Chinese Journal of Natural Medicines

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Citation: Jiaxin Zhang, Xinhao Zhu, Chaofeng Zhang, Wangning Zhang, Jiangwei Tian, Research progress on new techniques and methods for identifying active ingredients in traditional Chinese medicine, Chinese Journal of Natural Medicines, 2025, 23(10), 1153–1170. doi: 10.1016/S1875–5364(25)60982–4.

View online: https://doi.org/10.1016/S1875–5364(25)60982–4

Chinese Journal of Natural Medicines 23 (2025) 1153-1170

Contents lists available at ScienceDirect

Chinese Journal of Natural Medicines

journal homepage: www.cjnmcpu.com/

Review

Research progress on new techniques and methods for identifying active ingredients in traditional Chinese medicine

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ARTICLE INFO

Article history:
Received 21 June 2024
Revised 27 September 2024
Accepted 9 October 2024
Available online 20 September 2025

Keywords:
New techniques and methods
Active ingredients
Molecular probe
High-content screening

ABSTRACT

Recent years have witnessed significant advances in the development of novel techniques and methodologies for identifying active ingredients in traditional Chinese medicine (TCM), substantially advancing research and development efforts. Spectrum-effect correlation analysis, affinity ultrafiltration, high-content screening (HCS) imaging, and cell membrane chromatography (CMC) have emerged as essential tools, effectively linking TCM chemical constituents to their biological effects, thereby enabling efficient active ingredient screening. Additionally, molecular interaction analysis provides deeper insights into TCM-biomolecule interaction mechanisms, enhancing understanding of its therapeutic potential. Computer-aided techniques facilitate TCM active ingredient identification, optimizing the screening process for efficiency and cost-effectiveness. Molecular probe technology, as an emerging methodology, enables precise and rapid screening for novel therapeutic drug discovery. Ongoing technological advancement in this field indicates promising future developments, potentially leading to more effective and targeted TCM-based therapies.

1. Introduction

In the dynamic field of pharmaceutical research, the systematic screening of active ingredients within traditional Chinese medicine (TCM) holds significant academic and practical implications that extend beyond cultural boundaries [1,2]. TCM, an ancient medical system founded on millennia of empirical knowledge, comprises a vast array of botanical extracts, minerals, and natural substances, each possessing therapeutic potential for various ailments [3]. Nevertheless, the complex interactions among multiple compounds within TCM formulations create substantial challenges in identifying the specific active ingredients responsible for therapeutic effects.

The importance of screening for active ingredients in TCM encompasses multiple critical aspects. Primarily, it provides scientific validation for TCM therapeutic claims [4,5]. Through systematic isolation and characterization of compounds that produce specific pharmacological responses, researchers establish empirical evidence supporting TCM integration into modern medical practices and global healthcare systems. This research is essential for advancing international recognition and acceptance of TCM within the medical community, bridging traditional and modern medicine. Additionally, active ingredient screening enhances the understanding of TCM's pharmacological mechanisms [6,7]. Elucidating the molecular targets and signaling pathways affected by these compounds provides crucial insights into their modes of action and influenced biological processes. This knowledge advances understanding of disease pathogenesis and facilitates the development of targeted therapeutic strategies aligned with individual patient requirements, supporting the advancement of precision medicine. Moreover, TCM active ingredient exploration presents significant opportunities for drug discovery and innovation. TCM compounds, being natural derivatives, often possess unique chemical structures and biological activities that serve as valuable lead compounds for novel therapeutic agent development. This resource provides an alternative to conventional drug discovery methods, which primarily utilize synthetic chemistry and high-throughput screening approaches. TCM's biodiversity offers potential therapeutic solutions for various diseases, including those lacking effective current treatments. The identification of active ingredients in TCM represents a crucial endeavor bridging traditional and modern medicine [8]. This research validates TCM's therapeutic efficacy while advancing pharmacological science, drug discovery, and personalized medicine. Continued investment in research aimed at identifying TCM's active components shows considerable promise for enhancing global health outcomes and expanding medical knowledge [9].

Achieving these objectives with enhanced scientific rigor requires implementing a comprehensive, interdisciplinary approach. This framework incorporates advanced analytical methodologies, thorough pharmacological evaluations, and sophisticated bioinformatic analyses, as illustrated in Fig. 1. Recent advances in spectrum-effect correlation analysis, high-resolution affinity chromatography, and related techniques have enhanced the efficiency of screening and isolating active ingredients from TCM. These methodologies enable researchers to analyze complex TCM

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https://doi.org/10.1016/S1875-5364(25)60982-4

formulations' composition, identify bioactive compounds, and understand their pharmacological mechanisms. By determining these compounds' modes of action and therapeutic targets, researchers gain comprehensive insights into TCM's holistic approach and effectiveness. The systematic screening of active ingredients in TCM represents a significant advance in understanding this enduring medical system and utilizing its therapeutic potential. Through rigorous investigation and validation of these ingredients' efficacy, this research contributes to developing innovative therapeutic agents with potential implications for modern medicine.

2. Screening of active ingredients in TCM based on spectrum-effect correlation

In 2002, Li et al. introduced an innovative research methodology correlating the efficacy of TCM with spectral efficiency, establishing connections between changes in chemical components in fingerprint or characteristic spectra and pharmacological effects [10]. The spectrum-effect relationship concept adopts a holistic approach, where fingerprint spectra emphasize the complete component spectrum, encompassing comprehensive quality information. This research aims to obtain activity data for all fingerprint peaks or peak groups through both offline and online activity detection methods. Following the acquisition of activity data, researchers construct an active fingerprint spectrum corresponding to the chemical component fingerprint peaks in TCM [11–15]. Statistical analysis or computational processing then combines various chemical and biological fingerprint information to generate a multi-information graph model that illustrates the material foundation underlying TCM efficacy [16, 17].

Zheng et al. performed extensive chemical analyses on different proportions of Duijinsan extracts using high-performance liquid chromatography-mass spectrometry (HPLC-MS) [18]. Through the integration of spectral-effect relationship analysis and molecular docking, they identified 5 predicted active ingredients. Liu et al. examined the bioactive components of Fritillariae Bulbus (FB) in treating non-small cell lung cancer (NSCLC), utilizing spectral-effect relationship analysis and proteomics techniques [19]. Through partial least squares regression (PLSR), they identified six potential active ingredients, providing significant insights into FB's anti-NSCLC activity mechanisms and facilitating novel therapeutic development. Li et al. analyzed the spectrum-effect relationship of the Renshen-Fuzi herbal pair to identify the effective combination for anti-heart failure effects [16]. They discovered a combination of nine ginsenosides and three aconite alkaloids that demonstrated comparable efficacy to the Renshen-Fuzi herbal pair. Huang et al. developed and validated a fingerprint spectrum method using UPLC-quadrupole (Q)/time-of-flight (TOF)-MS, identifying 70 common peaks in 10 batches of Qi Yu San Long Decoction (QYSLD) [20]. Qiao et al. screened potential active ingredients in QYSLD through spectrum-effect relationships using grey relational analysis (GRA), PLSR, and back propagation neural network analysis (NNA) [21]. Deng et al. established a novel strategy for identifying pharmacodynamic states of active ingredients in multi-directional TCM based on multi-indicator spectral efficiency grayscale [22]. They evaluated pharmacological effects by measuring anti-bacterial, anti-inflammatory, and anti-coagulant activities of 14 *Astragalus* batches. GRA revealed key pharmaco-

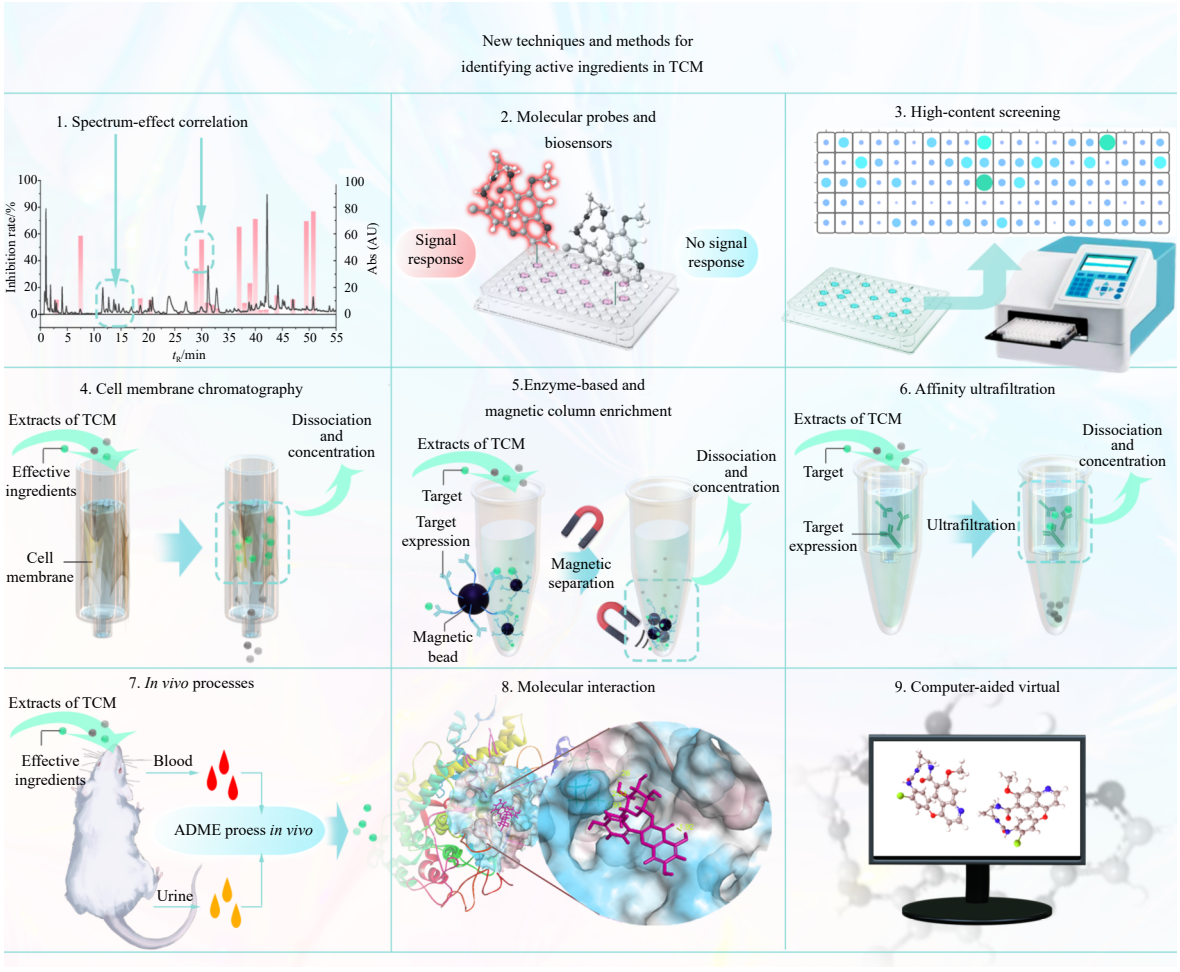

Fig. 1 Research progress in new techniques and methods for identifying active ingredients in TCM.

dynamic components in *Astragalus* with anti-bacterial, anti-inflammatory, and anti-coagulant properties.

Although spectrum-effect relationship research in TCM has been established for many years, established research methodologies remain in development. Several aspects of current research warrant further investigation. Critical challenges in spectrum-effect correlation studies include establishing characteristic peak information in chemical and biological fingerprint spectra, and linking chemical component characteristics from fingerprint spectra with biological effect-related information [23–25]. Consequently, this paper summarizes its correlation methods (Table 1).

Table 1 The summary of spectrum-effect correlation analysis methods.

Analysis method	Definition	Advantages	Disadvantages	Ref.
CA	A statistical analysis method that studies the correlation between random variables.	Measures the closeness, size, and direction of correlation.	Cannot explain the synergistic effect between components and pharmacological effects.	26
MLR	Establishes a dependency relationship between variables based on observed data, analyzes the inherent patterns of the data.	Reflects the combined effects of various chromatographic peaks on pharmacological indicators.	Multicollinearity's influence is unavoidable in some analyses.	27
NNA	A processing method developed from neuropsychology and cognitive science research, utilizing mathematical methods with high parallel computing ability, self-learning ability, and fault tolerance.	Strong nonlinear mapping ability, without the need to establish mathematical models.	A highly complex and intricate operation.	28
GRA	Judges the closeness of a connection based on the similarity of the geometric shapes of sequence curves.	Requires less data, has low demands, and is based on simple principles.	Difficult to quantify each peak's full pharmacological impact.	21, 26–28
PCA	Analyzes a few mutually independent principal components formed through the linear transformation of multiple interrelated variables.	Reduces analysis variables to clarify the main influencing factors in each group of variables.	Unable to quantify the correlation strength or establish a mathematical model between the two sets of analytical variables.	29
PLSR	Finds the best functional fit for a set of data by minimizing the sum of squared errors.	Small calculation volume, high prediction accuracy, no need to eliminate samples, easy to qualitatively interpret.	Correlation and model for all variables, including inactive, are unspecified.	28, 30, 31
CCA	Finds linear combinations of variables in two sets and uses the correlation between these composite variables to reflect the overall correlation between the two sets of indicators.	Studies focus on representative variable pairs with strong correlations, abstracting the intricate interdependencies between the two variable sets.	Unable to account for the correlations among variables within the same variable group.	32

CA: Correlation analysis; MLR: Multiple linear regression; NNA: Neural network analysis; GRA: Grey relational analysis; PCA: Principal component analysis; PLSR: Partial least squares regression; CCA: Canonical correlation analysis.

3. Screening of active ingredients in TCM based on molecular probes and biosensors

In the mid-18th century, Stokes pioneered the concept of "fluorescence" and elucidated the luminescent mechanism of quinine, the first well-defined small-molecule fluorophore [33]. In 1856, Perkin synthesized the fluorescent dye aniline purple, marking the beginning of artificial fluorescent dye synthesis [34]. In 1962, Shimomure et al. isolated green fluorescent protein (GFP) from the jellyfish genus Aequorea victoria [35]. GFP, one of the earliest fluorescent probes, has been widely applied in biomedical research. Subsequently, fluorescent probe development progressed from GFP to organic small-molecule fluorescent probes, with the initial probe emerging in the 1980s. Researchers have defined fluorescent probes as molecular devices that selectively interact with guest molecules through chemical or physical interactions, producing fluorescent signals for quantitative and qualitative detection of target molecules. As fluorescent probe detection technology advances, numerous function-specific fluorescent probes for discovering active ingredients have emerged (Fig. 2). During the past five decades, fluorescent probes have demonstrated utility in biological cell imaging, medical pharmacology, environmental analysis, and food science [36–42].

Zhang et al. developed an on-off molecular fluorescent probe (NBD-P) to visualize and monitor GSH fluctuations *in vitro* and *in vivo* [43]. NBD-P was applied to screen active ingredients in *Tripterygium wilfordii*, a promising anti-cancer TCM. Liang et al. designed a magnetic nano-fluorescent probe for sensitive detection of singlet oxygen ($^1O_2$) [44]. The research team utilized the probe (NMF-RP) to screen five plants for potential natural photosensitizers. The investigation confirmed that the $^1O_2$ generated by the screened photosensitizers induced Pseudomonas aeruginosa death, demonstrating the probe's potential for screening active ingredients in TCM for photodynamic therapy applications. Zhai et al. designed an on-off fluorescent probe (NHPF) for dynamic monitoring of glucuronosyltransferase UDP glucuronosyltransferase family 1 member A1 (UGT1A1) and imaging of endogenous UGT1A1 in living cells [45]. Eight compounds exhibited high inhibitory activity, with licorice isoflavone D showing the strongest inhibition based on IC_{50} values. Tian et al. developed an enzyme-activated near-infrared fluorescent probe (DAND) for quantitative detection and real-time imaging of endogenous fatty acid amide hydrolase (FAAH) in various living cells [46]. The research group employed this probe to conduct visual high-throughput fluorescent imaging of 93 TCM, identifying pepper as the most potent inhibitor. Ning et al. developed a two-photon fluorescent probe (BN-1) for semi-quantitative detection of CYP3A (cytochrome P450 3A) and imaging of endogenous CYP3A [47]. Using this probe, six TCMs were screened for their MBI effects on CYP3A, with Eudiae Fructus demonstrating the most significant effect. Sixteen components were isolated from the HPLC of Wuzhuyu and identified as limonin, evodiamine, and rutaecarpine, exhibiting strong MBI effects on CYP3A.

Our research group has made substantial progress in screening TCM active ingredients using optical probes. Acute kidney injury (AKI) represents a clinical syndrome characterized by a rapid decline in renal function due to various factors. Renal injury typically involves redox imbalance and excessive oxidative stress. Zhang et al. developed a highly sensitive and specific photoacoustic (PA) imaging probe (AB-DiOH) targeting the key redox reaction (ClO^-/GSH) during AKI progression [48]. This probe enables real-time and non-invasive AKI monitoring and facilitates screening of renoprotective drugs. Through *in vitro* and *in vivo* experiments, astragalin, a novel candidate drug, has demonstrated effectiveness in reducing cellular cytotoxicity and enhancing renal function after injury (Fig. 3A). Hydroxyl radicals (•OH) represent critical free radicals in oxidative stress, directly participating in oxidative stress reactions and inducing cellular and tissue damage. Gao et al. designed an activatable fluorescent/PA probe (CDIA) for sensitive and selective imaging of •OH in AKI [49]. The

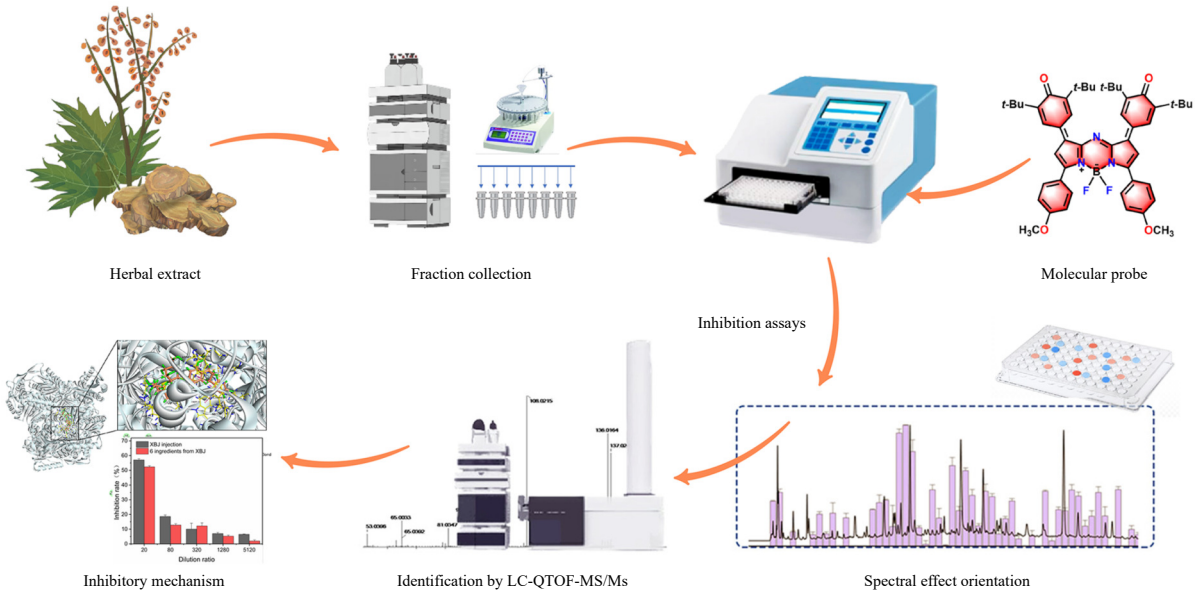

Fig. 2 Schematic diagram of the process for screening active ingredients in TCM based on optical probes.

probe demonstrates excellent screening capability for natural product •OH scavengers, identifying puerarin as a natural antioxidant and elucidating its chemical regulatory mechanism in AKI amelioration (Fig. 3B). Additionally, renal fibrosis represents a pathophysiological process involving progressive kidney function decline from health to injury, damage, and eventual dysfunction. During renal fibrosis, accumulation of renal tissue injury and uncontrolled fibrotic matrix deposition result in increased viscosity both intracellularly and extracellularly. Zhang et al. developed a viscosity-sensitive near-infrared fluorescence (NIRF) and PA imaging probe (BDP-KY) to detect abnormal viscosity changes during fibrosis and screened effective components of rhubarb, identifying potential anti-renal fibrosis compounds such as emodin-8-glucoside and chrysophanol-8-*O*-glucoside [50]. These compounds effectively reduce viscosity levels during renal fibrosis in a unilateral ureteral obstruction mouse model, potentially addressing the market gap for effective anti-renal fibrosis drugs (Fig. 3C). TCM compound prescriptions constitute the primary clinical application form in TCM, and identifying pharmacodynamic substances in TCM compound prescriptions remains a research challenge. Li et al. developed an aggregation-induced emission (AIE)-based fluorescent probe for monitoring thrombin activity [51]. This probe was integrated with UHPLC-FC and UHPLC-Q-TOF/MS to establish a spectrum-effect-oriented strategy for the separation, preparation, screening, and identification of thrombin inhibitors in Xuebijing injection (XBJ). Using this sensor, six compounds with high anti-coagulant activity (gallic acid, chlorogenic acid, oxypaeoniflorin, caffeic acid, senkyunolide I, and salvianolic acid B) were successfully screened from XBJ, providing a novel approach for identifying pharmacodynamic substances in TCM compound prescriptions (Fig. 3D). Han et al. constructed a simple, economical, rapid, and selective fluorescent sensor (TPE-S-TLG sensor) based on an AIE probe (TPE-Ph-In) and the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Mpro substrate (S-TLG), successfully screening for six active ingredients (protocatechuic aldehyde, chlorogenic acid, hydroxysafflor yellow A, caffeic acid, isoquercitrin, and pentapolysaccharide alose) in XBJ, exhibiting up to 90% inhibition of SARS-CoV-2 Mpro in XBJ [52]. This technique demonstrates broad applicability for detecting disease-related proteases and screening active ingredients in XBJ (Fig. 3E).

Despite advancements, fluorescent probe screening faces several challenges. First, the effectiveness of reactive fluorescent probes may be compromised by interference from other substances. This necessitates immediate efforts to improve selectivity and achieve specific binding between probes and their intended targets [53–56]. Second, the design and preparation of fluorescent probes require complex theoretical research and extensive validation experiments, often incorporating interdisciplinary knowledge [57–59]. Obtaining suitable probes typically requires testing multiple candidates, making the process efficient yet demanding substantial preliminary work. Finally, beyond studies where fluorescent probes are self-prepared under controlled experimental conditions, current research exploring these probes in high-throughput screening techniques depends on screening from previously successful probe preparations [60–63].

4. Screening of active ingredients in TCM based on high-content screening (HCS)

The HCS system employs automated cellular imaging analysis to examine the status and modifications of target cells in multiwell plates, preserving cellular structure and function at the single-cell level, overcoming the limitations of traditional high-throughput imaging's dependence on single endpoint measurements. HCS can simultaneously generate statistically significant and reliable data regarding the spatial/temporal distribution, expression intensity of intracellular target proteins, cell and organelle morphology, complex phenotypes, and the classification of multiple cell subpopulations [64–66]. Chemical constituents of TCM represent an essential source for identifying active lead compounds, and high-content technology provides an effective approach for comprehensive screening of active ingredients [67, 68]. Drug screening can be performed based on changes in overall cellular phenotype, including cell and organelle morphology, metabolism, adhesion and migration, cell cycle regulation, and apoptosis. Scientists commonly use this approach to develop *in vitro* models of relevant diseases, fluorescently label key phenotypes, and subsequently evaluate drug efficacy and screen for active ingredients using HCS technology. Moreover, target-based screening involves initial target identification, fluorescent labeling for visualization, and subsequent large-scale screening [69–71].

4.1. Application at the cellular level

Yan et al. aimed to identify novel inhibitors of the mTOR sig-

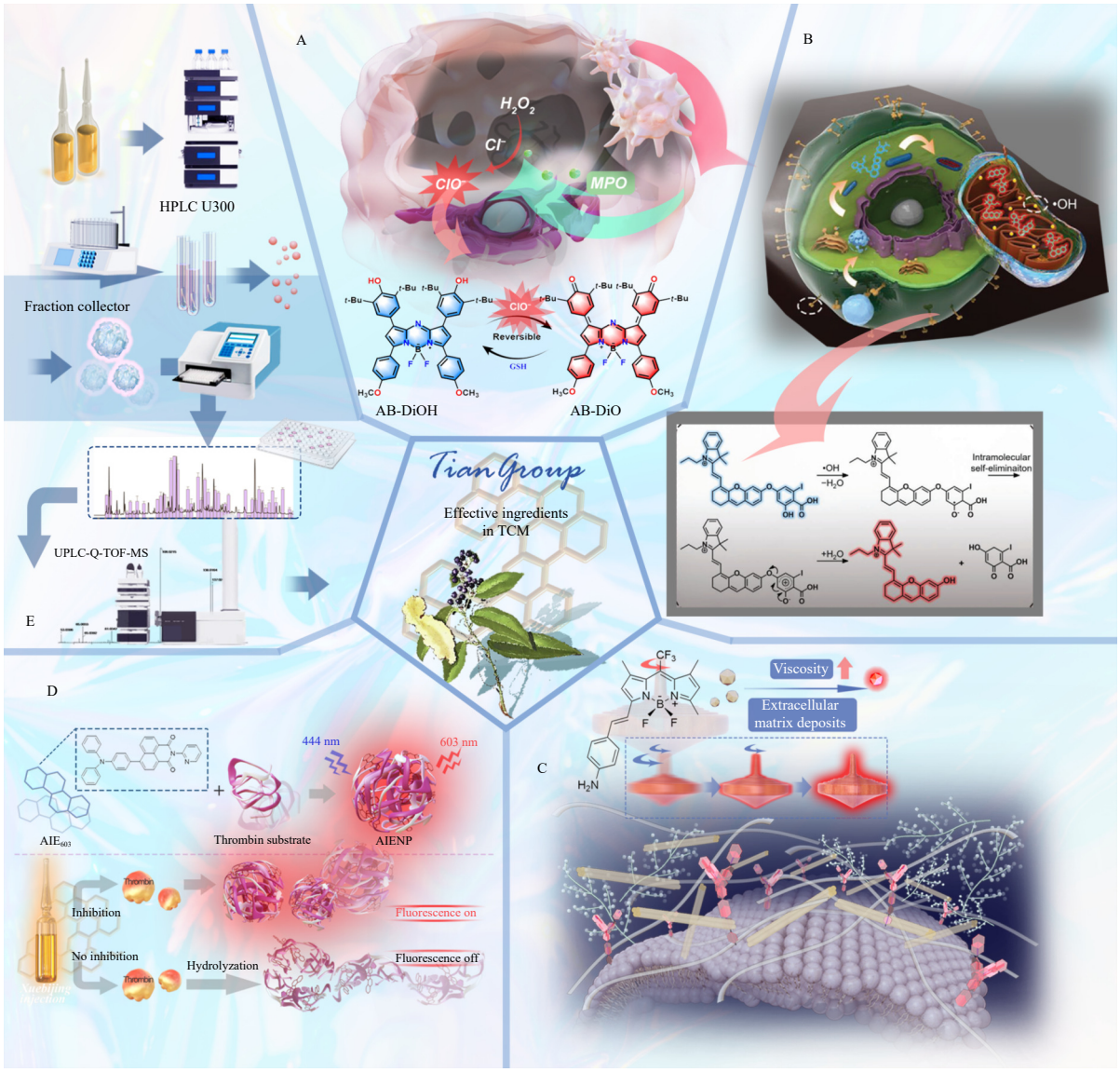

Fig. 3 Screening of active ingredients for renoprotection effects based on (A) ClO^-/GSH, (B) ·OH, and (C) Viscosity. The discovery of components in XBJ exhibiting inhibitory effects against (D) thrombin and (E) the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Mpro.

naling pathway from natural drugs [72]. They performed HCS of 1100 compounds from a natural compound library, applying them to MeF cells for 5 h. Compounds that increased the eIF4e [nuc:cyto] ratio by 1.5-fold were considered positive drugs, leading to the identification of nine compounds. Further evaluation of the candidate compounds in cancer cells demonstrated strong activity in inducing nuclear translocation of eIF4e for both compounds. Liu et al. performed gradient elution of Tongmaiyangxinwan extract, yielding 22 component systems [73]. Using HCS, they automatically collected and analyzed dual fluorescently labeled images, ultimately identifying glycyrrhizic acid, glycyrrhizin A, and glycyrrhizic acid A as active ingredients that inhibit endothelial-to-mesenchymal transition (EMT) in human proximal renal tubular epithelial (HK-2) cells, based on mass spectrometry analysis of common compounds among the components.

4.2. Application at the model organism level

Grissenberger S et al. explored the potential of zebrafish xenografts in high-throughput drug screening to discover novel combination therapies for Ewing sarcoma [74]. Through HCS of the xenografts in zebrafish larvae, combined with automated analysis of tumor size, they screened both single drugs and compound combinations. Their research identified three effective drug combinations targeting Ewing sarcoma cells: irinotecan in combination with myeloid cell leukemia 1 (MCL-1) or B-cell lymphoma-extra large (BCL-XL) inhibitors, particularly the dual inhibition of the anti-apoptotic proteins MCL-1 and BCL-XL, which showed efficacy in eliminating tumor cells within the zebrafish xenografts. Sturtzel et al. developed a workflow for drug screening in zebrafish xenografts using HCS [75]. They established an embedded method for HCS of 96-well format xenografts over several consecutive days. It offers an automated imaging and analysis strategy for zebrafish xenografts, including automated tumor cell detection and tumor size analysis over time. This efficient and cost-effective assay enables the quantification of anti-tumor efficacy of small compounds in large cohorts of a vertebrate model system *in vivo*.

HCS generates substantial volumes of data, making efficient and secure data storage and management essential. Researchers require comprehensive data processing and analysis capabilities to effectively utilize this information. Additionally, the automated analysis of extensive image datasets faces significant challenges, requiring improvements in related software and integration of artificial intelligence technologies to enhance image data processing. For solid organoid samples, current high-content sys-

tems demonstrate insufficient laser penetration efficiency and imaging resolution [76]. While reconstructed 3D images enable the extraction of parameters such as volume and area, they are restricted in achieving high-quality segmentation of cells within the spheroid's core, and precise protein localization remains challenging. These aspects are expected to be primary focus areas for optimization and enhancement in future HCS system development [77, 78].

5. Screening of active ingredients in TCM based on cell membrane chromatography (CMC)

CMC, developed by Professor Langchong He in 1996, integrates HPLC, cell biology, and receptor pharmacology [79]. Utilizing the specific affinity between drugs and membrane receptors, CMC converts the *in vivo* drug action process into a chromatographic process, enabling bionic studies of drug interactions *in vitro*. This method combines biomembrane and chromatography techniques, providing dual functions of receptor affinity (recognition) and chromatographic separation. CMC simulates the dynamic process of drug-membrane receptor interactions and is appropriate for screening active ingredients from complex systems [80].

Ding et al. modified silica gel with novel 3-aminopropyltriethoxysilane (APTES). The reaction between aldehyde groups on the silica gel and amino groups on the cell membrane formed a covalent bond, stabilizing the cell membrane on the silica gel surface [81]. This approach established a cancer stem CMC (CSCMC) column for screening anti-tumor active ingredients in *Salvia miltiorrhiza*. Tanshinone IIA, cryptotanshinone, and dihydrotanshinone I demonstrated retention on this model and showed efficacy against HepG2 tumor stem cells in cell proliferation and apoptosis experiments. Chai et al. developed a comprehensive 2D natural killer (NK)-92MI/CMC/HPLC-TOFMS system to screen for active ingredients in Astragalus that enhance NK cell activity and cytotoxicity as potential NK cell activators [82]. They synthesized 3-mercaptopropyltrimethoxysilane-modified silica gel to prepare NK-92MI CMC columns and identified isoastragaloside I and astragaloside IV, which enhanced the cytotoxicity of NK-92MI cells, representing immune activators of Astragalus. Gu et al. developed an efficient *in-situ* synthesis of membrane proteins (MPs) in the stationary phase and their unidirectional insertion into liposomes through a novel *in situ* MP affinity chromatography (iSMAC) method [83]. This method enabled screening of platelet-derived growth factor receptor beta (PDGFRβ) inhibitors from *Salvia miltiorrhiza* and *Schisandra chinensis*, identifying salvianolic acid B and gomisin D as effective PDGFRβ inhibitors. Cao et al. established an offline two-dimensional system combining cardiomyocyte membrane CMC-TOF/MS and HPLC-TOF/MS to analyze parent compounds and metabolites in rat urine samples after aconite root administration [84]. Lin et al. screened allergen-like components in Shenmai Injection using a multi-target CMC method (Fig. 4A) involving Mas-related G protein-coupled receptor (GPCR) X2, FcεRI, and H1 receptors, combined with an online LC-MS system [85]. Ginsenoside R_{b1}, R_{b2}, R_c, R_d, and 20(*S*)-ginsenoside R_{g3} were identified as allergen-like components (Fig. 4B). Wang et al. developed a novel comprehensive 2D APTES-modified MCF7-cell CMC/capcell-C18 column/TOF-MS system to screen for potential active ingredients in the Corydalis-Angelica sinensis drug pair [86]. This method successfully identified oxycorydaline (a *Corydalis* alkaloid) and isopimpinellin.

However, CMC presents limitations, including its inability to fully simulate complex *in vivo* environments, challenges in preparing biological cell membranes with short lifespans and low reproducibility, silica gel adsorption interference, and the limited quantity of active ingredients specifically retained by the CMC column [87].

6. Screening of active ingredients in TCM using enzyme-based and magnetic column enrichment

Enzyme immobilization technology comprises methods that physically or chemically constrain free enzymes within a defined spatial domain, enabling sustained catalysis of specific reactions and facilitating enzyme recovery and reuse. For screening active ingredients in TCM, magnetic bead enrichment employs magnetic particles as carriers. These particles enrich potential active ingredients by binding specific targets (proteins or enzymes) onto their surfaces, followed by incubation with TCM mixtures through receptor-ligand interactions. Chemical analysis techniques, such as LC-MS and NMR, then provide chemical information of the active ingredients, enabling structural inference [88]. The efficacy of screened compounds is subsequently verified through a series of pharmacological evaluations.

Magnetic beads, as an innovative carrier, demonstrate excellence in covalent enzyme immobilization. Magnetic nanoparticles, consisting of inorganic magnetic materials and various functional groups, exhibit high dispersibility in carrier liquids (polar or nonpolar solvents), forming stable colloidal solutions. Spherical in shape, magnetic beads range from 10 nm to 100 μm, featuring small particle sizes, uniform distribution, exceptional suspension stability, and large specific surface areas. Additionally, their surfaces can be conjugated with various active functional groups (e.g., carboxyl, amino, hydroxyl, aldehyde, and sulfhydryl groups), forming functionalized magnetic beads capable of binding multiple molecules [89–91].

Ye et al. effectively identified potential *β*-secretase inhibitors from Dendrobium officinale through direct covalent immobilization of *β*-secretase onto magnetic beads, coupled with UHPLC [92]. The results confirmed successful *β*-secretase immobilization and identified five potential inhibitor compounds (rutin, scoparone, naringenin, dendrobine, and erianin) *via* ligand fishing, establishing an efficient approach for screening secretase inhibitors from natural products. Tao et al. introduced multi-target affinity selection mass spectrometry (MT-ASMS) based on magnetic beads, integrating HPLC-MS for screening bioactive ingredients from plant medicines [93]. Tang et al. developed enzyme-immobilized magnetic beads (ACE-MB), providing a direct, cost-effective, and reliable screening method for ACE inhibitors using fluorescence detection, eliminating additional cutting procedures [94]. The enzymatic activity of successfully developed ACE-MB was verified through its Michaelis-Menten kinetic behavior towards hippuryl-histidyl-leucine using ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). This methodology was applied to screen ACE inhibitors within a library of 45 natural products (Fig. 5). The analysis identified and validated epiberberine and fangchinoline as compounds exhibiting significant ACE inhibitory activities. This research demonstrates the effectiveness of ACE immobilized magnetic beads screening, highlighting improvements in time efficiency and reagent usage. Li et al. fabricated immobilized enzymes with varying activity levels using nickel ion-functionalized magnetic mesoporous silica microspheres as support material [95]. Wang et al. assessed bioactive compounds from natural products using immobilized enzyme magnetic beads *via* HPLC-MS [96]. Xanthine oxidase, immobilized onto amino magnetic beads, was employed to screen extracts of fresh *Zingiber officinale* Roscoe, *Scutellaria baicalensis* Georgi, and *Pueraria lobata* Ohwi, leading to the identification of 12 potential xanthine oxidase ligands. Yi et al. utilized enzyme immobilization, molecular docking simulations, and HPLC-QTOF-MS to screen glutathione *S*-transferase inhibitors from natural products [97]. Magnetic mesoporous silica microspheres were synthesized with polydopamine layer surface modifications, enabling non-covalent interactions with glutathione *S*-transferase. Six potential inhibitors were identified from *Perilla frutescens*.

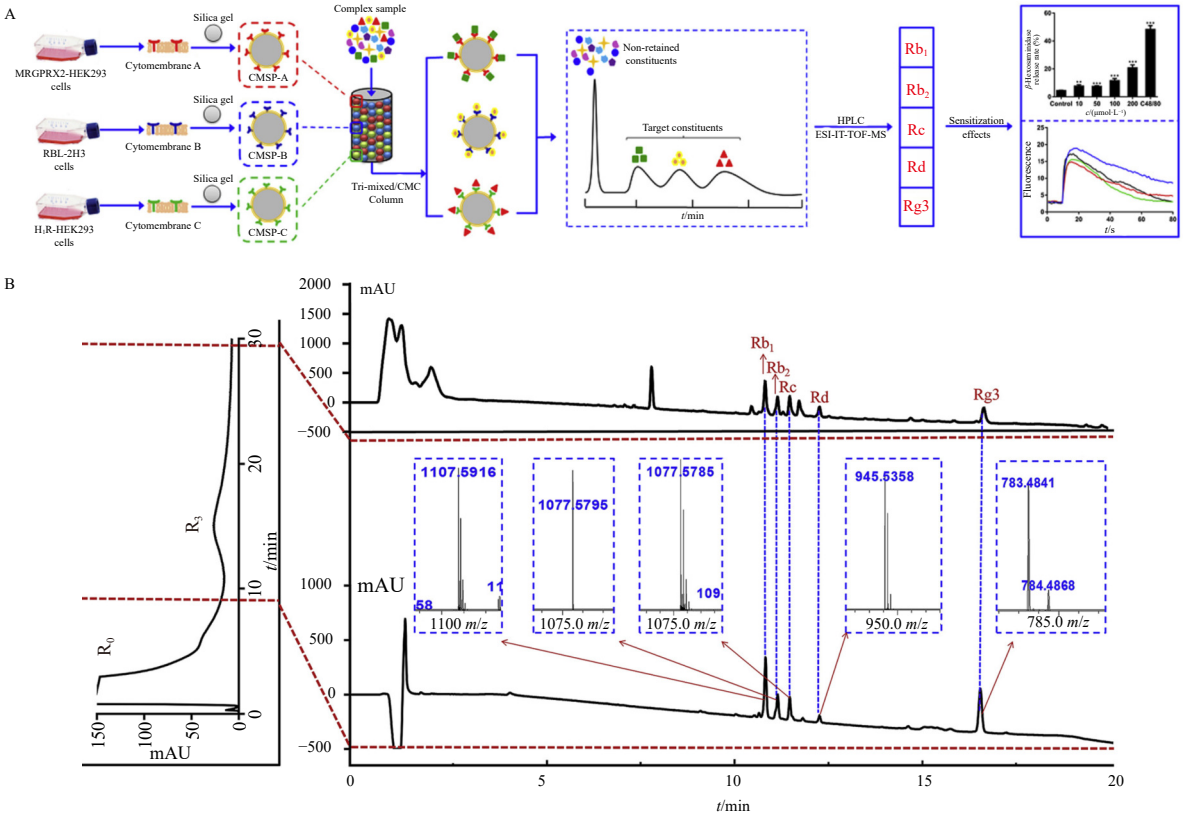

Fig. 4 (A) Schematic diagram of the process for screening active ingredients in TCM based on multi-targeted cell membrane chromatography. (B) Suitability of multi-targeted CMC online LC-MS system [85].

Enzymes demonstrate sensitivity to various environmental conditions, including temperature, pH, and ionic strength, frequently leading to inactivation or reduced catalytic efficiency. This sensitivity limits their application in complex environments. Furthermore, the production costs of certain enzymes remain high due to sophisticated bioprocessing requirements. The practical implementation of these enzymes demands specific reaction conditions and equipment, adding complexity to investment and engineering considerations.

7. Screening of active ingredients in TCM based on affinity ultrafiltration

Affinity ultrafiltration employs ultrafiltration membranes to isolate ligand compound molecules that specifically interact with biological macromolecules, including proteins and enzymes exceeding 10 kDa [98]. During incubation of the screening system with a target protein, small molecules with affinity activity bind specifically to the protein's active sites, forming receptor-ligand complexes, while non-binding compounds remain free. The ultrafiltration membrane's selective permeability allows unbound compounds to be eluted through a buffer solution. Appropriate dissociating agents facilitate protein denaturation, releasing the small molecule ligands (Fig. 6). High-speed centrifugation and LC-MS enable rapid analysis and identification of active small molecules. This technique aims to screen components that specifically bind to a given target, identifying ligands with distinct pharmacological activity and clear targeting mechanisms. The method proves particularly valuable for investigating synergistic enhancement properties of multi-components in TCM, where all components are simultaneously incubated with the receptor to simulate conditions of TCM's multi-component system effects. This approach enables high-throughput and efficient screening of active TCM ingredients [99–105].

Yang et al. investigated the active ingredients of Danggui Shaoyao San (DSS), a TCM formula comprising six herbs, for their effects on sodium retention in nephrotic syndrome [106]. The researchers employed BAU-UPLC-Q/TOF-MS to efficiently screen and analyze urokinase-type plasminogen activator (uPA)/plasmin-affinity compounds in DSS extracts. 1,2,3,4,6-*O*-Pentagalloylglucose demonstrated significant inhibitory activity against both uPA and plasmin. Zhang et al. employed affinity mass spectrometry to identify active ligands (5-HT_{2c} receptor) of GPCR in herbal extracts. Their research revealed that aporphine 1857 demonstrates strong selectivity for activating 5-HT_{2c} without activating 5-HT_{2a} or 5-HT_{2b} receptors [107]. Zhuang et al. utilized affinity ultrafiltration combined with LC-MS to screen bioactive components from the bark of *Millettia speciosa* Champ. using superoxide dismutase and xanthine oxidase as affinity probes [108].

Affinity ultrafiltration, as an emerging high-throughput screening technique, facilitates the screening of natural active ingredients from complex systems while maintaining native protein-ligand interactions in solution, offering an effective method for rapid identification of active ingredients in TCM [109]. However, this approach presents certain limitations, including false positive results from small molecules binding to non-functional target sites or non-specific binding to ultrafiltration membranes, substantial product losses during washing, limited mechanical strength of hydrophilic carriers, complex flow conditions, and inability to eliminate specific dissolved substances [110–115].

8. Screening of active ingredients in TCM using *in vivo*/*in situ*/*in vitro* processes

The absorption, distribution, metabolism, and excretion (ADME) process in TCM involves the movement of medicinal substances through these essential phases following administration, indicating their exposure *in vivo* and the corresponding physiolo-

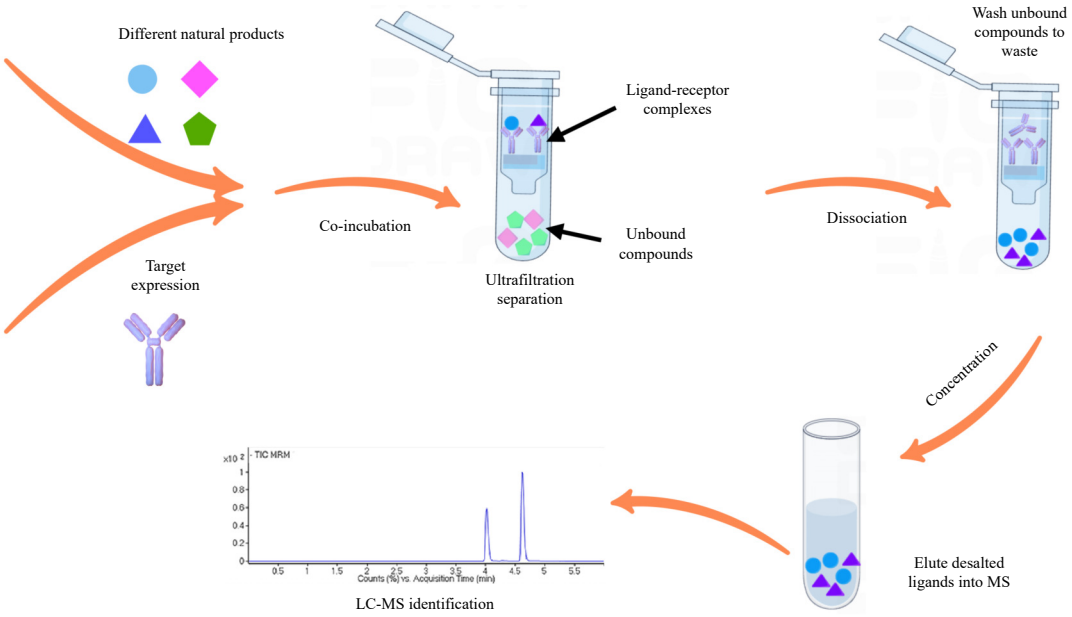

Fig. 6 Schematic diagram of the process for screening active ingredients in TCM based on affinity ultrafiltration.

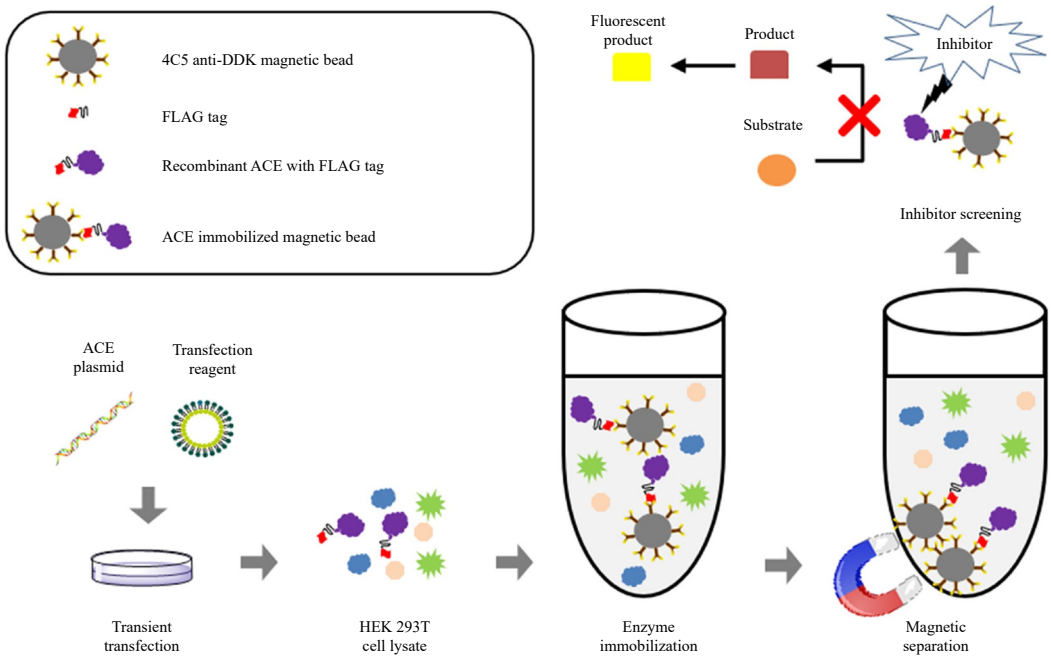

Fig. 5 Schematic diagram of the process used to screen active ingredients in TCM based on enzyme immobilization on magnetic beads [94].

gical response [116–118]. Oral administration represents the primary route in TCM, with absorption functioning as the critical initial step for drugs to enter the circulatory system and achieve therapeutic effects. Since the pharmacological effects or toxicity of most oral drugs depend exclusively on their absorbed components, intestinal absorption plays a crucial role in screening potential active ingredients and understanding drug mechanisms [119–121] (Table 2).

8.1. In vivo

Liu et al. conducted research utilizing a validated UFLC-MS/MS method to investigate the pharmacokinetics and oral bioavailability of three saikogenins (SGs) in rats [122]. The investigation developed a rapid UFLC-MS/MS method for the simultaneous quantification of four SGs (SGF, SGA, SGD, and SGG) in rat plasma. The validated method was effectively applied in pharmacokinetic studies of three SGs (SGF, SGD, and SGG) administered both orally and intravenously in rats. The absolute bioavailabilities of simultaneously quantifying four SGs (SGF) and SGD were determined to be 0.71% and 0.66%, respectively. Zhang et al. employed MS-based omics data processing methods to investigate chemical composition changes, blood absorption components, and brain entry components of Shuangxia Decoction, while examining its anti-insomnia mechanism through molecular docking technology [123]. Their analysis identified 49 chemical components and revealed 51 novel components from co-decoction. The research demonstrated that 7404 compounds from Shuangxia Decoction entered the bloodstream. The study rapidly identified 40 known compounds, while 15 novel compounds from co-decoc-

Table 2 Advantages and disadvantages of *in vivo*, *in situ*, and *in vitro* processes.

Research method	Specific technique	Advantages	Disadvantages	Ref.
In vivo	Blood and urine samples from whole animals at different time points	This reflects the comprehensive and realistic absorption of drugs, taking into account various factors encompassing physicochemical properties, physiological considerations, and dosage forms.	Extensive experimental durations, intricate operational procedures, and substantial individual variations pose challenges in accurately elucidating intestinal drug absorption mechanisms.	123
In situ	Intestinal perfusion, Intestinal loop, vascular cannulation, measuring nitrogen	Offers a more precise depiction of the genuine absorption of drugs within the intestine, facilitating the regulation of experimental conditions for enhanced accuracy.	Complex experimental operations and high costs pose challenges, while animal model limitations and anesthesia status introduce further complexities in ensuring valid results.	129, 133
In vitro	Everted gut sacs, using chamber, membrane vesicle, cell culture (Caco-2 cell model)	The streamlined operation and short duration facilitate high-throughput drug screening in early stages.	Significant tissue activity variations hinder reflecting true intestinal drug absorption.	134

tion were also absorbed into the blood. UPLC-MS/MS confirmed the blood absorption of 10 compounds and brain entry of 9 compounds. He et al. introduced the use of UPLC-Q/TOF and nuclear magnetic resonance spectroscopy to examine the *in vivo* metabolic profile of 20*S*-protopanaxatriol after oral administration [124]. This research provided the first evidence that the phase II metabolic pathway includes glucuronidation and cysteine conjugation. Huang et al. examined the effect of astragali on the *in vivo* disposition of doxorubicin (DOX) [125]. A network-based approach analyzed interactions between DOX metabolism and transport enzymes and the targets within the human protein-protein interaction (PPI) network of nine representative astragali components. Network analysis indicated that five of these nine astragali components were not proximate to DOX disposition-related modules. These findings suggest that astragali may reduce DOX-induced toxicity by modulating drug targets, rather than directly affecting drug disposition. LC-MS/MS was used to measure DOX concentration in rat plasma and six tissues: heart, liver, lung, kidney, spleen, and skeletal muscle. Wang et al. developed sustained-release microbeads containing four primary active ingredients from Danshen (*Salvia miltiorrhiza*) to modulate their *in vivo* processes [126]. Pharmacokinetic studies revealed that the four sustained-release microbeads within the compound Danshen capsule showed identical T_{max} values and similar extended mean residence time. Wang et al. investigated the complementary therapeutic effects of *Ginkgo biloba* extract (GBE) combined with statins for hyperlipidemia (HLP) treatment, examining both pharmacodynamic and pharmacokinetic aspects [127]. Their results demonstrate that combining statins and GBE produces superior improvements in lipid parameters, liver fat content reduction, and decreased abdominal fat cell size compared to statins alone. Additionally, lipidomics research has shown that GBE exhibits regulatory effects on abnormal lipid metabolism in HLP patients' livers. Yang et al. established a rapid, sensitive, and straightforward LC-MS method for the simultaneous quantification of drug concentrations in plasma samples from healthy and non-alcoholic fatty liver disease (NAFLD) rats [128]. This method was thoroughly validated for selectivity, linearity, and sensitivity, proving effective in monitoring pharmacokinetic profiles of Chlorogenic acid, kaempferol-7-*O*-*β*-D-glucoside, and ilexgenin A, three key bioactive ingredients of Ilex hainanensis extract. Oral administration of Ilex hainanensis extract significantly altered the pharmacokinetic behavior of chlorogenic acid, kaempferol-7-*O*-*β*-D-glucoside, and ilexgenin A in NAFLD rats.

8.2. In situ

Wang et al. utilized an *in vivo* unidirectional intestinal perfusion model to assess glycyrrhizin permeability at concentrations of 25, 50, and 75 μg·mL^{-1} across different rat intestinal segments, including the duodenum, colon, jejunum, and ileum [129]. Through analysis of solubility and permeability data, the study determined glycyrrhizin's biopharmaceutical classification. The findings demonstrated that glycyrrhizin displays minimal solubility across various pH levels and low lipophilicity. The permeability of glycyrrhizin across different rat intestinal segments exceeds 1.2 × 10^{-3} cm·min^{-1}, suggesting favorable absorption characteristics. The absorption of glycyrrhizin likely involves active transport or diffusion mechanisms. Based on these findings, glycyrrhizin is classified as a biopharmaceutical class II compound, characterized by low solubility and high permeability. Yang et al. conducted a detailed investigation using pharmacokinetic analysis, single intestinal perfusion, and a Caco-2 cell model to understand how rhein (RH) enhances rehmannioside D (RD) absorption [130]. Their research demonstrated that RH enhances RD absorption by modulating breast cancer resistance protein (BCRP) and multidrug resistance-associated protein 2 (MRP2) activity, thereby affecting intestinal epithelium permeability. The study also revealed competitive binding between RH and RD to BCRP and MRP2, with RH inhibiting these proteins' expression in the ileum, thus improving RD intestinal absorption.

8.3. In vitro

Yang et al. examined the alkaloid absorption characteristics of CR and evodiae fructus (EF) both individually and in combination, investigating their interaction in Zuojin Pill (6∶1 ratio) and Fanzuojin Pill (1∶6 ratio) [131]. The research analyzed bidirectional transport of extracts from these formulations and equivalent single-herb extracts across Caco-2 cell monolayers. Using LC-MS/MS, they identified 18 alkaloids in CR and EF. The Fanzuojin Pill combination significantly altered the bidirectional Papp values of CR alkaloids, decreasing the ER of five protoberberine alkaloids from CR while increasing the ER of two quinolone alkaloids from EF. Wang et al. implemented an *in vitro* intestinal absorption model with HPLC-PDA-MS to screen potential active ingredients in complex multi-component TCM systems [132]. They selected the Ussing chamber model, recognized for its effectiveness in rat oral administration studies, as the primary screening method for *Salvia miltiorrhiza*, *Astragalus propinquus*, *Plantago asiatica*, *Fallopia multiflora*, *Epimedium brevicornu*, Moutan Cortex, *Citrus reticulata* Blanco, and *Panax notoginseng*. The screening process identified 44 absorbed components as potential active ingredients.

9. Screening of active ingredients in TCM using molecular interaction techniques

Drug pharmacological effects primarily result from complex intermolecular interactions between drug molecules and their target receptors. TCM, with its multi-component, multi-target, and multi-pathway characteristics, presents substantial challenges in identifying pharmacodynamic substances and validating their target receptor interactions. This limitation represents a significant obstacle in modernizing TCM research. Recent advances in cross-disciplinary knowledge and technology integration have introduced various techniques for characterizing these intermolecular interactions, including surface plasmon reson-

ance (SPR), isothermal titration calorimetry (ITC), bio-layer interferometry (BLI), and microscale thermophoresis (MST) [135]. These methodologies provide valuable references and insights for exploring TCM mechanisms, improving quality standards, and developing novel applications, thereby supporting the advancement of TCM research and its applications [136] (Table 3).

9.1. Surface plasmon resonance (SPR)

SPR, a label-free optical detection technique, demonstrates exceptional capability in characterizing intermolecular interactions. This method enables real-time monitoring, rapid characterization, and quantitative analysis of dynamic molecular interaction processes. Through monitoring dynamic changes in SPR angle signals, researchers can determine the association constant (K_a) and dissociation constant (K_d) from detailed characterization of binding and dissociation reactions. The ratio of these constants yields the dissociation equilibrium constant (KD), which indicates the strength and specificity of intermolecular interactions between drugs and target molecules. Wang et al. investigated the TCM herb ginseng, focusing on the potential immunomodulatory programmed death-1 (PD-1) receptor [137]. Using SPR technology, they identified three ginsenoside compounds with PD-1 binding activity from ginseng. Subsequently, they employed cellular pharmacological methods to validate the PD-1 inhibitory activity of the representative compound, ginsenoside Rg1. This research provides valuable guidance for screening immune signaling pathway inhibitors from TCM using SPR technology. Furthermore, in addressing diseases such as Disease 2019 (COVID-19) and malignancies, researchers have successfully combined various techniques, including systems pharmacology data mining, computer molecular docking, and UHPLC-MS, with SPR technology [137]. This integrated approach has enhanced the efficiency of identifying bioactive compounds in TCM while effectively discovering specific drug targets. Such methodology enables precise characterization and verification of their interactions, providing substantial technical support for TCM applications in disease treatment.

9.2. Isothermal titration calorimetry (ITC)

ITC, a label-free, highly sensitive, and automated microcalorimeter, demonstrates exceptional capability in continuously and precisely monitoring heat variations during intermolecular interactions. This technology characterizes the calorimetric profiles of binding processes and provides comprehensive insights into affinity and thermodynamic parameters. These measurements prove essential for understanding the nature of intermolecular interactions and exploring their thermodynamic driving forces. Xu et al. utilized ITC technology to investigate the interaction between oxymatrine (OMT), the principal constituent of Sophora flavescens, and bovine serum albumin (BSA) [138]. Their research identified two distinct binding sites. Based on the thermodynamic differences between these sites, subsequent circular dichroism spectroscopy analysis confirmed that the interactions between OMT and BSA during these binding processes could modify the secondary structure of the target protein, resulting in changes to the relative abundance of different structural units. This study addresses a significant gap in current research regarding OMT's key targets, presenting novel perspectives for its application and development. Peng et al. applied ITC technology to investigate the molecular basis of the interaction between tripterine and heat shock protein 90 [139]. Their research elucidated the mechanism underlying tripterine's therapeutic efficacy in cancer and neurodegenerative diseases. These findings provide valuable experimental evidence illuminating the complex mechanisms of drug-target interactions between bioactive compounds in TCM and their respective targets, offering significant insights into their pharmacological actions.

9.3. Bio-layer interferometry (BLI)

Through continuous monitoring of light interference signals on the biosensor surface and their conversion into response signals, BLI accurately characterizes the interaction parameters during the binding process between two molecules. This methodology offers several advantages, including resistance to crude samples, label-free operation, rapid simplicity, and data reliability. Its robustness with crude samples makes it particularly suitable for investigating pharmacodynamic substances and target interaction mechanisms in complex crude samples, such as TCM. Guo et al. combined BLI technology with UHPLC-MS to dynamically monitor the binding and dissociation capabilities between the TCM compound Kai-Xin-San and biotin-labeled amyloid *β*-protein (Aβ) [140]. The subsequent analysis of the dissociated complex using liquid chromatography coupled with high-resolution mass spectrometry identified poricoic acid C, dehydrotumulosic acid, and tumulosic acid from the *Poria cocos* component of Kai-Xin-San as the primary Chinese medicine components showing strong affinity towards Aβ. Li et al. employed BLI technology to characterize the binding affinity between the *Salvia miltiorrhiza* Radix et Rhizoma-Gingseng Radix compound and its three constituent components: total phenolic acids, total saponins, and *Gingseng* polysaccharides [141]. They screened the target peptide from a human lung cancer cDNA library using phage display technology. Their research revealed that Gingseng polysaccharides and total phenolic acids from *Salvia miltiorrhiza* Radix et Rhizoma were the primary contributors to the binding of the compound to the target peptide. This provides more precise experimental evidence for TCM applications in modern targeted

Table 3 Advantages and disadvantages of molecular interaction methods.

Technique	Detection Principle	Information acquired	Advantages	Disadvantages	Ref.
SPR	Dynamic change of SPR angle	K_a, binding rate, dissociation rate	Features include high sensitivity, real-time analysis, no sample labeling, non-destructive testing, and accurate quantification.	The high cost, sensitivity to interfering factors such as sample composition and temperature, and difficulty in distinguishing non-specific adsorption are issues.	146–149
ITC	Thermal dynamics	K_a, binding enthalpy, ΔS, number of binding sites	High sensitivity, high precision, no sample labeling required, thermodynamic information obtained, compatibility with any buffer additive, small sample dosage, the ability to characterize weak binding, non-destructiveness, fast response time, and short experimental time.	The enzyme is sensitive to temperature, buffer, and pH levels.	150–152
BLI	Optical interference signal changes	K_a, binding rate, K_d	Simple to use, suitable for complex, viscous, coarse samples, the volume effect is minimized, requires no sample labeling, and offers fast detection speed.	Lower sensitivity, limited precision in determining kinetic parameters, limited capability to measure tightly bound ligands and fast binding rates, diffusion-limited measurements, and limited ability to measure fast dissociation rates.	153–155

lung cancer treatment research.

9.4. Microscale thermophoresis (MST)

MST is a technique that enables real-time monitoring of molecular directional migration in a temperature gradient environment, primarily for determining parameters such as the dissociation constant (K_d) for biomolecular binding. Its advantages include sample non-immobilization, rapid detection time, high sensitivity, and operational simplicity. In this method, fluorescently labeled receptors and target analytes are placed in a thin capillary tube. An infrared laser within the MST instrument generates a localized temperature gradient, inducing directional movement of the molecules within the tube. The fluorescence intensity of the labeled receptor varies within this gradient over time. The K_d value for the intermolecular interaction can be precisely determined by correlating a series of ligand concentrations with the standardized fluorescence intensity. Chen et al. utilized MST, along with Enzyme-Linked Immunosorbent Assay and Co-Immunoprecipitation techniques, to examine the interaction between the T cell activation inhibitor immunoglobulin V domain (VISTA) and immunoglobulin domain 8 (VSIG-8) [142]. Their research provided the first evidence of the interaction between VSIG-8 and VISTA and its subsequent inhibition of T cell function. Huang et al. subsequently employed MST technology, combined with multi-spectral and molecular docking analyses, to determine the K_d values of dihydromyricetin and myricetin in their interactions with bovine lactoferrin [143]. Their research demonstrated that bovine lactoferrin exhibits enhanced transport capacity for myricetin, attributed to its higher affinity compared to dihydromyricetin, resulting in improved absorption and bioavailability of myricetin *in vivo*. This technique substantially enhances the methodological toolkit for investigating interactions between TCM pharmacodynamic substances and their target molecules.

Given the unique characteristics of various analytical samples and specific research objectives, researchers may employ one or multiple molecular interaction techniques for cross-validation purposes. This approach enables a comprehensive investigation and validation of the complex interactions between TCM bioactive compounds and their respective target proteins. This methodology aims to develop a deeper understanding of TCM pharmacological mechanisms, thereby revealing its scientific foundations and promoting its advancement in modern drug discovery [144, 145].

10. Screening of active ingredients in TCM based on computer-aided virtual

Recent years have witnessed rapid advancement in techniques for screening active ingredients in TCM. These techniques, leveraging specific "receptor-ligand" interactions, enable rapid identification of TCM small-molecule active ingredients that bind to specific targets. These methods facilitate the identification of active ingredient structures [156-165]. These techniques are essential for understanding the material basis of TCM efficacy and developing novel TCM drugs, thus complementing traditional drug discovery methods. The evolution from traditional screening methods to diverse screening models, facilitated by advanced computer technology, has enabled the screening of active ingredients in complex TCM [166]. This development provides new candidate compounds for drug development and establishes novel research directions. Computer-aided virtual screening technology incorporates disciplines including chemistry, molecular biology, pharmacology, statistics, and computer science (Table 4). Through mathematical and computer modeling, this technology enables active ingredient screening in TCM and preliminary mechanism investigation. Virtual screening technologies are categorized into two main types: receptor-based macromolecular virtual screening, also termed structure-based virtual screening, which requires well-defined receptor biomacromolecules and includes molecular docking; and ligand-based small-molecule virtual screening, which achieves active ingredient screening through chemical component similarity analysis. The latter approach aims to establish correlations between component structural characteristics and biological activity, encompassing methods such as machine learning, pharmacophore modeling, and molecular similarity assessment [167]. The computer-based virtual screening workflow comprises several essential steps: database curation, methodology selection based on receptor structure information availability, implementation of either ligand-based or receptor-based screening, and biological experimental validation. Computer-based virtual screening has emerged as a valuable tool in TCM active ingredient screening.

Table 4 The advantages and disadvantages of computer-aided methods.

Modeling principles	Technique types	Classify	Advantages	Disadvantages	Ref.
Receptor-based virtual screening	Molecular docking	Rigid docking based on the "lock-and-key" principle, semi-flexible docking, and flexible docking based on the principle of "induced-fit theory".	Core technology for virtual screening, a mature theoretical framework, availability of various software, and the ability to simulate ligand-receptor binding states, resulting in more accurate outcomes.	It requires a well-defined receptor structure, involves relatively high computational cost, and takes longer calculation time.	169, 196
Ligand-based virtual screening	Machine learning	Supervised learning, semi-supervised learning, unsupervised learning, and reinforcement learning.	The system is capable of optimizing models through continuous learning, achieving high accuracy.	The system has a high dependence on training data, lacks interpretability of models, and is prone to overfitting.	197–207
	Molecular similarity methods	Structural similarity search and active fragment search.	The screening speed is extremely fast, with no strict requirements on target naming.	The scope and accuracy of results are limited, requiring further improvement.	208

10.1. Molecular docking and network pharmacology

Molecular docking technology, a fundamental technique in computer-aided drug design, serves two primary functions: examining the interactions between drug actives and targets, and enabling the discovery and optimization of lead compounds [168]. Also known as molecular docking virtual screening technology, it operates by screening chemical components from TCM or natural product databases against specific protein targets to identify compounds that demonstrate selective binding to target proteins. Through decades of development, molecular docking, initially utilized as a virtual screening technology in early drug research, has achieved substantial maturity and sophistication in its theoretical framework. Currently, due to its efficacy, rapidity, and accessibility, this technology has attracted considerable interest from TCM researchers and maintains a vital role in the field. The molecular docking framework consists of two primary phases: initially, determining potential binding modes between receptors and ligands through conformational search; subsequently, evaluating the generated conformations using various scoring functions, which are classified into four categories: force field-based, knowledge-based, experience-based, and machine learn-

ing-based. Selecting an appropriate scoring function is essential for determining the optimal conformational binding mode. As a valuable research tool, molecular docking should be implemented in TCM active ingredient investigation while respecting the distinct characteristics of traditional medicine. This implementation requires careful consideration of potential challenges at each experimental stage, including data preparation and processing before docking, parameter selection during docking, and model validation and assessment after docking [169]. Data quality, particularly regarding the three-dimensional structural data of TCM active ingredients and target proteins, is crucial for improving screening accuracy. The parameter configurations of molecular docking software must be optimized for specific conditions to generate more precise simulation results. Furthermore, virtual screening results require experimental validation to confirm their actual activity and effectiveness. Through meticulous control of molecular docking parameters, experimental errors can be minimized substantially, ensuring model validity and result reliability. Sun et al. investigated the novel immune checkpoint protein, VISTA, and identified a lead compound, A1, through molecular docking and MST measurements, demonstrating strong affinity binding to the VISTA protein [170]. Following comprehensive structural optimization analysis, the structure-activity relationship of this compound series was determined, leading to the optimization of compound A4, which demonstrated the highest VISTA protein affinity. Significantly, compound A4 exhibits strong antitumor activity, effectively inhibiting the binding affinity between VISTA and its ligand protein, resulting in tumor cell elimination through restored T-cell anti-tumor activity. The identification of this novel VISTA inhibitor using molecular docking techniques presents substantial opportunities for advancing inhibitor development and target function exploration.

Molecular docking frequently integrates with other methodologies to enhance the identification of matching drugs and their targets, thereby improving screening efficiency and accuracy. A notable integration involves its combination with network pharmacology, which incorporates mathematics and bioinformatics to understand the holistic and systematic properties of TCM in treating complex diseases. Network pharmacology initially identifies effective ingredient targets of TCM, and the relationship between ingredients and targets is clarified through an "active ingredient-target-pathway" network. Subsequently, molecular docking techniques refine the screening of active ingredients based on identified targets. Through network pharmacology analysis, Zhi et al. constructed a "drug-component-target" network for Jin-Si-Wei (JSW) in treating AD [171]. This network comprised seven drugs, 363 active ingredients, and 116 targets. The targets were mapped to corresponding GO and KEGG enrichment terms and validated experimentally. Molecular docking techniques identified five key targets within the network: ALB, APP, PTGS2, ACHE, and GABRA1, which are integral to neurotransmitter function. These targets demonstrate significant roles in AD and exhibit strong binding affinity with JSW's key active ingredients. Similarly, Fan et al. identified GSK3β as a crucial target for renal fibrosis in DN through a PPI network focusing on tripterygium glycosides (TP) [172]. Molecular docking with TP validated GSK3β as a target, revealing strong and stable interactions at its active site. The study concluded that TP regulates the GSK3β/Nrf2/HO-1 pathway, reducing renal fibrosis in diabetic nephropathy patients. Qi et al. utilized network pharmacology, molecular docking, molecular dynamics simulation, and SPR to investigate novel targets of sitagliptin in type 2 diabetes mellitus (T2DM) management [147]. Their analysis identified angiotensin-converting enzyme 2 (ACE2) as a novel target associated with dipeptidyl peptidase IV (DPP4). Semi-flexible molecular docking simulations validated sitagliptin's binding capacity to ACE2, while molecular dynamics simulations assessed the complex's stability. SPR analysis elaborated on the binding characteristics, providing evidence for ACE2 as a potential sitagliptin target in T2DM treatment. Xiao et al. developed an analytical strategy based on network pharmacology to understand sea buckthorn flavonoids (SF) in HLP management [173]. Their component-target-disease network identified 12 bioactive flavonoid compounds and 60 targets relevant to SF and HLP. Experimental validation demonstrated that SF extract and specific bioactive compounds affected lipid metabolism-related gene expression. Molecular docking simulations showed isorhamnetin's strong binding affinity with the PPAR-γ ligand-binding domain. Wang et al. analyzed Fufang Xueshuantong (FFXST) in IS treatment through network pharmacology [174] identifying STAT1, STAT3, and HIF1A as significant targets. Molecular docking confirmed associations between active ingredients and core targets. Zhou et al. analyzed Qingfei Dayuan granules using multiple databases and molecular docking, revealing potential activity against SARS-CoV-2 [175]. Wu et al. employed network pharmacology and molecular docking to predict therapeutic targets for anti-inflammatory extract (AF-ext) from Ainsliaea fragrans Champ in treating anti-primary dysmenorrhea (PD) [176] with subsequent target validation through activity screening and immunoblotting.

Molecular docking techniques, utilizing known compounds that are readily available for purchase or synthesis, offer distinct advantages, particularly in screening effective drug components through computer software integration [177–180]. This technology addresses the limitations of pharmacological experiments and has gained widespread application across various aspects of TCM, providing effective solutions for active ingredient screening. Nevertheless, certain challenges remain. First, there is insufficient research validating the accuracy of small molecule databases used in molecular docking. Second, the computational nature of these experiments presents challenges in precisely determining protein binding sites [180–184].

10.2. Machine learning

Machine learning fundamentally operates by extracting structural and physicochemical features from compounds. These features, combined with appropriate algorithms, enable the construction of models for compound screening. The field currently encompasses supervised, semi-supervised, unsupervised, and reinforcement learning methodologies [185]. Each learning approach utilizes specific algorithms: supervised learning employs decision trees, support vector machines (SVM), linear regression, and naive Bayes, while unsupervised learning primarily utilizes K-nearest neighbors and clustering algorithms [186–190]. Through continuous model refinement, machine learning achieves high accuracy in screening results. Deep learning, an emerging branch of machine learning, utilizes artificial neural network (ANN) algorithms to construct models through supervised, unsupervised, or hybrid learning approaches. It demonstrates particular effectiveness in processing large, complex activity datasets, substantially improving research accuracy [185]. These advantages position deep learning as a promising tool for screening TCM active ingredients. Qi et al. utilized bioinformatics techniques, including least absolute shrinkage and selection operator (LASSO) regression and SVM-RFE algorithms, to identify lymphangiogenesis-associated core genes in MI [191]. Their analysis predicted potential TCMs for MI treatment. The machine learning algorithms successfully identified four diagnostically significant core genes (*PFKFB3*, *IL1B*, *IL1RN*, *ADM*) and predicted therapeutic TCMs, including Ginseng and Astragalus, based on these targets. Similarly, Yang et al. investigated core genes and immune cell relationships in IS using bioinformatics and machine learning approaches. Through LASSO regression, random forest, and SVM methods, they identified five Hub genes: *ABCA1*, *FGL2*, *GPR18*, *MFGE8*, and *PROS1*. Their research identified several potential molecular drug

sources for IS treatment, including Rhei Radix et Rhizoma, Curcumae Longae Rhizoma, Curcumae Radix, Notogingseng Radix et Rhizoma, Salviae Miltiorrhizae Radix et Rhizoma, and Eudiae Fructus.

10.3. Molecular similarity assessment

Molecular similarity methods enable the screening of compounds with similar physicochemical properties and biological activities through precise structural feature matching [192]. These methods comprise two main categories: structural similarity search and active fragment search [193]. Both approaches follow a similar modeling process, beginning with activity-specific compound selection to create a comprehensive dataset. The process then involves selecting molecular structure parameters, such as molecular shape indices and electrotopological indices, for modeling purposes [194]. Following model completion, similarity parameters are calculated. Popular platforms implementing this technology include MolPrint 2D, ChemMappe, eSHAFTS, and Me2Explorer [195]. While molecular similarity methods provide rapid active ingredient screening, relying solely on similarity for biological activity prediction has limitations. Therefore, a comprehensive evaluation of compound structure, properties, and mechanisms of action remains essential during screening. Computer-aided virtual screening techniques effectively integrate various disciplines, reducing workload, experimental duration, inefficiencies, and resource waste associated with traditional toxicity testing methods. While virtual screening plays a crucial role in TCM active ingredient screening, it should complement rather than replace traditional research methods, serving to accelerate TCM active substance research progress.

In conclusion, computer-aided virtual screening technology represents an essential tool in identifying TCM active ingredients, fundamentally relying on data analysis and application. However, implementation challenges persist. First, data limitations exist, as insufficient TCM compositional information fails to meet rigorous screening requirements. Second, while computer-aided virtual screening serves as a crucial method for identifying TCM active ingredients, its results cannot supersede pharmacological research findings. Ultimately, pharmacological experimental results remain the definitive standard for evaluating TCM medicinal substance screening.

11. Outlook and trends in screening systems for natural products

In the academic domain of screening active ingredients in TCM, substantial progress has been achieved in recent years, driven by the emergence of advanced technologies and methodologies. These developments have enhanced both the efficiency and accuracy of the screening process while advancing the modernization and internationalization of TCM research [209–218]. Particularly noteworthy is the progress in implementing advanced drug target identification techniques, which enable swift and precise identification of bioactive compounds within TCM formulations. Moreover, contemporary analytical instruments such as HPLC-MS and GC-MS have been instrumental in accurately identifying and quantifying chemical constituents in TCM preparations [219–224]. This progress has substantially enhanced the chemical profiling of TCM, facilitating a more thorough understanding of its chemical complexity. Additionally, pharmacological experiments and biotechnological methods have yielded valuable insights into the biological activities and underlying mechanisms of TCM compounds. These investigations have markedly enhanced our understanding of TCM's therapeutic potential and supported the rational design and refinement of TCM formulations.

The field of TCM active compound screening demonstrates considerable promise for future innovations and breakthroughs. The incorporation of advanced technologies, including artificial intelligence, big data analytics, and genomics, is anticipated to facilitate more targeted and efficient screening of bioactive compounds. Furthermore, the implementation of innovative experimental models and platforms will enhance the capacity to evaluate TCM compounds' efficacy and safety. Interdisciplinary collaboration among TCM researchers and scientists from fields such as pharmacology, biotechnology, and computational science will accelerate advances in TCM active compound screening, potentially leading to the identification of novel therapeutic agents beneficial to human health.

12. Conclusion

In summary, the academic landscape of TCM research has experienced a fundamental transformation, driven by significant advances in the development of novel screening techniques and methodologies. These developments have focused on establishing precise correlations between TCM chemical constituents and their biological effects through techniques including spectrum-effect correlation analysis, affinity ultrafiltration, HCS imaging, and CMC. This precise mapping has expedited the identification of effective ingredients, while molecular interaction analysis has provided crucial insights into the mechanisms underlying TCM's therapeutic potential. Additionally, the integration of computer-aided and molecular probe technologies has optimized the screening process, enhancing efficiency and cost-effectiveness. Consequently, the field is well-positioned for future breakthrough discoveries, ready to advance into a new era of targeted and effective TCM-based therapies.

Funding

This work was supported by the National Natural Science Foundation of China (Nos. 22175078, 52373287, 82404846, and 22467002), the Natural Science Foundation of Jiangsu Province of China (No. BK20241597), and the Fundamental Research Funds for the Central Universities (No. 2632024TD05).

Declaration of competing interest

These authors have no conflict of interest to declare.

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