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•Review•

Modern research thoughts and methods on bio-active components of TCM formulae

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[ABSTRACT] TCM formulae are the important guidances for clinical application of traditional Chinese medicines, which follow the principles of diagnosis and treatment in TCM. Elucidating the bio-active components of TCM formulae is the key to the modernization and internationalization of traditional Chinese medicines. With the rapid development of modern instruments and technology, many new theories, methods and strategies are emerging, which upgrade the research of TCM formulae into a higher level. Only when the medicinal efficacy, bio-active components, function mechanism of TCM formulae are understood, we can guarantee TCM safety and quality control. In this paper, we summarized the latest modern research thoughts and methods on bio-active components of TCM formulae including formula decomposition study, serum pharmacology and serum pharmacochemistry, association analysis, biochromatography, network pharmacology, metabolomics and proteomics, so as to provide reference for the research and development of TCM in the future.

[KEY WORDS] Chinese medicine; TCM formulae; Bio-active component; Morden research methods
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Introduction

As early as the primitive society, our ancestors started to use herbs to treat illnesses and cure sicknesses. At first, they used single herbs alone; after many years of medical practice, they realized that multi-herb-combined remedies might achieve better effects. Then, traditional Chinese medicine (TCM) formulae were gradually developed, which then gradually become the main form of TCM for clinical treatment.

Basic research on bio-active components of TCM formulae in China began in the 1970s. Generally, natural pharmaceutical chemists follow Western medical research models to separate bio-active ingredients from each herb. Compared with single herbs, it is still a critical challenge to research on bio-active components of TCM formulae, due to their complex chemical composition. Even though a single herb contains a variety of chemical components. For example, liquorice contains more than 60 chemical constituents, including 18 types of flavonoids, terpenoids, and 22 types of amino acids, 14 types of alkaloids, coumarins, cinnamic aldehydes, and inorganic salts, and each active ingredient exerts various biological activity [1]. They can work together to exert synergistic, additive or antagonistic effects. Therefore, a TCM formula is a complex network system, which challenges researchers to fully understand its underlying mechanism of action. With the development of modern science and technology, TCM scholars continue to put forward new thoughts and methods to investigate the bio-active components of TCM formulae from different perspectives.

Modern Research Thoughts and Methods

Formula decomposition study

Formula decomposition study is guided by basic TCM theories. According to the formulating principles, it is performed by gradually subtracting one or several herbs from one formula to observe the change of therapeutic effect, so as to explain their corresponding roles in the formula. The main purpose of formula decomposition study is to clarify the com-

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patibility of a formula, screen the main components or active ingredients in the formula, determine the optimal compatibility ratio of the herbs, and simplify the formula to promote the research of new drugs.

ZHAO *et al*. [2] investigated the effects of Baihe Shugan Anshen decoction and its decomposed formulae on the HPA axis and monoamine neurotransmitters in rats with anxiety and depression-like behavior. As a result, compared with the Baihe Shugan Anshen decoction group, the effects of decomposed Baihe Anshen and Baihe Zhimu decoctions on the rats were reduced, the contents of 5-HT and NE in the hippocampus were down-regulated, and the content of NE in the venlafaxine group increased ($P < 0.05$). Based on the idiosyncratic drug-induced liver injury model mediated by immune stress, it was found that Epimedii Folium and Psoraleae Fructus were the major herbs of Xianling Gubao that led to liver injury. The liver injury caused by the full formula was less serious than that caused by Epimedii Folium and Psoraleae Fructus alone, which suggested that the other four herbs (Dipsaci Radix, Anemarrhenae Rhizoma, Rehmanniae Radix, and Salviae Miltiorrhizae Radix et Rhizoma) can prevent/alleviate the liver injury. According to formula decomposition study, the effect of Salviae Miltiorrhizae Radix et Rhizoma was the most significant [3].

Sepsis is a leading cause of both morbidity and mortality among surgical patients. Huanglian Jiedu decoction (HLJDD), a well-known Chinese herbal formula, has long been used for the treatment of sepsis. In XU *et al*. group [4], they subtracted one herb each time, reformulated the remaining herbs of HLJDD and obtained four HLJDD variants forms **1**−**4**, corresponding to removal of Phellodendri Chinensis Cortex, Scutellariae Radix, Gardeniae Fructu and Coptidis Rhizoma, respectively. Metabolomics approach combined with histological examination, biochemical measurement and molecular biological studies were used to investigate the effects of HLJDD and its four variants on a cecal ligation and puncture (CLP) model of sepsis, which were compared to decipher the formulating principles of HLJDD (Fig. 1) [4]. The results showed that HLJDD exhibited the strongest therapeutic effects on the CLP model, compared with the four variants, which was ascribed to its most significant enhancement of the cholinergic anti-inflammatory pathway and inhibition of the HMGB-1/TLR4/NF-*κ*B signaling pathway. Most of all, metabolites specifically changed between groups of HLJDD and its four variants were related with the exceptional treatment effects of HLJDD.

Xinshenghua granule (XSHG) is a popular remedy commonly used for the treatment of lochiostasis after delivery. PANG *et al*. investigated the roles of herb pairs containing Angelicae Sinensis Radix (Danggui) upon the formula by evaluating the coagulation and hemorheology in acute blood stasis rats. As a result, various herb pairs containing Danggui played different roles in improving the abnormality of hemorheology and coagulation, and the herb pair Danggui−Yimucao (Danggui−Yimucao > Danggui−Chuanxiong > Danggui−Honghua > Danggui−Zhigancao > Danggui−Taoren > Danggui−Jiangtan) was particularly important for the formula, which was consistent with the characteristics of XSHG and the pharmacological effect of Yimucao [5].

Serum pharmacology and serum pharmacochemistry

The concepts of serum pharmacology and serum pharmacochemistry were originally put forward by Shinichi Tashiro in the 1980s [6], which provide a methodology for exploring the efficacy of Chinese herbal medicines and discovering possible active constituents related to the specific effect *in vivo*. Recently, serum pharmacology has been developed for investigating the pharmacological effects of traditional Chinese medicines *in vitro*. After oral administration of herbs

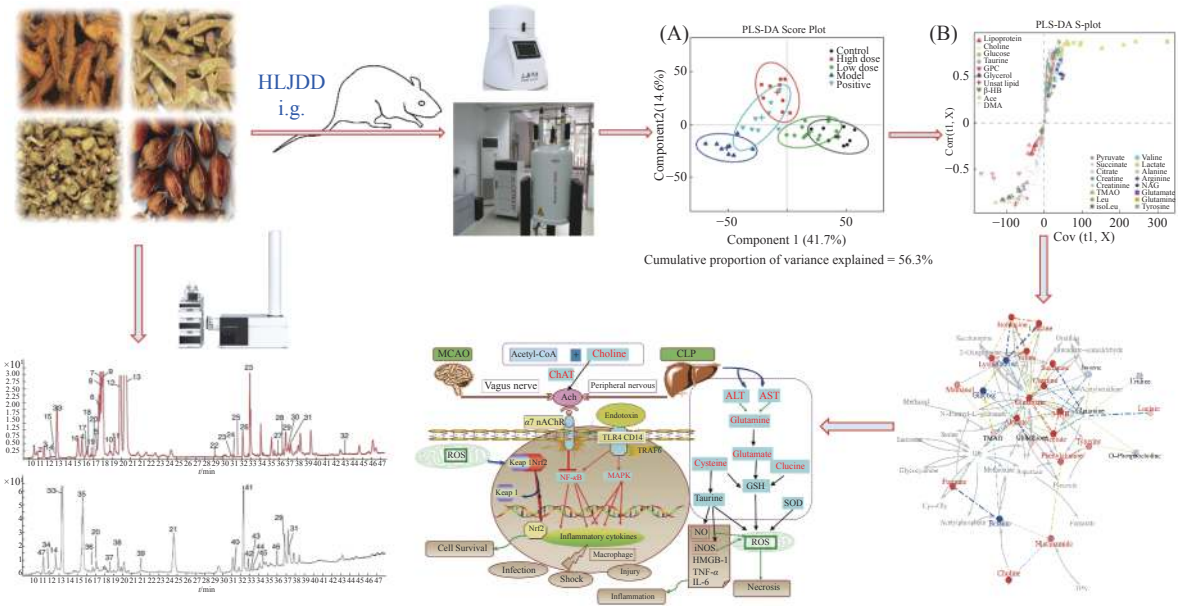

Fig. 1 Deciphering the mechanism of HLJDD on the treatment of sepsis by formula decomposition

to animals, blood samples are collected at specific time points and the resultant serum is used to evaluate the pharmacological effects using *in vitro* models.

Serum pharmacology has the advantages of preventing the physical and chemical properties of crude drugs from interfering with experimental results *in vitro*. Compared with traditional pharmacology methods where crude drugs are directly added into cells or organs *in vitro*, serum pharmacology establishes the bridge between *in vitro* and *in vivo* experimental methods for pharmacology studies, which are reasonable and suitable for TCM formulae. Nevertheless, the constituents that actually exert pharmacological effects in the serum are still not clear, and it is unable to clarify the material basis of the efficacy of TCM formulae. Accordingly, serum pharmacochemistry was developed, which can exactly compensate for this defect, and a specific concept "serum pharmacochemistry of traditional Chinese medicines" formulated by Chinese scholar WANG Xi-Jun [7]. Based on the research methods of traditional medicinal chemistry, serum pharmacochemistry of traditional Chinese medicines can analyze and identify the changed components in the medicated serum after oral administration of traditional Chinese medicines, in order to elucidate the metabolism and dynamics of substances absorbed into the blood from traditional Chinese medicines to exert a therapeutic effect on the organism and clarify the correlation between the active constituents and the traditional effects of herbal medicines. This method implements a comprehensive analysis from the perspective of constituents absorbed into the blood, which explains the active substances of TCM formulae in a scientific manner, and eliminates the interference of numerous components in herbal medicines and the interactions between the medicine and the organism. A study investigated the anti-inflammatory, antibacterial effects and serum pharmacochemistry of Qiwei Xiaoyan decoction (QWXYD), which has been widely used as an anti-inflammatory drug [8]. The results demonstrated that QWXYD exhibited significant anti-inflammatory and antibacterial effects. Additionally, calycosin-7-glucoside, sennoside A, aloeemodin and rhein were determined as the biologically active components in rat serum which may play a critical role in the anti-inflammatory activity of QWXYD. Although serum pharmacochemistry is an effective method to explore the potential active ingredients of traditional Chinese medicines, the active ingredients found cannot be directly related to the clinical effectiveness of traditional Chinese medicines due to the method limitations. Many challenges still remain in revealing the effective material basis that exactly reflects the clinical efficacy. Therefore, an innovative approach that integrated serum pharmacochemistry of traditional Chinese medicines with metabolomics, named chinmedomics, was established by WANG Xi-Jun [9], which may compensate for the abovementioned shortcoming of serum pharmacochemistry. Chinmedomics is defined as a comprehensive strategy that discovers syndrome biomarkers and active chemical compositions from traditioanl Chinese medicines *in vivo* and evaluates formula efficacy, in order to explore the constituents highly associated with clinical efficacy by means of analyzing the correlation between the endogenous biomarkers and the active constituents (Fig. 2), which can reveal the pharmacodynamic material basis of TCM formulae [10]. This method emphasizes the whole system of TCM formula rather than individual parts and focuses on the complex interactions of the components of TCM formula, which is consistent with the holistic view of TCM and facilitates to understand the efficacy of herbal medicines. Chinmedomics has been successfully applied to numerous studies for the scientific interpretation of key issues in the development of herbal medicines. For instance, a classical Chinese herbal formula Yinchenhao decoction (YCHD), which has long been used for the treatment of hepatic injury (HI) and jaundice

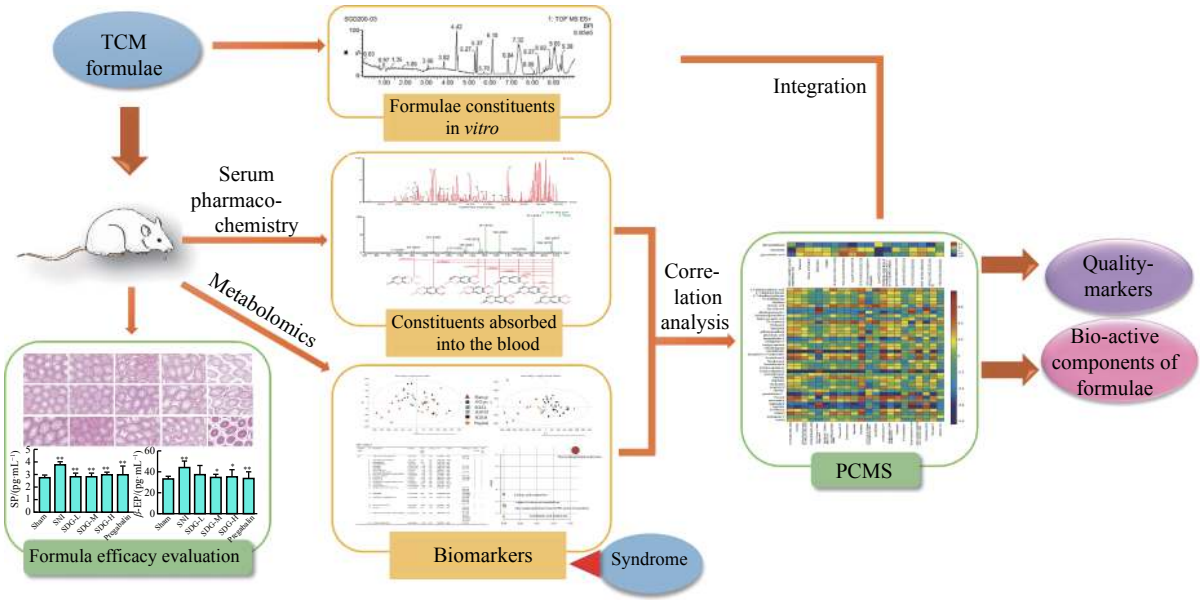

Fig. 2 Scheme of chinmedomics-based strategy

syndrome, was systematacially investigated to clarify the effective material basis using the strategy of chinmedomics [11]. It was found that a total of 69 compounds were identified in YCHD, where 41 of them were absorbed into the blood. Furthermore, 34 biomarkers in the urine were identified from dampness-heat jaundice syndrome. Of note, the plotting of the correlation between marker metabolites and serum constituents (PCMS) method was used to perform a correlation analysis between the urine biomarkers and *in vivo* components, which indicated 9 core-components as the effective substances responsible for YCHD efficacy. As a result, 12 potential targets were identified based on the integrative strategy. Chinmedomics can provide an approach for exploring the integration concept of the holistic effects of a TCM formula, which will greatly promote Chinese herbal research, and facilitate the modernization and extend the application of TCM formulae in modern healthcare industry.

Association analysis

A TCM formula is a holistic treatment system, which contains multiple components and involves multiple targets and integrated adjustment, making it difficult to elucidate the therapeutic bio-active components. Therefore, a series of association analysis methods have been explored, which can be divided into three steps: (1) establishing the chemical fingerprint of a TCM formula; (2) evaluating the pharmacological action by selecting proper pharmacological models and indicators; and (3) employing suitable statistical tools to discover the material basis by correlating components and efficacy. According to previous studies, association analysis can be categoried into different types, such as Pearson or Spearman correlation analysis, grey relational analysis (GRA), multivariate linear regression (MLR), partial least-squares regression (PLSR), and artificial neural network (ANN). Brief introduction of each method is showed in Table 1, and the bio-

Table 1 Introduction and application of the main association analysis methods

Association analysis method	Brief introduction	Advantages and disadvantages	Examples
Pearson or Spearman correlation analysis	The degree of closeness and positive or negative correlation between two or more variables are reflected by calculating the magnitude and direction of the correlation coefficients [12].	It can determine the closeness and direction of variables, but neglects the synergistic action of multiple components as well as the integrity of TCM.	Yinchenhao decoction: 2-ethyl-2-hexenal, isofraxidin, 2,5-dimethyl-7-hydroxy chromone, 6,7-dimethoxy coumarin, geniposide, capillarisin, neochlorogenicacid, chimaphylin, isorhamnetin-3-glucoside highly correlated with the therapeutic effect for dampness-heat jaundice syndrome were determined as effective components [13].
Grey relational analysis	The correlation between variables are determined based on the similarity of geometric curves of various factors, and the correlation coefficient and grey relational grade are calculated.	It can handle small data with insufficient information, but is deficient in describing the contribution of multiple components to the integrated efficacy.	Yuanhu Zhitong tablets: protopine, *α*-allocryptopine, and corydaline were considered to be noticeably correlated with bioactivity since their GRAs were all > 0.75 [14].
Multivariate linear regression analysis	The relationships between several independent variables and one dependent variable are modeled by fitting determined data to linear equations [15].	It can measure the contribution of each component to efficacy, but the influence of multiple linear relations between independent variables is difficult to avoid.	Artificial *Calculus bovis*: cholic acid, taurocholate sodium, and hyodeoxycholic acid were identified as the major effective components [16].
Partial least-squares regression analysis	Linear combination of independent and dependent variables is transformed into new integrated and independent variables with the maximum of original information for regression analysis. The importance of variables can be evaluated by variable importance for the projection values [17].	It is skilled in processing small samples and multivariate correlational variables, but the accuracy of the prediction model is difficult to ensure in reference to redundant nonlinear systems.	Chuanxiong Rhizoma and Cyperi Rhizoma herbal pair: ferulic acid, senkyunolide I, senkyunolide A, 3-nbutylphthalide, *Z*-ligustilide, Z-3-butylidenephthalide, nookatone, levistilide A, and *α*-cyperone were considered as anti-migraine compounds [18].
Artificial neural network	Using ingredients and efficacy data as the input and output variables to construct the network structure, then training and testing the samples and predicting efficacy by ingredient information [19].	It is suitable for nonlinear and nonparametric problems and can approximate any continuous function to any desired accuracy, but is deficient and risky for small sample data for overfitting due to the principle of empirical risk minimization.	Angelicae Sinensis Radix and Chuanxiong Rhizoma herb pair: aromatic acids exerted the effects of NTB and ADBS while phthalide lactones exhibited the effects of RMRP [20].

active components of TCM formulae have been identified using these methods.

In our previous study, artificial neural network (ANN) was employed to analyze the correlation between the content of main components and their integrated effects utilizing 21 samples of Angelicae Sinensis Radix, Chuanxiong Rhizoma, and their combination. Results showed that both aromatic acids and phthalide lactones of the herb pair exhibited the effects of nourishing and tonifying blood (NTB), activating blood circulation and dissolving blood stasis (ADBS), regulating menstruation and relieving pain (RMRP); aromatic acids laid particular stress on NTB and ADBS, while phthalide lactones laid particular stress on RMRP [20]. This strategy explains the effective substances of different efficacy of prescriptions and their contribution to the overall efficacy. Using different statistical models may get different results. At present, there is no specific mathematical model that can comprehensively reflect the relationship between chemical fingerprint and pharmacological effects. Therefore, the hybrid application of mathematical statistics is regarded as an incentive strategy to ensure the maximum of information and the accuracy of experimental results. In one case, Pearson correlation analysis and PLSR were used in combination to determined the effective components of Shaoyao Gancao decoction (a decoction of Paeoniae Radix Alba and Glycyrrhizae Radix et Rhizoma Praeparata Cum Melle, SGD). Through the high resolution of UPLC-Q-TOF/MS technology, a total of 128 compounds were identified from SGD as the chemical fingerprint. By integrating the behavior indicators, biochemical parameters, and metabolomics data, the efficacy was evaluated with multi-index comprehensive method. Pearson correlation analysis was introduced to discover 14 components related to the effect, and PLSR was adopted to further identify five most related effective components including paeonol, arabinose, benzoic acid, hispaglabridin A, and paeonilactone C for the treatment of neuropathic pain [21]. It provides a powerful method for the discovery of the effective components of TCM formulae.

Up to data, several strategies have been applied for the study of material basis. As an extraordinarily integrated system, traditional Chinese medicines exhibit multiple pharmacological effects even synergy or antagonism by multi-components towards multi-targets. However, some studies ignored the synergistic role of multiple components and the minor components, while some neglected the integrated effects of multiple targets and pathways. Therefore, association analysis based on a comprehensive analytical approach integrating chemical, biological, and metabolic methods are recommended, which include the following four steps: (1) analyzing the chemical profiling of TCM formulae by high performance liquid chromatography (HPLC), liquid chromatography/multiple stage mass spectrometry (LC-MS^n), or gas chromatography/mass spectrometry (GC-MS), so as to construct the complete profile of chemical components and obtain qualitative and quantitative data; (2) evaluating the pharmacological effects, where whole animals are the most feasible models to reflect the integrity of traditional Chinese medicines, and multi-index comprehensive method are recommended for comprehensive elucidation of efficacy combined with behavioral index, biochemical parameters, and omics data; (3) performing association analysis between chemical components and biological effects to discover the candidate components, where the mixed use of mathematical statistical tools are encouraged to ensure the maximization of information and the accuracy of results; and (4) verifying the pharmacological effects of candidate components, so as to clarify the material basis. The schematic flow is shown in Fig. 3. With the development of association analysis, the black box of TCMs will be slowly opening up, and the standardization and modernization of TCMs will be accelerated.

Biochromatography

Biochromatography is a new chromatographic technology that combines bioactive materials such as target proteins (including receptors and enzymes, etc.), cell membranes or cells with carriers as stationary phases, using the principle of specific binding between bioactive substances and the characteristics of liquid chromatography [22]. The screening and separation of the tested substances by traditional chromatography is based on the physical and chemical properties. Although the separation efficiency is high, it cannot give the information related to the biological activity of the components to be tested. The stationary phase ligands of biochromatography are bioactive substances, so the substances to be tested specifically combine with the stationary phase ligands through hydrophobic force, van der Waals force, electrostatic interaction and so on. Through screening the active components of traditional Chinese medicines by this method, the effective substances of traditional Chinese medicines can be identified by analyzing the changes of the effects before and after the removal of active components, combined with the analysis of the interaction between components. According to different stationary phases, biochromatography is mainly divided into molecular biological chromatography (MBC) and cell membrane chromatography (CMC).

With the development of modern molecular biology, especially the close combination of molecular biology with biomedicine and pharmaceutical chemistry, MBC is being developed. It is a new chromatographic technique which combines receptors, plasma transport proteins, enzymes, DNA and other biological macromolecules with physiological functions with carriers as stationary phases, while the principle of molecular specific recognition was used to investigate the interaction of biomolecules, so as to discover new physiologically active substances, and understand the mechanism of traditional Chinese medicines and the material basis of the actions of compound prescriptions [23]. It is fast and simple, with the characteristics of good reproducibility, high measurement accuracy of chromatographic system, small coefficient of variation, and is therefore suitable for the screening and research of the material basis of traditional Chinese medicines.

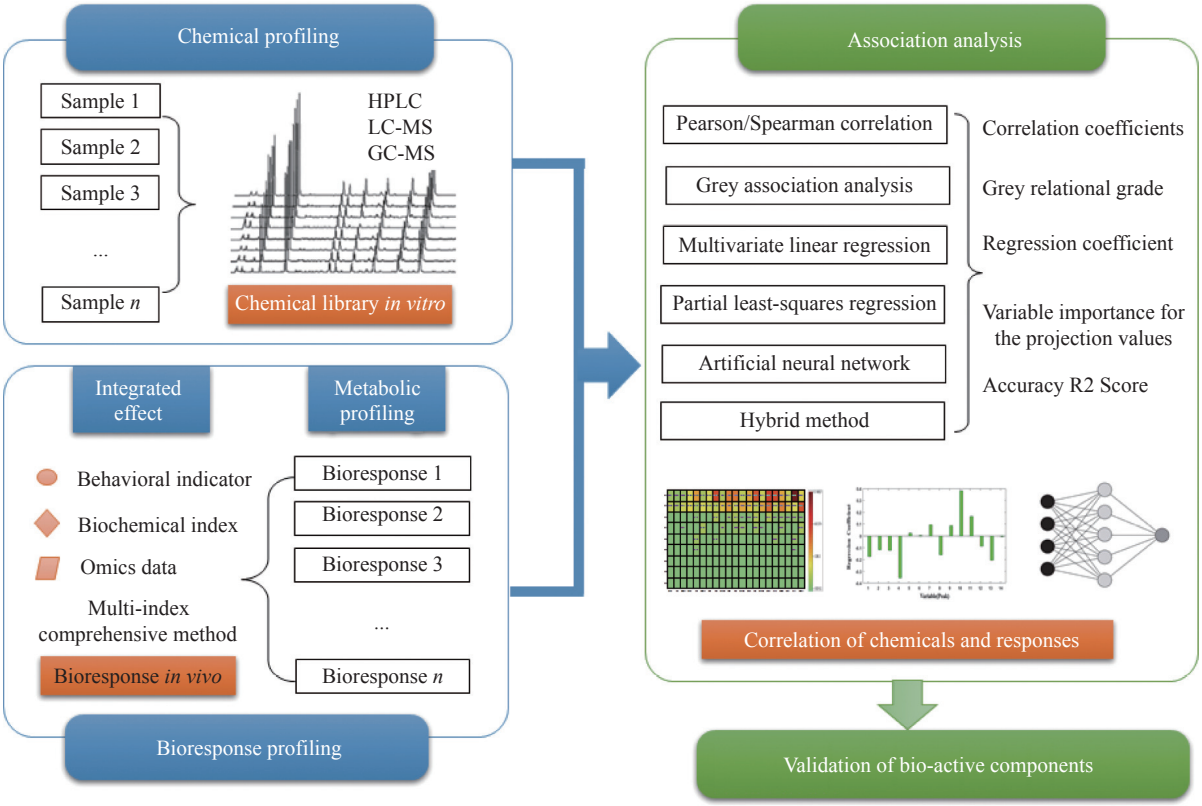

Fig. 3 Schematic flow of TCM material basis study based on association analysis

LI *et al*.[24] used an immobilized *β*2-adrenoceptor (*β*2-AR) affinity column to separate the bioactive compounds in the water-extract of Shaoyao Gancao decoction, which were then identified using quadrupole time-of-flight mass spectrometry. Results showed that the bioactive compounds in the water extract of Shaoyao Gancao decoction that bound to *β*2-AR were paeoniflorin and liquiritin, which had only one binding site on the immobilized *β*2-AR, with the affinity constants of $(2.16 \pm 0.10) \times 10^4$ and $(2.95 \pm 0.15) \times 10^4$ L·mol^{-1}, respectively. In addition, WANG *et al*.[25] also used the porcine recombinant immobilized *β*2-AR for selective screening and high-precision identification by Q-TOF-MS. It was found that ferulic acid, hydroxysafflor yellow A, and naringin were confirmed to be the bioactive compounds in Huoxue capsules that specifically bound to *β*2-AR. Therefore, the *β*2-adrenoceptor affinity chromatography is a useful tool for identifying potential *β*2-AR ligands in natural products used in TCM.

Chromatographic techniques based on molecular biology can be used not only to investigate the material basis of formula efficacy, but also to explain drug distribution, excretion, metabolism, activity, toxicity and biotransformation *in vivo*. These methods will exhibit a significant impact on the basic research of traditional Chinese medicines in the future.

CMC combines animal or plant active cell membranes to the activated silica gel surface to form cell membrane stationary phase, while drugs act as mobile phase to flow during chromatography, with different retention characteristics according to different active components and degrees of action, so as to separate, purify and screen the active components of traditional Chinese medicines and investigate the targets and receptors of each component[26]. Since it was first reported by Professor HE Lang-Chong's research group of Xi'an Jiaotong University in 1996[27], this technique has been increased used for screening flux from "offline" to "online". From "multi-target" to "specific target (receptor)", the specificity and sensitivity increase; from "unknown" target to specific receptor, high expression cell line target and receptor function become more clear. Virtual docking, molecular docking and drug affinity target stabilization techniques such as those based on cloud computing and molecular biology have been applied in cell membrane chromatography to achieve effective identification and confirmation of targets[28].

Through continuous improvement and modification, the above technique has developed nearly 30 cell membrane chromatographic models, and has been successfully applied in screening the active components of more than 40 kinds of traditional Chinese medicines or compound formulae. A large number of studies have shown that the new cell membrane chromatography preparation technique combined with full two-dimensional chromatography tandem high resolution mass spectrometry is very suitable for the screening of active components in complex systems.

WU *et al*.[29] screened the potential anti-osteoporotic active components in Liuwei Dihuang decoction by cell membrane chromatography/ultra high performance liquid chroma-

tography-time of flight mass spectrometry (CMC/UPLC-TOF/MS). Using the water extract of Liuwei Dihuang decoction (90 g·L^{-1}) as the sample, the retained components of cell membrane chromatographic column (osteoblast membrane chromatographic stationary phase) were quickly identified by CMC/UPLC-TOF/MS analysis, and 16 potentially active components in Liuwei Dihuang decoction were obtained with high selectivity. Then, the affinity of catalpol, paeonol and oleanolic acid to cell membrane chromatographic stationary phase was analyzed by chromatography, and the high affinity intensity and content of catalpol were selected to verify the efficacy *in vivo* and *in vitro*. It was found that catalpol significantly stimulated the growth of mouse osteoblasts and increased the mineralized area of bone in the head of osteoporotic zebrafish. YU *et al*. [30] developed a method combining the erythrocyte membrane binding assay with solid-phase extraction and UPLC-QTOF-MS/MS to screen for potential bioactive compounds that reduce the blood consistency in Buyang Huanwu decoction (BHD). Calycosin, paeoniflorin, 6-hydroxy behenol-3,6-di-*O*-glucoside and calycosin-7-*O*-*β*-D-glucoside were found to bind the red blood cell (RBC) membranes. Furthermore, the identified ingredients promoted the activity of Na^+-K^+-ATPase, sialic acid and superoxide dismutase and reduced the content of cholesterol on the RBC membrane, suggesting that these BHD ingredients protect the activity of RBCs. Based on these results, the RBC membrane binding assay combined with SPE and mass spectrometry is a novel and effective approach for screening potentially anti-erythrocyte lesion constituents in traditional Chinese medicines.

Biochromatography has unique advantages in complex systems, especially for screening the effective ingredients of traditional Chinese medicines, extracting active components and investigating the material basis of TCM compound formulae, and has been applied to a certain extent (Fig. 4). However, there are also some problems, such as high preparation cost, short service life of chromatographic column, difficulty in commercialization, and being unable to simulate the complex metabolic environment *in vivo*. Therefore, the screening of active components is not comprehensive. With the development of follow-up research, biochromatography will have a broader application space in the field of screening active components of traditional Chinese medicines.

Network pharmacology

With the gradual rise of interdisciplinary subjects such as system biology, bioinformatics, artificial intelligence, and big data science, TCM research has changed from a single and isolated mode to a multi-faceted and systematic research mode [31]. Utilizing the "network" to regain the "whole" generates an unprecedented opportunity for the systematic research of TCM. Therefore, network pharmacology evolves as a systematic paradigm and has widely employed to decipher the potential active ingredients and underlying mechanisms of TCM [32]. In general, the conventional TCM network pharmacology analysis starts with identification of active ingredients present in a TCM formula and their plausible corresponding targets following a database-based strategy and culminates with the investigation of the signaling pathways and sub-networks regulated by the formula to evaluate its effects on disease-associated gene sets or networks [33, 34]. Nowadays, computer algorithms play an essential role to meet the data-dependent needs in the various aspects of TCM network pharmacology. Specifically, machine-learning algorithms for predicting ADME (absorption, distribution, metabolism, and excretion) parameters and targets are useful to screen active ingredients in TCM and to identify putative targets [35, 36]; net-

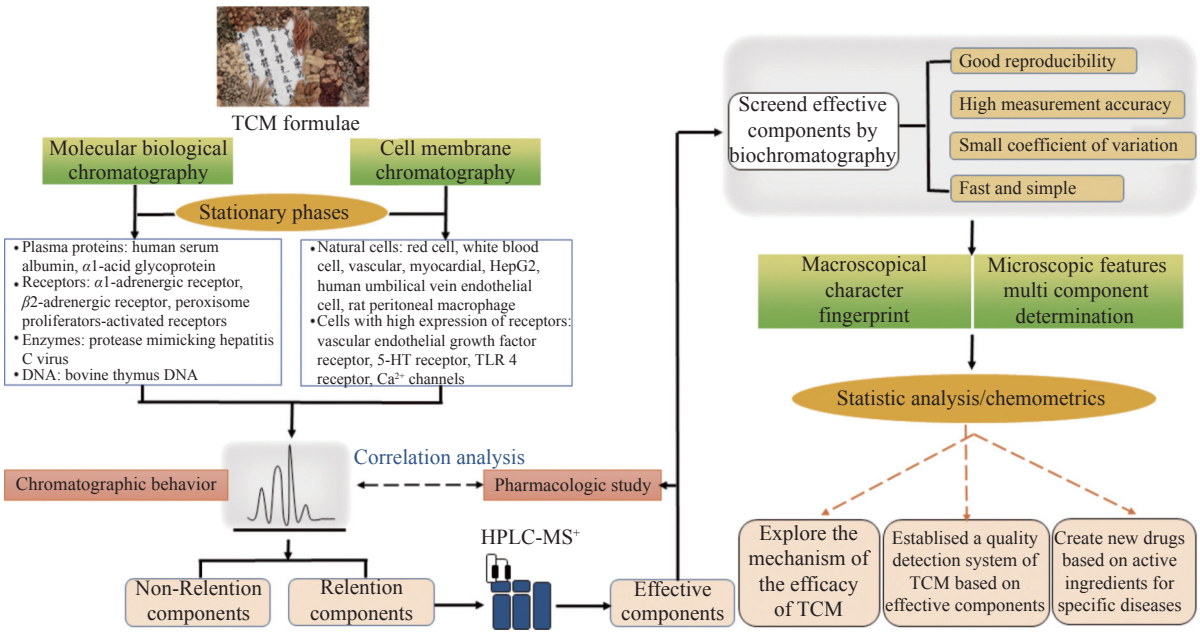

Fig. 4 Schematic flow of biochromatography for investigating the bio-active components of TCM formulae

work propagation-like algorithms can recognize proteins influenced by a TCM formula within a network [37]; and algorithms for finding hub nodes in networks facilitate the identification of core ingredients and targets of TCM [38-40].

In the past decade, several specialized ADMET-associated databases and servers were established, such as admetSAR 2.0 (http://lmmd.ecust.edu.cn/admetsar2/) [41], ADMETlab (http://admet.scbdd. com/) [42], SwissADME (http://www.swissadme.ch) [43], vNN-ADMET (https://vnnadmet.bhsai.org/) [44], DrugMint (http://crdd.osdd.net/oscadd/drugmint/) [45], XenoSite (http://swami.wustl.edu/ xenosite/p/quinone) [46], pkCSM (http://structure.bioc.cam.ac.uk/pkcsm), and FAF-Drugs (http://fafdrugs4.mti.univ-paris-diderot.fr) [47]. For better understanding, major physicochemical properties, drug-likeness score, ADMET properties, ecotoxicity endpoints, and major *in silico* modeling tools involved in the corresponding databases and servers are summarized [48] in Table 2. Techniques such as k-nearest neighbor (k-NN), support vector machines (SVM), random forest (RF) and artificial neural networks (ANNs) are the examples of machine learning methods that have recently excelled in the investigation of ADMET properties [49]. Several examples of TCM network pharmacology based on ADMET to screen and identify active ingredients are also presented. WANG *et al*. proposed a computational systems pharmacology approach for investigating the molecular mechanisms of Tianma Gouteng decoction for the treatment of Alzheimer's disease (AD) [50]. Ingredients with human intestinal absorption (HIA) and blood brain barrier (BBB) penetration classified as negative by admetSAR websever were considered poorly absorbed by the intestine and could hardly penetrate the BBB, and hence 494 out of 731 herbal ingredients in Tianma Gouteng decoction were left for further analysis. Then, 12 active compounds to be most significantly related to Alzheimer's disease were identified by Fisher's exact test. Through network pharmacology-based investigation of the constituents of Mahuang Fuzi Xixin decoction, Caco-2 cell permeability, HIA, and oral bioavailability (OB) limits F (F-20% and F-30%) of the corresponding compounds were screened by ADMETlab and 20 active ingredients with favorable absorption properties were identified [51]. Furthermore, a novel system pharmacology model including pharmacokinetic parameters, pharmacological data, and component contribution scores was constructed to decipher the potential therapeutic mechanism of Zhizhu pills on functional dyspepsia by WANG *et al*. [52]. The properties of components were retrieved from TCMSP, including molecular weight (MW), OB, Caco-2 permeability (Caco-2), drug-likeness (DL), Moriguchi octanol-water partition coefficient (LogP) (MLogP), the number of acceptor atoms for H-bonds (nHAcc), the number of donor atoms for

Table 2 The physicochemical properties, drug-likeness rules, ADMET parameters and modeling algorithm of commonly employed databases and servers for ADMET prediction

In silico databases and servers	Physicochemical properties	Drug-like rules	ADMET parameters	Modeling algorithm
admetSAR 2.0	MW, log P, HBA, HBD, RB	Lipinski	CacoP, BBB, HIA, OB, P-gp±, PPB, BPB, CYPI/S, Cl, $t_{1/2}$, AOT, AT, CAR, Herg, DILI, EI, MT, ED, CAR, *etc*.	SVM, kNN, RF, CNN,
ADMETlab	log P, log D, log S, *etc*.	Lipinski, Ghose, Oprea, Veber, Varma	CacoP, BBB, HIA, OB, 10 CYPI/S, P-gp±, PPB, VD, Cl, $t_{1/2}$, SkinSen, AOT, AT, Herg, HepTox, *etc*.	SVM, RF, RP, PLS, NB, DT
XenoSite	/	/	SOM, epoxidation, QF, reactivity and UGT mediated SOM	ANN
SwissADME	MW, HBA, HBD, RB, nA, nHA, MR, log P, log S, *etc*.	Lipinski, Ghose, Veber, Egan, Muegge	GI absorption, 5 CYPI/S, SP, HIA, BBB, PAINS, Log K_p, synthetic accessibility, structural alert	MLR, SVM
vNN-ADMET	/	/	MT, CytTox, CarTox, HepTox, Herg, 5 CYPI/S, P-gp±, DILI, DDI, *etc*.	vNN
DrugMint	MW, log P, HBA, HBD, RB, *etc*.	DrugMint models	/	SVM, PCA, MACCS keys
pkCSM	MW, log P, RB, HBA, HBD, TPSA	Lipinski	CacoP, HIA, P-gp+/, VD, BBB, 7 CYPI/S, Cl, AT, Herg, AOT, AT, SkinSen, HepTox, *etc*.	LBM
FAF-Drugs	MW, HBA, HBD, RB, TC, MPSA, log P, *etc*.	Lipinski, Egan, Veber, PhysChem	Yes, with PAINS and structural alerts	QED

H-bonds (nHDon), and topological polar surface area (TPSA). Furthermore, GI absorption, a parameter retrieved from SwissADME, was also included to evaluate whether a drug can be well absorbed in the gastrointestinal tract. Therefore, 61 active ingredients were screened out of the 378 ingredients of Zhizhu pills.

The widespread application of network pharmacology has unveiled the mystery of TCM to some extent by constructing the relationship of "drug-ingredient-target-disease". With the integration of computational techniques into network pharmacology, the efficiency of data mining and the accuracy of active ingredient identification and target fishing have been improved, and the "drug-ingredient-target-disease" network has been more systematically and comprehensively explained to reflect the chemical basis and action mechanisms of TCM. The basic network properties combined with computational algorithms, offer a powerful approach for systematic identification of the candidate active ingredients in traditioanl Chinese medicines. Generally, networks are composed of nodes and edges, and a node can represent an ingredient, a protein, a gene, a disease phenotype and so on. The connection between two nodes is called an edge, which can represent the interaction of ingredient-target, protein-protein, or transcription regulation. Network topology analysis is aimed to determine the bioactive ingredients, crucial targets, and metabolic pathways by extracting targets or target combinations, drugs or drug combinations. In network topology, node centrality is a widely used measurement with three main metrics: degree, closeness, and betweenness. The degree of a node is defined by the number of connected edges. In a directed network, in-degree and out-degree represent the number of adjacent head and tail endpoints, respectively. A hub is a high degree node in a network. Closeness is related to the position of nodes in a network with the boundary nodes having small closeness. The closeness of a node equals the inverse of the total sum of all the shortest paths to every other node. The betweenness of a node represents the frequency of the given node's participation in all shortest paths. A node with high betweenness might suggest a primary role in information diffusion. Hence, we can evaluate the centrality of the nodes based on three general measurements: degree, betweenness, and closeness to identify the core ingredients in biological networks. Moreover, for the purpose of reducing the false positive rate and improving the false discovery rate, computational algorithms have been integrated with the basic network properties to identify the hub nodes (ingredients and targets, etc.). As a demonstrative example, YUE *et al*. first obtained a total of 31 active ingredients of Danggui Honghua herb pair from database using the threshold (OB ≥ 30%, Caco-2 ≥ −0.4, and DL ≥ 0.18), and then a contribution index (CI) was proposed to identify top ingredients based on network topology property (degree) and efficacy weight (relevant literature). The CI was proposed and calculated by eqs (1) and (2):

$$NE(j) = \sum_{i=1}^{n} d_i \tag{1}$$

$$CI(j) = \frac{c_j \times NE(j)}{\sum_{i=1}^{m} c_i \times NE(i)} \times 100\% \tag{2}$$

Where n is the number of targets associated with ingredient j; d_i is the degree of target i associated with ingredient j; c_i is the number of blood stasis syndrome-related literature of ingredient i; and m is the number of ingredients. Six bio-active ingredients including hudroxysafflor yellow A, safflor yellow A, safflor yellow B, *Z*-ligustilide, ferulic acid, and *Z*-butylidenephthalide were indicated due to the most contribution to the blood-activating and stasis-dissolving effects.

As mentioned earlier, WANG *et al*. performed a computational network pharmacology approach for investigating the active ingredients of Tianma Gouteng decoction in treating AD. In the study, HIA and BBB were selected as the threshold from retrieved active ingredients from database due to the characteristic of AD. Then, a computational algorithm with Fisher's exact test was presented to determine the AD-related ingredients. As only a very small portion of natural products have known targets, the number of an ingredient's targets related to AD conforms approximately to hypergeometric distribution and its probability mass function is showed as follows:

$$P(X = k) = \frac{\left(\frac{K}{k}\right)\left(\frac{N-K}{n-k}\right)}{\left(\frac{N}{n}\right)} \tag{3}$$

where N is the total number of genes, K is the total number of AD-related genes, n is the number of predicted genes, k is the number of AD-related genes in predicted genes and $P(X = k)$ is the probability of k AD-related genes occurring in predicted genes for an ingredient. However, the constructed network did not cover all protein-coding genes. Therefore, in a certain network, N was the number of protein-coding genes it covered, K was the number of all AD-related genes in the network and n was the number of predicted genes. Then, the Fisher's exact test was implemented to assess the significance of enrichment of AD-related genes in 20 predicted and known genes for each ingredient, and every ingredient had k AD-related genes. The frequency distribution of k in the background set was calculated and approximated roughly to the probability distribution of the background set:

$$P(X = k) = \frac{NP_k}{NP} \tag{4}$$

where NP is the number of all collectable compounds and NP_k is the number of ingredients having k AD-related genes. Then, if an ingredient has k AD-related genes where P ($X = k$) < 0.01, the ingredient is probably enriched onto AD. *P*-value was calculated and adjusted by Benjamini–Hochberg method, and used to rank all ingredients. Top-ranked ingredients were presumed to be critical components of this formula in treating AD.

The unclear bioactive ingredients of TCM is one of the

key issues leading to the bottleneck of TCM research, so a comprehensive method that can identify multi-ingredients is urgently required. Network pharmacology provides an easy method by mapping chemical compounds into the disease-gene network to seek potential bio-active ingredients. The schematic flow is shown in Fig. 5. To date, network pharmacology has been applied for investigating many traditional Chinese herbs and herbal formulae for the dissection of the corresponding chemical basis. Even some unavoidable limitations stand in the research of network pharmacology, it provides a multi-dimensional research strategy for the identification of bio-active ingredients of TCMs.

Metabolomics

Metabolomics, or alternatively metabonomics, as an emerging field of biochemical research, is a complementary technique to genomics, transcriptomics, and proteomics. It refers to comprehensive assessment and simultaneous profiling of endogenous metabolic changes in living systems. This approach offers a global analysis of low molecular weight metabolite level changes in biological samples and has shown great promise as a means to identify endogenous metabolites of drug efficacy. Direct qualitative and quantitative measurements of metabolite expression in the urine, serum, plasma, and tissue are essential for the study of biological processes in normal and disease states. In order to figure out the number and name of metabolites in a biological sample, a large separation science plays an important role in metabolomic research. An increasing number of modern techniques such as gas chromatography (GC), high performance liquid chromatography (HPLC), capillary electrophoresis (CE), nuclear magnetic resonance (NMR), and supercritical fluid chromatography have been used for metabolomics analysis [53].

HE *et al*. [54] explored the beneficial effects of Huanglian Jiedu decoction (HLJDD) on diabetic encephalopathy in db/db mice by UPLC-Q-Orbitrap HR-MS/MS based untargeted metabolomics analysis. As a result, Morris water maze test revealed that HLJDD effectively improved the learning and memory of db/db mice. Brain histological and biochemical examination indicated that HLJDD protected against neurodegeneration and oxidative stress in db/db mice. Meanwhile, a total of 21 potential biomarkers with significant differences were identified between the model group and the control group using untargeted metabolomics strategy. Among them, 11 metabolites showed a trend towards the normal levels after HLJDD intervention. These metabolites were mainly involved in glycerophospholipid metabolism, fatty acid β-oxidation, linoleic acid metabolism, glucose metabolism and glutathione metabolism based on the metabolic pathway analysis, which were regulated in DE model mice after HLJDD intervention. Generally, these results demonstrated that HLJDD exhibited beneficial effects on DE, which was mediated through ameliorating the metabolic disorders.

GUAN *et al*. [55] investigated the preventive effect of Sijunzi decoction (SJZD) on mitomycin *C*-induced immunotoxicity in rats by ^{1}H NMR and MS-based untargeted metabolomic analysis. As a result, 8 biomarkers in plasma samples, 19 in urine samples and 10 in spleen samples were identified to be primarily involved in amino acid metabolism, carbohydrate metabolism and lipid metabolism. The most critical pathway was alanine, aspartate and glutamate metabolism. The variations in biomarkers revealed the preventive effect of the immunotoxicity of SJZD on mitomycin C, which were important for exploring the possible metabolic mechanism.

Proteomics

The rapid growth of the proteomics field has resulted in

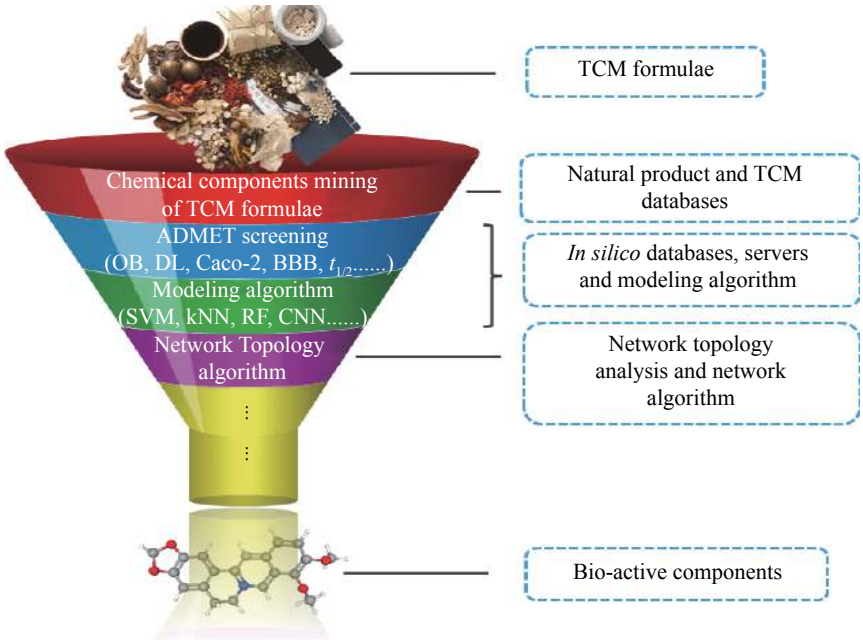

Fig. 5 Flowchart of screening the bio-active components in TCM formulae based on network pharmacology

an array of new tools for investigating TCM, while proteomic analysis also facilitats the globalization of TCM. Being aware of the intrinsic relationship between TCM and systems biology, some researchers have already proposed that proteomics approaches may be helpful in validating TCM theories. Proteomics researches provide the basis for investigating the action mechanism and active components of TCM formulae, while providing a platform for comparing protein expression between different pathologies, and for screening novel targets for TCM formula intervention.

YAN *et al*. [56] investigated the therapeutic mechanism of Sijunzi decoction on spleen deficiency syndrome by label-free mass spectrometry-based proteomics. The results showed Sijunzi decoction significantly restored damaged liver tissue and enzymatic activity. The DEPs of Sijunzi decoction-intervened spleen deficiency syndrome mainly regulated various metabolic processes involved in cellular responses to enzyme activity for catalysing, transforming and transferring. The four pathways (bile secretion, pyruvate metabolism, cysteine and methionine metabolism, and fatty acid metabolism) might be closely related with the intervention mechanism of Sijunzi decoction on spleen deficiency syndrome. Protein H2afx (H2AFX), interferon stimulated gene 15 protein (ISG15), and signal transducers and activators of transcription1 (STAT1) proteins were predicted to play important roles in Sijunzi decoction action networks. Our study provides novel perspectives for revealing the therapeutic mechanism of Sijunzi decoction on spleen deficiency syndrome.

LUO *et al*. [57] investigated the effects of Yiqi Huayu Jiedu decoction (YQHYJD) on lipopolysaccharide-induced acute respiratory distress syndrome in rats on the basis of tandem mass tag-based proteomics. The results showed YQHYJD alleviated the LPS-induced pathological damage of lung tissue in rats. There were 134 DEPs between the YQHYJD treatment and model groups. The genomes pathway analysis revealed that the DEPs were closely related to immune system pathway. The mass spectrometry analysis revealed that YQHYJD exhibited a protective effect on lung tissue by significantly up-regulating hematopoietic cell kinase (Hck), phospholipid phosphatase 3 (Plpp3), myristoylated-alanine rich C-kinase substrate (Marcks), and actin-related protein 2/3 complex subunit 2 (Arpc2), which were related to Fc gamma receptor-mediated phagocytosis pathway. Therefore, YQHYJD can alleviate the lung injury of ARDS rats by regulating the Fc gamma receptor-mediated phagocytosis pathway, which is related to immune system.

Discussion

As a complex system, the bio-active components of TCM formulae should be broad chemical constituents, which include organic compounds, inorganic compounds and trace elements. TCM formulae depend on these chemical components to exhibit main, multi-target, organic, overall and synergistic therapeutic effect. It is necessary to scientifically elucidate the efficacy of TCM formulae, and strictly adhere to the combination of chemical composition research and pharmacological research. A composition research without the guidance of theraputic effects is a pure academic investigation, while a pharmacological study without the interaction of chemical constituents can only be a low repetition research. Therefore, based on pharmacological research, we can determine the effective chemical section and constituents related to pharmacodynamic effects. The chemical composition of TCM formulae can be comprehensively investigated from the levels of medicinal herbs, effective sections and effective components, so as to provide a more realistic evidence for explaining thhe chemical constituents, efficacy, and mechanism of actions of TCM formulae.

Due to complex compatibility, one TCM formula may exert diverse therapeutic effects. Similarly, some TCM formuale may exhibit various effects, which can be used for the treatment of a variety of disorders. However, it is necessary to grasp the property and the main effect of TCM formulae after compatibility for in-depth studies. For example, according to previous studies, the effects of Liuwei Dihuang pills (decoction) were reported in more than 3000 articles, where more than 100 diseases were treated, which involves regulating multiple systems such as the kidneys, gonad, reproductive system, and immune system, and can improve immune function, remove oxygen free radicals and enhance the activity of antioxidant system, improve DNA damage repair capability, enhance bone strength, improve bone metabolism, delay skin and renal aging. Liuwei Dihuang pill (decoction) is one of the classic representatives of traditional Chinese medicines, and it is necessary to grasp the main effect of “nourishing kidney yin”, combined with the function of the kidneys of traditional Chinese medicines and the function of modern medical neuroendocrine immune regulation network. Therefore, in-depth studies are required from the perspectives of the inner secretion of animal models, neuroendocrine immune network, pharmacology, and the mechanism of action.

Conclusions and Prospects

With the rapid development of science and technology, more and more techniques and methods have been widely utilized, such as gene chip technology, Caco-2 cell technology, chemometrics, artificial intelligence and so on. These techniques and methods have been widely used in investigating the bio-active components of TCM formulae. As the composition of TCM formulae is complex, it is difficult to comprehensively explain the bio-active components invovled. However, these techniques and methods are still essential in the related studies. With the integration of medicine, biology, chemistry, mathematics and computer science, it is believed that the research of bio-active components of TCM formulae in China will eventually make a breakthrough under the guidance of the basic theories of TCM, making a great contribution to TCM modernization.

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