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Systematic identification of chemical components in Fufang Shuanghua oral liquid and screening of potential active components against SARS-CoV-2 protease

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ABSTRACT

Coronavirus disease (COVID-19) caused by SARS-COV-2 infection has been widely prevalent in many countries and has become a common challenge facing mankind. Traditional Chinese medicine (TCM) has played a prominent role in this pandemic, and especially TCM with the function of “heat-clearing and detoxifying” has shown an excellent role in anti-virus. Fufang Shuanghua oral liquid (FFSH) has been used to treat the corresponding symptoms of influenza such as fever, nasal congestion, runny nose, sore throat, and upper respiratory tract infections in clinic, which are typical symptoms of COVID-19. The content of chlorogenic acid, andrographolide and dehydrated andrographolide as the quality control components of FFSH is not less than 1.0 mg/mL, 60 μg/mL and 60 μg/mL respectively. In this study, UPLC-Q-TOF-MS/MS was employed to describe the chemical profile of FFSH. Virtual screening and fluorescence resonance energy transfer (FRET) were used to screen the effective components of FFSH acting on SARS-CoV-2 main protease (Mpro). As a result, 214 compounds in FFSH were identified or preliminarily characterized by UPLC-Q-TOF-MS/MS, and 61 active ingredients with potential inhibitory effects on Mpro were selected through receptor-based and ligand-based virtual screening. In particular, quercetin, forsythoside A, and linoleic acid showed a good inhibitory effect on Mpro in FRET evaluation with IC50 values of 26.15 μM, 22.26 μM and 47.09 μM respectively, and had a strong binding affinity with the receptor Mpro (6LU7) in molecular docking. CYS145 and HIS41 were the main amino acid residues affected by small molecules in the protein binding domain. In brief, we characterized, for the first time, 214 chemical components in FFSH, and three of them, including quercetin, forsythoside A and linoleic acid, were screened out to exert beneficial anti-COVID-19 effects through CYS145 and HIS41 sites, which may provide a new research strategy for TCM to develop new therapeutic drugs against COVID-19.

1. Introduction

Coronavirus disease (COVID-19), named by WHO, is an infectious disease caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) virus [1], which is the most widespread global pandemic that mankind has ever encountered [2]. In this pandemic, China has responded quickly, and the situation of pandemic prevention and control continues to develop actively [3]. One of the indispensable reasons is the unique advantage and important role of traditional Chinese medicine (TCM), especially Chinese patent medicine (CPM), such as
Jinhua Qinggan granules and Lianhua Qingwen capsules, which have been proved to be effective in clinical treatment [4]. CPM derived from Chinese medicine prescriptions may be a treasure trove of effective drugs for the treatment of COVID-19.

Fufang shuanghua oral liquid (FFSH), as a clinical experience prescription of TCM for the treatment of upper respiratory tract infection [5], is composed of four herbal drugs, including Lonicera japonica Thunb (Jinyinhua, JYH) 30 g, Forsythia suspensa (Thunb.) Vahl (Lianqiao, LQ) 30 g, Andrographis paniculata (Burm. f.) Nees (Chuanxinlian, CXL) 30 g and Isatis indigotica Fort (Banlangen, BLG) 30 g. FFSH is the exclusive dosage form of Beijing Kangyi Pharmaceutical Co., Ltd., and the content of chlorogenic acid, andrographolide and dehydrated andrographolide as the quality control components is not less than 1.0 mg/mL, 60 μg/mL and 60 μg/mL respectively. FFSH has the effects of clearing away heat, detoxifying, and reducing pharyngeal swelling [6]. Likewise, it can relieve fever, asthma, sore throat and other symptoms, which are typical symptoms of COVID-19. Therefore, FFSH may have the potential to alleviate the symptoms of COVID-19. Current research on FFSH mainly focuses on quality control [7], and there is so far no report on whether FFSH could be used for the treatment of COVID-19. Among targets of SARS-CoV-2, it has been reported that the main protease (Mpro) is an essential non-structural protein of SARS-CoV-2, which plays a crucial role in viral replication and transcription [8]. Mpro consists of 306 amino acids and has hydrolase activity after forming a dimer. The active form of Mpro consists of two mutually perpendicular dimers, and each dimer consists of three structural domains that closely related to the catalytic site, the chymotrypsin-like skeleton and the proteolytic activity [9]. Therefore, Mpro is a ‘hot’ target for drugs discovery, which has been proved by the inhibitory effect of Lonicera japonica Thunb [10], Andrographis paniculata (Burm. f.) Nees [11] and Isatis indigotica Fort [12] on Mpro in the literature. It is worth investigating whether these potential active components in FFSH could treat COVID-19 by inhibiting Mpro.

In this paper, UPLC-Q-TOF-MS/MS were used to analyze and identify the chemical components of FFSH, virtual screening was used to simulate the effect of the chemical components of FFSH on Mpro protein, and the fluorescence resonance energy transfer (FRET) method was used to experimentally verify the binding of small molecules to proteins, which aimed to clarify the material basis of FFSH and the molecular mechanism of its antiviral activity, and to provide a new strategy for the treatment of COVID-19.

2. Materials and methods

2.1. Materials and reagents

FFSH was provided by Beijing Kangyi Pharmaceutical Co., Ltd (Beijing, China). The chemical standards contained in FFSH were purchased from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China). DMSO was purchased from Vetec (Sigma-AIDRICH brand). Forsythiaside A, chlorogenic acid, cryptochlorogenic acid, andrographolide, cymaroside, neandrographolide, isochlorogenic acid A, forsythin, forsythoside E were purchased from the TopScience Limited Liability Company (Shanghai, China). The distilled water was purchased from the GuangzhouWatsons Food & Beverage Co., Ltd. (Beijing Beverage Branch), Methanol and Acetonitrile were purchased from the Merck Company (Darmstadt, Germany).

2.2. Sample preparation and solutions

Fufang shuanghua (0.5 g) dry powder was accurately weighed into a conical flask with cap, and ultrasonically extracted in 50 mL of 70% methanol-waters (V/V) solution for 30 min before centrifugation at 12,000 g for 10 min using a centrifuge (Thermo Scientific, Waltham, MA, USA). The supernatant was filtered through a 0.22 μm PTFE filter (Agilent Technologies, USA) and stored at 4 °C prior to analysis.

2.3. UPLC-Q-TOF-MS/MS analysis

Chromatography was performed on a Waters Acuity UPLC I-Class system (Waters Corp., Milford, USA) with a Waters Acuity UPLC HSS T3 column (2.1 × 100 mm, i.d. 1.8 μm; Waters, USA) maintained at 40 °C. The mobile phases consist of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B). A gradient elution program was used as follows: 0–4 min, 2–6% B; 4–18 min, 6–20% B; 18–21 min, 20–24% B; 21–30 min, 24–35% B; 30–33 min, 35–98% B. The flow rate was set at 0.5 mL/min, and a 1-μL aliquot was set as the injection volume.

Mass spectrometry analysis was performed on a Waters SYNAPT G2HDMS system (Waters Corp., Milford, USA) equipped with an electrospray ionization (ESI) source in both positive and negative ion modes. The optimal parameters were set as follows: capillary voltage, 2 kV; cone voltage,40 V; resolution gas (N2) flow, 900 L/h; source temperature, 100 °C; resolution temperature, 450 °C; scanning time and interval, 0.2 s; scan range, m/z 50–1500; trap collision energy, 20–50 eV; lock mass, [M+H]+ 556.2775 and [M–H]− 554.2615. The data were collected in the MS8 continuum mode using Masslynx 4.1 software (Waters Corp., Milford, USA).

2.4. Integrated application of ligand-based and receptor-based virtual screening

In order to screen potential Mpro-inhibiting chemical components in FFSH, the components characterized by UPLC-Q-TOF-MS were subjected to ligand-based and receptor-based integrated analysis through the D3 Targets-2019-nCoV web server (https://www.d3pharma.com/D3Targets-2019-nCoV/D3Similarity/index.php). The chemical structures (SDF format) of these components were drawn by ChemDraw v18.0 (Cambridge Biosoft). Uploaded the SDF formats to the virtual screening module of D3Similarity and D3Docking respectively, and checked the “Mpro protein” (3 C-like protease) option. The molecular similarity between these components and the molecules in the subset of the ligand-based database was evaluated after the molecular energy of these components was minimized by MMFF94 force field. We chose the ‘2D × 3D’ scheme as the ranking scheme of molecular similarity, and selected the components with similarity scores > 35% as ligand-based virtual screening components. In the D3Docking module, the protein activity pockets were defined by D3 Pockets on the website, and pockets with the PPV > 200 Å2 were selected for molecular docking with energy minimization components. We selected the components with docking free energy < −10.5 and the results of molecular docking were shown by Discovery Studio 2019 Client software (Accelrys, San Diego, CA). D3Similarity is a ligand-based virtual screening and D3Docking is a receptor-based virtual screening. D3Docking can also obtain the potential binding modes of small molecules and proteins, which is a supplement to D3Similarity.

2.5. Mpro enzyme assay

2.5.1. Compound library screening

In order to verify the potential antiviral active constituents in FFSH, FRET experiments were carried out. The fluorogenic substrate (MCA-ALVQSGFR-Lys(Dnp)-Lys-NH2) was prepared at 100 μM in dimethyl sulfoxide (DMSO). Individual standard stock solution (10 mM) of components obtained by virtual screened was prepared by accurately weighing the required amounts into separate volumetric flasks and dissolving in DMSO, and then diluted with Tris-Cl Buffer (50 mM, pH 7.3) to 200 μM solution.

40 μL fluorogenic substrate (100 μM) and 40 μL Mpro solution (12.5 μL/m, provided by Institute of Microbiology, Chinese Academy of Sciences) was sequentially dispensed to each well of the black 96 F-bottom microplates (Greiner Bio-One, Monroe, NC, USA), and incubated for 10 min at room temperature. After addition of 20 μL fluorogenic substrate
again, the fluorescent signal was read at 320 nm immediately after excitation at 405 nm by a Multi-Mode Microplate Reader (BMG CLARIONstar Plus). Calculated the inhibition rate with Microsoft Excel: reaction rate with compound / reaction rate without compound.

2.5.2. Determination of gradient inhibition rate

The compounds with inhibition rate greater than 50% in the first step were selected. The compounds solution was diluted twice with Tris-HCl Buffer from the maximum 1 mM, and the experimental method was consistent with the screening of compound library. The IC$_{50}$ value and dose-response curve were calculated and plotted by GraphPad Prism 8.0.1.

2.6. Statistical analysis

UNIFI 1.8 (Waters, Milford, MA, USA) was used for visualization,
3. Results

3.1. Optimization of the extraction, LC and MS conditions

High performance liquid chromatography has the characteristics of high pressure, high efficiency, high speed, high sensitivity, high selectivity, etc., and is widely used in pharmaceutical, environmental protection, food and other industries, which is an important method for separation analysis nowadays [13,14]. In this article, we used UPLC to increase the throughput, sensitivity, and peak capacity of the analysis.

In order to obtain the optimal extraction method, different solvents including 30% methanol, 70% methanol and 90% methanol were compared, and 70% methanol showed better extraction efficiency of the target analytes.

In UPLC analysis, conditions, especially mobile phase, elution gradient, column temperature, play a critical role in achieving a reliable separation and appropriate ionization. Formic acid is usually added to the aqueous solution to enhance protonation and improve sensitivity in ESI mode. As mobile phase organic solvents, acetonitrile and methanol were compared, and acetonitrile showed better chromatographic separation of the target analytes. Accordingly, 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) was selected as the binary mobile solvent system, with the optimized gradient elution described in Section 2.3 UPLC-Q-TOF-MS/MS analysis.

In MS analysis, parameters, especially the spray voltage, capillary temperature, heater temperature and auxiliary gas flow rate, play an important role in the ion response of the compounds. In full-scan mode, the sensitivity and selectivity usually do not reach the optimal level at the same time, and thus there should be a balance. The optimal conditions described in Section 2.3 UPLC-Q-TOF-MS/MS analysis were selected based on our previous experience with the best responses for most of the analytes.

3.2. Mass fragment analysis of compounds in FFSH

The base peak intensity (BPI) chromatograms of FFSH corresponding to the positive and negative ion modes were shown in Fig. 1A and B. The MS² data obtained by UPLC-Q-TOF-MS/MS were processed using the UNIFI 1.8 software automatically to tentatively characterize the components by matching the detailed information with the in-house library. In addition, these characterized components were confirmed by reference standards and literature. As a result, a total of 214 target compounds were screened out in both positive (Table S1) and negative (Table S2), of which 67 originated from Lonicerajaponica Thunb., 56 from Andrographispaniculata Burm. f. Nees, 64 from Forsythia suspensa Thumb. Vahl and 33 from Isatis indigotica Fort. (Fig. 1C), including 37 flavonoids, 36 diterpene lactones, 26 organic acid, 23 Lignans, and 92 others (Fig. 1D).

3.2.1. Identification of organic acids and derivatives

The characteristic cracking of organic acids is the sequential loss of CO₂, H₂O and CO, which is helpful for further identification. As shown in Fig. 2A, compound 70 was identified as chlorogenic acid after comparison with the reference substance. Its quasi-molecular ion peak was at RT = 7.62, m/z 355.1158 [M+H]⁺, and the main fragments were m/z 163.0454 [M-H-C₆H₅O₉]⁻, m/z 145.0328 [M-H-C₆H₅O₉-H₂O]⁻, and m/z 135.0500 [M-H-C₆H₅O₉-H₂O-OH]⁻, which was consistent with the literature report [15].

3.2.2. Identification of phenylethanol glycosides

Phenylethanol glycosides, composed of phenethyl alcohol and sugar bases, are widely distributed in nature and have significant pharmacological activities, such as antibacterial, anti-inflammatory, anti-tumor, anti-viral, anti-oxidant, liver protection, and analgesic [16]. As shown in Fig. 2B, compound 119 was identified as forsythoside A. Its quasi-molecular ion was at RT = 15.21, m/z 623.2166 [M+H]⁺, and the main fragments were m/z 461.1609 [M-H-C₆H₅O₉]⁻, m/z 443.1738 [M-H-C₆H₅O₉-H₂O]⁻, m/z 179.0361 [M-H-C₆H₅O₉-H₂O-C₃H₇O₂]⁻ and m/z 161.0271 [M-H-C₆H₅O₉-2H₂O-C₃H₇O₂]⁻, which matched the chemical standards and were consistent with the literature report [17].

3.2.3. Identification of flavonoids

Flavonoids are a type of polyphenol compounds, generally referring to a series of natural products with a basic structure of C₆-C₃-C₆ two benzene rings connected by three carbon chains. As shown in Fig. 2C, compound 114 was identified as isoquercitrin. The quasi-molecular ion peak was at RT = 14.46, m/z 463.0800 [M+H]⁺, and the main fragments were m/z 301.0300 [M-H-GLC]⁻, m/z 271.0197 [M-H-GLC-CH₃O]⁻ or m/z 255.0361 [M-H-GLC-H₂O-OC]⁻ or m/z 285.0323 [M-H-GLC-OH]⁻, which was consistent with the literature report [18].

3.2.4. Identification of diterpene lactones

Diterpene lactone compounds are distributed among many Chinese medicines, such as Ginkgo biloba, tripterygium wilfordii, which have antibacterial, anti-inflammatory, anti-tumor, anti-viral, and hepatoprotective properties. It had the effect of promoting cholesterol and treating cardiovascular and cerebrovascular diseases [19]. As shown in Fig. 2D, compound 161 was identified as andrographolide after comparison with the reference substance [20]. The quasi-molecular ion was at RT = 21.09, m/z 351.2160 [M+H]⁺, and the main fragments were m/z 333.2054 [M-H₂O]⁻, m/z 315.1964 [M-H₂-H₂O]⁻, m/z 297.1832 [M-H-3H₂O]⁻, m/z 285.1842 [M-H₂-H₂O-CH₃O]⁻, and m/z 257.1520 [M-H₂-2H₂O-CH₃O-CO]⁻.

3.3. Screening of compounds in FFSH with inhibitory effect on SARS-CoV-2 Mpro

D3Similarity and D3Docking were used to screen 214 chemical constituents in FFSH respectively, and 61 of them were selected. Fluorescence resonance energy transfers, as a common method of high-throughput screening experiments [21,22], was employed to screen the inhibitory effect of 23 chemical standards on SARS-CoV-2 Mpro (Fig. 3A), and 23 of them was mapped to the results of the virtual screening section (Fig. 3B). Baiacaline was selected as the positive control drug, and its IC₅₀ value was equivalent to the reported value [23]. Moreover, compounds with an inhibition rate above 50% at a single concentration were selected to measure the gradient inhibition rate and obtain the IC₅₀ value (Fig. 4.). As shown in Fig. 3B, the three selected compounds had a good effect on Mpro in the enzymatic activity inhibition experiment, with IC₅₀ values less than 50 μM. Quercetin and forsythoside A had higher similarity scores and docking free energy, while linoleic acid had a slightly lower similarity score.

According to the molecular docking results, baiacaline and positive drug could form hydrogen bonds with the amino acid residues of HIS41, CY5145, SER144, LEU141 and HIS163 (Fig. 4A) to increase the stability of the combination. Quercetin was able to form hydrogen bonds and conjugation effects with the amino acid residues of MET165, ARG188, GLU166, PHE140, HIS163, HIS41 and CY5145 in 6LU7 (Fig. 4B),
Fig. 2. Fragmentation diagram of the components in Fufang shuanghua oral liquid. (A) Chlorogenic acid, (B) Forsythoside A, (C) isoquercitrin, (D) Andrographolide.
increasing the stability of the structure. Forsythoside A was able to bind to sites, such as the amino acid residues of THR190, GLU166, ASN142, THR26, SER144, HIS163, GLN189 and HIS41 in 6LU7 (Fig. 4C), forming a large number of hydrogen bonds with good affinity. It could also form Pi-Sulfer with amino acid residues of CYS145, and form a conjugation effect with the amino acid residues of LEU167, PRO168 and MET165 (Fig. 4C). Linoleic acid was bound to amino acid residues, such as HIS41, PHE140, SER144, CYS145, HIS163 and MET165 in 6LU7 (Fig. 4D), which indicated that four ingredients had good Mpro binding affinity. Therefore, forsythoside A, quercetin, linoleic acid were the three most potent compounds to inhibit SARS-CoV-2 Mpro activity in our present study.

4. Discussion

During the SARS-CoV-2 pandemic in Wuhan, TCM has participated in the treatment of COVID-19 throughout the whole process. It has played a prominent role in shortening the hospital stay [24], reducing the conversion rate from mild to severe symptoms [25], and reducing mortality [26]. Especially in recovery phase of COVID-19 patients, TCM has greatly reduced the probability and severity of patients-related sequelae. Hence, TCM may be a potentially effective drug for the treatment of COVID-19, but clarifying the material basis was the first step. At present, UPLC-Q-TOF-MS/MS is a powerful analytical tool for chemical components of TCM. A total of 214 compounds were identified or tentatively characterized in FFSH by using UPLC-Q-TOF-MS/MS, of which 67 originated from JYH, 56 from LQ, 64 from CXL, and 33 from BLG, including 37 flavonoids, 36 diterpene lactones, 26 organic acid, 23
Lignans, and 92 others. Interestingly, most of the identified compounds come from *Lonicera japonica* Thunb and *Andrographis paniculata* (Burm. f.) Nees (Fig. 1C). It’s reported that chromogranin A contained in *Lonicera japonica* Thunb improved the expression of IL-10 and IL-6 in LPS-induced RAW264.7 cells. Luteolin reduced the phosphorylation of IkB and P38 kinase, the activity of NF-κB and the expression of IL-6 in RAW264.7 cells to block these effects of CGA. Phenylethanoid, the main bioactive component of *Forsythia suspensa* (Thunb.) Vahl, was shown to have anti-inflammatory, antioxidant, antibacterial and antiviral effects [27]. *Isatis indigotica* Fort mainly contained alkaloid compounds, and were commonly used in clinical treatment of viral infectious diseases, such as influenza, and can also treat fever, dizziness, cough, sore throat, caused by lung heat [28]. *Andrographis paniculata* (Burm. f.) Nees, mainly contains diterpene lactones, and is usually used to treat respiratory diseases. As fever, inflammation, respiratory infection, are the most common symptoms of COVID-19, FFSH may have a potential therapeutic effect on COVID-19.

It is well known that the interaction between chemical component
and target protein is a prerequisite for the drug onset. Therefore, computerized screening combined with experimental validation has been proved to be an effective method for screening potential pharmacological components from a large number of components at present. In recent years, the merged analysis based on ligand and receptor virtual screening is an effective tool for drug discovery, which can greatly improve the success rate of drug discovery. This is because that ligand-based virtual screening is limited by known compounds, while receptor-based virtual screening is limited by conformation or binding sites. The two kind of screening are prone to false negative results each, and they complement each other in a comprehensive manner. In this article, virtual screening and FRET were used to screen the effective ingredients on Mpro. Consequently, quercetin, forsythoside A and linoleic acid were selected, which were consistent with the published literature [29,30]. Quercetin, forsythoside A and linoleic acid could form hydrogens and covalent bonds with Mpro, and the active residue sites were CYS145 and HIS41, which were also the main binding sites of green tea polyphenols [31] and corticosteroids [32] with Mpro. It could effectively inhibit the replication of the virus and stop the progression of disease after acting on CYS145 and HIS41.

In addition, SARS-CoV-2 triggered an inflammatory storm, which was an important cause of death for many patients during the current pandemic. It’s reported that flavonoids have the function of resisting the inflammatory storm induced by SARS-CoV-2 [33]. Equally, forsythoside A exerts anti-inflammatory effects by interfering with MAPK and NF-kB signaling pathways [34]. Besides, linoleic acid could regulate inflammation in a beneficial manner in vivo [35]. It could be seen that quercetin, forsythoside A and linoleic acid, which could not only directly act on Mpro through CYS145 and HIS41 to inhibit the replication of the virus and prevent the development of the disease, but also regulate the immune function of the body and improve the symptoms of COVID-19, were the potential active ingredients in FFSH against COVID-19.

5. Conclusion

In brief, we characterized, for the first time, 214 chemical components in FFSH, and three of them, including quercetin, forsythoside A and linoleic acid, were screened out to exert beneficial anti-COVID-19 effects through CYS145 and HIS41 sites, which may provide a new research strategy for TCM to develop new therapeutic drugs against COVID-19.

CRediT authorship contribution statement

Haiyu Xu, Ping Wang and Lifeng Fu conceived and designed the study. Hong Jiang, Yu-te Zhong and Li-ping Kang performed UPLC-Q-TOF-MS/MS analysis. Jie Chen and Guohua Wang performed fluorescence resonance energy transfer. Xin Li and Meng Yu performed virtual screening. Xin Li, Hong Jiang and Jie Chen also prepared the manuscript. Yu-te Zhong, Li-ping Kang, Guohua Wang and Meng Yu helped do data analysis. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2022.115118.

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