried out on an Agilent G4212B HPLC system (Agilent Technologies, Santa Clara, CA, USA). The chromatographic separation was performed on an ACE Excel C18 column of 100 × 2.1 mm packed with 3 μ m particles (ACE Corp., ACT, UK). The mobile phases were composed of (A) water containing 0.1% (v/v) Glacial acetic acid and (B) methanol with a flow rate of 0.35 mL/min. The column temperature was kept at 40 °C. The wavelength of the monitor was set at 205 nm and the injection volume was 1.0 μ L. The gradient elution program for GQP was carried out as follows: 10%-12%B for 0-1.5 min, 12%-13%B for 1.5-2.5 min, 13%-16%B for 2.5-4 min, 16%-20%B for 4-8 min, 20%-30%B for 8-23 min, 50%-50%B for 23-35 min, 50%-90%B for 35-40 min. The post-run equilibration time of gradient elution was 5 min.

Liquid chromatography (LC)-Mass spectrometry (MS) analysis was carried out to confirm the peak identities. The identification was performed on an Agilent G6530B Q-TOF mass spectrometer system equipped with a diode array detector (DAD) and electrospray interface (ESI) (Agilent Technologies, Santa Clara, CA, USA). This MS system was operated in negative ionization modes with the mass scan range set at m/z 100-1200. The mass spectral parameters were a gas temperature of 300° C; a gas flow of 6 L/min; a nebulizer pressure of 45 psi; a capillary voltage of 4000 V; a Nozzle voltage of 500V. The chromatographic conditions were the same as those described above. Agilent Mass Hunter version B.06.00 software was used for data acquisition and processing. Peaks were identified based on comparison of retention times and MS spectra with standards.

GQP Samples and Serum Preparation

GQP were ground to a fine powder and suspended with 0.5% sodium carboxymethyl cellulose (CMC-Na). The concentration of GQP was 3.7 g/kg and 1.85 g/kg, which was used as sample solution for gastric administration in mice. A distilled water vehicle control was given to the blank mice. Blood samples (500 μ L, 3.7 g/kg) were collected from the retroorbital sinus 1 h after oral administration and centrifuged at 3000 rpm for 15 min. Then, 600 μ L acetonitrile was added to the 200 μ L serum samples, vortexed, and then centrifuged at 12000 rpm for 10 min. The supernatant solution was transferred to another tube and dried with nitrogen gas. The residue was added 1 ml mobile phase to dissolve and centrifuged at 12000 rpm for 15 min. The supernatant was taken and sampled for analysis.

Collection of key targets proteins of chemical compositions

Then we determined the compound structural formulas of chemical components that were identified in HPLC-TOF-MS and used the Chemdraw software to draw these formulas. We save the structural formulas File as mol2 format. Each 3D structure was put onto the Pharm Mapper online server ([https:// 59. 78. 96.61/ pharmmapper/](https://59.78.96.61/pharmmapper/)) to predict the effective target of the drug molecule, and the targets were scored and sorted according to the similarity matching results. The obtained target information was then searched and transformed through Uni Prot KB in the Uni Prot database ([https:// www. UniProt. org/](https://www.UniProt.org/)) to obtain all the target protein information related to the chemical composition, which provided the data support for predicting the follow-up pathway.

Collection of key targets proteins of disease and Biological function enrichment analysis

Gene Cards server (<https://www.genecards.org>) was used to predict and analyze disease-related protein genes. Based on delayed diarrhea caused by irinotecan, we define the disease as diarrhea to fit the characteristics of delayed diarrhea. The first 200 gene targets with a score greater than 18 were selected and then matched with the predicted compound targets of Pharm Mapper. Furthermore, the STRING system ([https:// string-db.org](https://string-db.org)) was used to filter the relevant pathway and analyze the pathway containing different targets and a significance level of target enrichment in some pathways. The statistically significant enrichment pathway ($P < 0.05$) was selected as the data to build the "drug-pathway-disease" network. Next, combined with the KEGG database and GO analysis ([https:// www. genome. jp/ Kegg/ pathway. Html](https://www.genome.jp/Kegg/pathway.Html)), the gene analysis function stored in the KEGG database was utilized to note and analyze the pathway for construction of the follow-up network pharmacology.

Construction of the target network

The prediction of targets and pathways based on diarrhea was carried out in a virtual environment. The visualization of network analysis can explain the relationship between "chemical composition-target-pathway-disease." The obtained target-pathway information correspondence is input into Cytoscape 3.6.1 to construct the protein-target and anti-diarrhea target protein-protein interaction (PPI, $P\text{-value} < 0.01$). In the PPI diagram, the protein of component-disease is represented by nodes and the edge connection between nodes. After that, the contribution degree and degree of nodes are evaluated by Network Analyzer. To evaluate the contribution degree of the node, the degree of the node is the number of edges connected with the node. The larger the degree value, the more nodes, and edges associated with the node in the network, indicating that the node contributes more to the network.

Western blot (WB) analysis

Tissue protein from colons was collected by homogenization in a 5-fold volume of ice-cold RIPA lysis buffer and centrifugation at 3000 rpm for 30 min. The BCA method was utilized to determine the protein concentration in supernatants with bovine serum albumin as a standard. Protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Millipore, USA). The membrane was blocked for 1 h and subsequently incubated with primary antibodies Kelch-like ECH-associated protein 1(Keap1, 1:1000), Nuclear factor

erythroid 2-related factor 2 (Nrf2, 1:1000), or Heme Oxygenase-1 (HO-1, 1:1000) at 4°C overnight. The corresponding horseradish-peroxidase-conjugated secondary antibody (Cell Signaling Technology) was used to incubate with the membrane for another 1 h. The membrane was reacted with western blot ECL detection reagents and visualized with Tanon-5200 Chemiluminescent Imaging System (Tanon Science & Technology). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Results were qualified with the ImageJ software.

Statistical analysis

Data were presented as mean $\pm$ SD. Statistical difference was determined by one-way ANOVA followed by the Tukey-Kramer post hottest (version 20.0; SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to be statistically significant.

Results

Administration of an extract of GQP protects against gut toxicity, including delayed-onset diarrhea in mice

Observing symptoms is an important part of disease diagnosis. Mice treated with CPT-11 (55 mg/mL) in four consecutive days developed gut toxicity, including diarrhea, significant weight loss, loose stools, haematochezia. As it was shown in Fig. 2, On the 10th day, the average body weight for the control, model, LPM, GQP/H, and GQP/L group was 29.4, 21.83, 22.84, 24.58, and 24.4 mg respectively. The treatment with LPM, GQP/H, and GQP/L could significantly increase the mice body weight ($p < 0.05$), compared with the model group. A diarrhea score was used to assess the severity of diarrhea in mice. The diarrhea score of control, model, LPM, GQP/H, and GQP/L group was 0.13, 1.74, 0.87, 1.05, 1.15 respectively ($p < 0.05$). The treatment with LPM, GQP/H, and GQP/L could separately distinctly inhibit the incidence of diarrhea. Interestingly, the general state of the colon of LPM, GQP/H, and GQP/L was almost the same as that of control group. Furthermore, as it was shown in Fig. 2E, the control group exhibited normal tissue structures with no histopathological changes. While, the model group showed serious gut injury, expressing as full of inflammatory cell infiltration, loss of crypts, and submucosal. In contrast, the colons of the mice treated with LPM, GQP/H, and GQP/L showed only mild infiltration of inflammatory cells to the mucosa, minimal loss of crypts and a slight reduction in goblet cells compared with those of the models.

Identification of prototype constituents and metabolites

In order to identify definitive effective compounds for GQP, we will need to have a deeper understanding of the major constituents, metabolites, and active ingredients of this formulation [19, 20]. The HPLC and MS conditions were optimized in our previous study; we found that the methanol-water system displayed a more powerful capacity to separate the major constituents in these samples. In this previous study, a total ion chromatogram (TIC) comparison was carried out on GQP, drug-containing serum, and

corresponding blank substrates to distinguish prototypical and metabolic compositions under the same aforementioned HPLC-Q-TOF-MS conditions [10]. The positive ion mode and negative ion mode were selected for Q-TOF-MS analysis. Representative TICs of GQP are shown in Fig. 3. Using this method, we identified nineteen unique chemical constituents in mouse serum, including sixteen compounds in the serum sample that were identified as prototype constituents, as well as three additional compounds that were metabolites of GQP. For a better understanding of the fragmentation behavior of compounds, nineteen compounds were evaluated as shown in Data Supplement table S1; findings include accurate mass measurements for protonated molecules of these compounds, parts per million, errors, Rts, formulas, and original sources for GQP. Their fragmentation ions are also included.

As shown in Fig. 3, peak 2 was detected in the positive ion mode with an Rt of 4.26 min and has a precise molecular weight m/z of 416 Da; this compound was derived from puerarin. The main fragment ions were analyzed by MS/MS screening and were observed at m/z 417.11 Da $[M + H]^+$ ions in the positive ion mode; its MS/MS data were revealed as m/z 399 Da with loss of a neutral fragment of 18 Da ($-H_2O$), and m/z 351 and m/z 267 in the positive ion spectrum. Based on the obtained MS and MS/MS data, the molecular formula of this species was tentatively identified as $C_{21}H_{20}O_{10}$ and inferred to be a derivative of puerarin. The mass spectrum and proposed fragmentation pathway of puerarin are shown in Fig. 4A. Similarly, peak 12 was detected at an Rt of 19.69 min with an m/z of 447.09 Da $[M + H]^+$. The main MS/MS data revealed a compound of m/z 271 Da after the loss of a neutral fragment of 176 Da ($-C_6H_8O_6$) and a loss of 18 Da ($-H_2O$) with m/z of 253 Da $[M-(GluA-H_2O)]^+$; Its formula was determined as $C_{21}H_{18}O_{11}$ and was identified as baicalin (Fig. 3B). Finally, Peak 10 was identified at an Rt of 15.39 with m/z of 336.12 Da $[M + H]^+$ and characterized as $C_{20}H_{17}N_4O_4$. The MS/MS data revealed an m/z of 321 Da with a loss of 15 Da ($-CO$) in comparison with M10 (336 Da). This compound was identified as berberine. These fragmentation rules are in accordance with previous data; fragmentation pathways are shown in Fig. 4A, B, and C.

Construction of a Compound-Target Network (C-T network) and a Compounds-Target-Pathway-Disease Network (C-T-D network)

Firstly, with the help of database, candidate compounds from the HPLC-Q/MS associated with their related targets was constructed. As shown in Fig. 5, the network included 46 nodes (seventeen candidate compound and twenty-nine candidate targets) and one hundred and two edges. Amongst 29 targets, the top three identified were Keap1, Nrf2 and HO-1. In general, compounds presenting with a higher degree are more pharmacologically important and suggest biases towards specific drug compounds in GQP networks. Next, since diarrhea is the main adverse event associated with the administration of CPT-11, we gathered targets associated with diarrhea from open source databases. And then the identified compounds, known targets and pathways associated with diarrhea disease were uploaded to the STING database to identify the functional partnerships and interaction between them. Protein interaction with a confidence score 0.9 or higher were imported into Cytoscape v3.6.1 to generate the compounds, protein and pathway interaction network. As shown in Fig. 6, the putative GQP targets and known therapeutic targets within the diarrhea network included 184 nodes and 613 edges. Furthermore, pathways related to

diarrhea induced by CPT-11 were identified and included to reveal potential mechanisms underlying these observations. To identify the hub nodes and essential proteins in network, the topological parameters of the nodes degree were calculated by Network Analyzer. Detailed information are shown in Supplemental Data S2. It could be found that the interactions with compounds, targets, pathway varies a lot, some candidate targets were hit by only one candidate component, while others could be modulated by multiple components among them. Keap1, Nrf2 and Ethylene-responsive transcription factor ESR1 (ESR1) are amongst the core targets with the highest degrees; these targets interact with, and can be inhibited by seven distinct compounds in GQP with direct impact on pathways associated with cancer, endocrine resistance, proteoglycans in cancer and epithelial cell signalling in *Helicobacter pylori* infection pathways.

GO Function and KEGG Pathway Enrichment Analysis

In addition, to investigate the mechanisms underlying the clinical efficacy of GQP, GO enrichment, and KEGG pathway analysis was performed. GO enrichment was constructed from the perspective of the biological process (BP analysis), cellular components (CC analysis), and molecular function (MF analysis). The top 20 GO entries were selected based on three aspects of BP, CC, and MF analysis (Fig. 7). The results indicate that target protein functions are related to the regulation of programmed cell death, positive regulation of molecular function, and regulation of cell population; additional pathways included proliferation in BP analysis, identical protein binding, phosphatidylinositol-4,5-bisphosphate 3-kinase activity, protein binding, and enzyme binding in MF analysis and localization in the extracellular space, extracellular region and membrane rafts in CC analysis.

Additionally, to verify further that the biological process related to the target protein were associated with the occurrence of diarrhea, the top of 20 signalling pathways identified by P-value were screened using KEGG pathway analysis (Fig. 7). Various signalling pathways, including those associated with cancer, endocrine resistance, epithelial cell signalling in *H. pylori* infection, oestrogen signalling, colorectal cancer, Mitogen-activated protein kinase (MAPK) signalling and Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor resistance were also associated with diarrhea and ranked the top-level (Supplemental Data S3). The results of GO and KEGG pathway analysis suggest that the active components in GQP, which may be involved with the aforementioned signalling pathways, may be responsible for alleviating intestinal toxicity associated with the administration of CPT-11.

Predicted mechanisms of GQP to alleviate diarrhoea induced by CPT-11

The mechanisms by which GQP promotes the alleviation of intestinal toxicity remain unclear. Therefore, we attempted to probe the potential mechanism of action via an analysis using the network pharmacology approach. According to the KEGG pathway analysis of the C-T-D network, we combined the top 20 pathways to devise a pathway related to diarrhea using the potential interaction targets between disease and compounds. Consequently, the potential mechanisms that would promote the relief of diarrhea due to the actions of GQP may relate to its role in biological processes, including proliferation,

apoptosis, and inflammation; these pathways involve interactions involving the Keap1/Nrf2 pathway, MAPK, and TNF-mediated signalling (Fig. 8).

Experimental validation of potential targets and GQP suppresses colonic inflammatory levels and oxidant stress

To further validate the results obtained *in silico* and to explore the important role of the potential targets of GQP, we analyzed the levels of 3 target proteins, Nrf2, Keap1 and HO-1, in colon tissues of mice from the different experimental groups by WB to confirm the treatment of diarrhea of GQP. As shown in Fig. 9, the protein levels of both Keap1, Nrf2 and HO-1 were significantly decreased in CPT-11 treated mice when compared to normal mice. However, the administration of GQP reversed this decrease of Keap1, Nrf2 and HO-1 activity. Generally, Nrf2 is a master regulator of cellular oxidative levels against environmental stresses[21]. The Keap1/Nrf2 system has been shown to play important roles in maintaining the integrity of the intestinal mucosal barrier, and already has become recognized as the body's dominant defence mechanism against environmental insults[22]. Our goal was to evaluate the impact of GQP on colonic inflammation in mice with CPT-11-induced diarrhea. The results from the enzyme-linked immunosorbent assay reveal that treatment with CPT-11 leads to severe inflammation of the colon tissue in mice. We compared these results with the baseline and found that levels of pro-inflammatory cytokines, including IL-1 β and TNF- α , were significantly elevated in the colon tissue of mice treated with CPT-11. Interestingly, the treatment of diarrhea in CPT-11-induced mice with either LPM or increasing concentrations of GQP resulted in diminished levels of inflammatory cytokines to a point similar to those observed in the control group. As shown in Fig. 9C and 9D, the GQP extract exhibited an equivalent therapeutic efficiency compared with LPM and significantly reduced local concentrations of IL-1 β and TNF- α ($p < 0.05$); these results indicated that GQP is capable of significant anti-inflammatory activity. Additionally, as oxidative stress and inflammation are always associated with each other, the use of antioxidants can be employed to be protective against inflammatory diseases. The MPO levels are often used to evaluate neutrophil function and activity in inflamed tissues[23]. The antioxidant status of colon tissue samples was evaluated via an assessment of the activities of glutathione peroxidase (GSH-Px) and MPO. As shown in Fig. 9E and 9 F, GSH-PX activity in supernatant from mouse colon tissue was dramatically reduced in response to the administration of CPT-11 ($p < 0.05$); opposite results were obtained with respect to colon MPO activity ($p < 0.05$).

Discussion

Chemotherapy-induced side-effects or toxicity exhibit a high incidence rate in clinics and is urgent need to develop new treatment strategies[24]. CPT-11 therapy is associated with a host of debilitating off-target toxicities, of which gut toxicity, characterized by severe diarrhea. TCM as adjunctive therapy to inhibit Chemotherapy-induced side-effects. Some TCMs (e.g. PHY906) have exhibits significant effects on relieving patients' discomfort brought[25]. The severe intestinal mucositis suffered in mice treated with

CPT-11 can be seen in Fig. 2 and. CPT-11 also induced severe inflammatory processes, causing tissue injury and affecting the animal's body weight gain and diarrhea score.

GQP is a classic TCM prescription in treating gastrointestinal diseases, such as dysentery, enteritis, ulcerative colitis[26]. In the previous study, Gegen Qinlian decoction extract has been shown to alleviate diarrhea induced by CPT-11[10]. We also speculated that a Chinese patent possess a similar therapeutic effect. Based on this, we implemented a similar animal experiment to corroborate the preventive activity of GQP treatment remarkably reduces the occurrence of diarrhea, remit body mass decrease and increase the diarrhea score in Fig. 2. However, the effective chemical compounds and mechanism of GQP on gut toxicity are not well understood. As we all known, the identification of network targets, based on widely existing database, is one of the core issues used to reveal the mechanism of traditional Chinese medicine, and could help people to form a better understanding of TCM works[27, 28]. Here, a network pharmacology with serum pharmacochimistry was combined to reveal the potential mechanisms underlying the effects of GQP. Nineteen compounds from GQP in the serum by HPLC-Q-TOF-MS were identified and were involved network construction. Therapeutic targets and the signaling pathways in which they were explored by reverse screening, C-T network and C-T-D construction, and pathway enrichment analysis[29]. Information from various online servers and database was integrated. The results that we obtained are very reliable[30], as they benefitted from multiple information sources and different target identification methods. 29 proteins were shown to be involved. Three key targets, which closely related to both the components and diarrhea disease. In addition, the Keap1, NRF2 and HO-1 protein expression and the content of TNF- α , IL-1 β , MPO, GSH-Px were determined to validate the prediction results of the network pharmacological investigations.

Meanwhile, according to the retrieval results of database and KEGG pathway, the potential mechanism of GQP on the diarrhea induced by CPT-11 was construction. As it was shown in Fig. 7, based on the predicted pathway of GQP, the Keap1/Nrf2 pathway is one of the most important defence mechanisms against oxidative and/or electrophilic. Many agents were found to improve colitis and colitis-associated colorectal cancer via Keap1/Nrf2 activation[31–33]. The Keap1 released Nrf2 allowing its nuclear translocation and activation of NRF2 genes and the downstream anti-oxidative response stresses[34]. It is generally believed that oxidative stress, which is accompanied by intestinal accumulation of hydrogen peroxide (H₂O₂) and other reactive oxygen species (ROS), plays an important role in intestinal inflammation[35, 36]. Therefore, activation of the Nrf2-dependent anti-oxidative response is a promising strategy in gut toxicity treatment. By the way, the MAPK signalling pathway[37] is involved in various cellular functions, including cell proliferation, differentiation, and migration; these pathways promote cell growth and are capable of activating other protein kinases as well as transcription factors[38]. MAPK signalling also promotes the expression and release of various pro-inflammatory cytokines such as TNF- α , IL-1 β , and interleukin-6 (IL-6)[39, 40]. GQP-mediated regulation of the MAPK pathway may be achieved by inhibiting the actions of its constituents, including RR, RC and RS. Likewise, it has been reported that berberine [38] can significantly attenuate cytokine-stimulated phosphorylation of ERK and JNK together with endo-nuclear transcription factors, including c-Jun or CREB[41]. Furthermore, the administration of

daidzein reduces the expression of TNF- α and p-JNK and results in markedly decreased levels of pro-inflammatory cytokines, including TNF- α , IL-6, and IL-1 β [42]. Caspase (Casp)-dependent apoptosis is the best-characterized modality of programmed cell death[43]. Inhibition of cellular apoptosis plays a vital role in the pathogenesis of diarrhoea secondary to CPT-11 along with excessive cell proliferation; the integrity of the intestinal mucosa is maintained by a critical balance between apoptosis and cell regeneration[44]. This balance is disrupted in the inflamed intestine, most notably when the epithelium is exposed to several toxins and proinflammatory cytokines that may promote both apoptosis and cellular necrosis[45]. Consequently, a significant increase in the frequency of epithelial cell apoptosis contributes to impaired intestinal barrier function[46]. Apoptotic cell death can be mediated by various signalling pathways, including the Casp cascade and the stress-activated protein kinase pathway[47]. Furthermore, Casp occupies a significant position in network pathway analysis, which included Casp-3, Casp-7 and Casp-9[46]. Berberine can induce apoptosis by upregulating caspase-3-mediated degradation as well as baicalin-induced apoptosis by suppressing Bcl-2 levels followed by activation of Casp-9 and Casp-3[48]. Generally, it is possible that the compounds characteristic of GQP can exert the anti-diarrhea effect via their collective impact on the NRF2 nuclear translocation to upregulate NRF2-mediated antioxidant response by directly inhibiting Keap1-Nrf2 interaction. And it is also restraining MAPK pathway and inhibiting the expression of IL-2, IL-1 β , ERK, and jun; they may also block apoptosis by activating Casp-3, Casp-7, and Casp-9. The TNF-mediated signalling by down-regulating TNFR1 and TNFR2, and thereby inhibiting the expression of downstream inflammatory factors. These factors may all contribute to the mechanisms associated with GQP-mediated reduction in toxicity associated with the administration of CPT-11.

Conclusions

This study featured integrated strategies used to discover effective compounds for GQP, using HPLC-Q/TOF-MS and network pharmacology, which to speculate potential mechanisms for relieving diarrhea induced by CPT-11. We first demonstrated that the administration of GQP limited the gastrointestinal toxicity induced by CPT-11; this was largely due to GQP-mediated inhibition of proinflammatory cytokines. In addition, major constituents of GQP were identified in mouse serum after oral administration; these were identified by an established HPLC-Q/TOF-MS protocol. A total of 19 compounds were identified, including 16 prototypical elements and three metabolic derivatives. A C-T network and C-T-D network were constructed based on these findings, and core mechanisms were queried via the use of both KEGG pathway and GO analysis, following mechanisms-predicted of GQP to alleviate diarrhea induced by CPT-11. In summary, this study provides important insights, which may further our understanding of the mechanism of action of diarrhea induced by CPT-11.

Abbreviations

GQP

Gegen Qinlian Pill; CPT-11:irinotecan; GO:Gene Ontology; KEGG:Kyoto Encyclopedia of Genes and Genomes; LPM:loperamide; KM mice:Kunming mice; IACUC:Institutional Animal Care and Use Committee; DAI:Disease activity index; H&E:hematoxylin and eosin; IL-1 β :Interleukin-1 beta; TNF- α :Tumor necrosis factor-alpha; ELISA:enzyme-linked immunosorbent assay; MPO:myeloperoxidase; GSH-PX:glutathione peroxidase; LC-MS:Liquid chromatography-Mass spectrometry; DAD:diode array detector; ESI:electrospray interface; CMC-Na:carboxymethyl cellulose; PPI:protein-protein interaction; SDS-PAGE:sulfate-polyacrylamide gel electrophoresis; Keap1:Kelch-like ECH-associated protein 1; Nrf2:Nuclear factor erythroid 2-related factor 2; HO-1:Heme Oxygenase-1; GAPDH:Glyceraldehyde-3-phosphate dehydrogenase; TIC:total ion chromatogram; ESR1:Ethylene-responsive transcription factor ESR1; C-T network:Compound-Target Network; C-T-D network:Compounds-Target-Pathway-Disease Network; BP analysis:biological process; CC analysis:cellular components; MF analysis:molecular function; MAPK:Mitogen-activated protein kinase; EGFR:Epidermal growth factor receptor; GSH-Px:glutathione peroxidase; H₂O₂:hydrogen peroxide; ROS:reactive oxygen species; IL-6:interleukin- 6; JUN:Transcription factor AP-1; CREB:Cyclic AMP-responsive element-binding protein; Casp:Caspase.

Declarations

Acknowledgment

Not applicable

Author's Contributions

J.Z. and Y.H conceived and designed the experiments; Y.W., Y.C., and Y.Y. conducted HPLC experiments, analyzed data, and prepared the manuscript; D.W., X.Y. and C.F. helped to prepare the manuscript and provided discussion.

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Availability of data and materials

Please contact corresponding authors for data requests.

Ethics approval and consent to participate

All in vivo experiments were carried out under the guidelines approved by the Institutional Animal Care (Approval No. 2014 DL-023) and Use Committee (IACUC) of Chengdu University of Traditional Chinese Medicine.

Consent for publication

Not applicable.

Competing interest

The authors declare no conflict of interest.

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Figures

Figure 1

The experimental flow of this study.

Figure 2

GQP alleviates CPT-11 induced gut toxicity symptoms. (A) The diagram of administration of mice. (B) Variation curves of mice body weight during the whole experiment. (C) The disease active index (DAI) curves of mice in each group. (D) The representative image of colon segments of mice in each group. (E) Microscopical images of sections of colons from each group stained with H&E (E $\times$ 100 magnification)

Figure 3

The total ion chromatograms of GQP in mice serum by HPLC/Q-TOF MS: (A) blank serum; (B) in the positive ion mode; (C) in the negative ion mode; (D) the sample of GQP

Figure 4

The fragment pathways of main kinds of compound. (E)The MS/MS spectra of the [M+H]⁺-ions at m/z 417 for puerarin and its proposed fragmentation pathways, (F) m/z 417 for baicalin and its proposed fragmentation pathways, (G) m/z 336 for berberine and its proposed fragmentation pathways

Figure 5

Compound-Target Network (C-T Network). A yellow triangle is the common target of all compounds. The purple hexagon represents the compounds from SR and the red hexagon represents the compounds from PR and the green hexagon represents the compounds from CR The blue hexagon represents the compounds from GR

Figure 7

Enriched KEGG pathways (A) and GO analysis including biological process (B), molecular function (C), and cellular component (D) of GQP

Figure 8

predicted pathway of GQP in the relieving diarrhea induced by CPT-11

Figure 9

GQP prevented the decrease of Keap1, Nrf2 and HO-1 targets in mice treated by CPT-11. Protein samples in each group were derived from the colonic tissues of 12 mice and Western blot analysis was repeatedly implemented for three times. Effects of GQP on pro-inflammatory cytokines (including TNF- α , IL-1 β) and oxidant stress in colon tissues of diarrhea model mice (n=12). ▲p<0.05 represented the statistically significant difference between model group and control group. *p<0.05 represented the statistically significant difference for groups with agent's treatment compared to model group.

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