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A novel systems pharmacology platform to dissect action mechanisms of traditional Chinese medicines for bovine viral diarrhea disease

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ABSTRACT

Due to the large direct and indirect productivity losses in the livestock industry caused by bovine viral diarrhea (BVD) and the lack of effective pharmacological therapies, developing an efficient treatment is extremely urgent. Traditional Chinese medicines (TCMs) that simultaneously address multiple targets have been proven to be effective therapies for BVD. However, the potential molecular action mechanisms of TCMs have not yet been systematically explored. In this work, take the example of a herbal remedy Huangqin Zhizi (HQZZ) for BVD treatment in China, a systems pharmacology approach combining with the pharmacokinetics and pharmacodynamics evaluation was developed to screen out the active ingredients, predict the targets and analyze the networks and pathways. Results show that 212 active compounds were identified. Utilizing these lead compounds as probes, we predicted 122 BVD related-targets. And in vitro experiments were conducted to evaluate the reliability of some vital active compounds and targets. Network and pathway analysis displayed that HQZZ was effective in the treatment of BVD by inhibiting inflammation, enhancing immune responses in hosts toward virus infection. In summary, the analysis of the complete profile of the pharmacological activities, as well as the elucidation of targets, networks and pathways can further elucidate the underlying anti-inflammatory, antiviral and immune regulation mechanisms of HQZZ against BVD.

1. Introduction

Bovine viral diarrhea (BVD), caused by bovine viral diarrhea virus (BVDV) (Carman et al., 1998), is an acute, highly contagious disease usually along with the occurrence of fever, diarrhea, pneumonia, mucosal lesions, or even sudden death. The prevalence of BVDV infection is not only determined in calves but also in other ruminant species such as pigs and sheep, which poses a serious impact on livestock (Carman et al., 1998). Western medicine has studied BVD for many years since it was originally described in 1946 (Houe, 1995), however, people continue to face challenges in treating BVD. Although vaccination (modified-live or killed) programs can provide some protection from BVD and the development of persistently infected fetuses, it alone cannot control or eliminate BVDV (Brock, 2003). Antiviral therapy, such as the administration of ribavirin, is effective to control BVDV (Buckwold et al., 2003; Ouzounov et al., 2002), but this therapy yields significantly higher sustained virologic responses (approximately 40%) (Gutfreund and Bain, 2000). In recent years, a large number of therapies have been investigated in attempt to fight BVD, however, it's still difficult to achieve the most ideal treatment effect.

Traditional Chinese medicines (TCMs) are effective to relieve complicated diseases in a multi-target/multi-component manner, which makes them unique among all traditional medicines (Qiu, 2015). And TCMs have been applied to the livestock industry for at least 1000 years (Stogdale, 2008). For instance, a series of formulas like wei-cang-san, jian-pi-san, sheng-yang-yi-wei tang, have been used for the treatment of different types of chronic diarrhea in horses (Xie et al., 1997). Note that Huangqin Zhizi (HQZZ) formula, a widely used herbal formula in Chinese medicine for BVD treatment, is applied in

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this work. This formula is consisted of 10 different herbs, *Radix Scutellariae* (RS., Huang-qin), *Cortex Fraxini* (CF., Qin-pi), *Cape Jasmine* (CJ., Zhi-zi), *Rheum Officinale* (RO., Da-huang), *Cortex Moutan* (CM., Mu-dan-pi), *Raw Rehmanniae Radix* (RRR., Sheng-di-huang), *Terminalia Chebula Retz* (TCR., He-zi), *Pomegranate Rind* (PR., Shi-liu-pi), *Cortex Magnoliae Officinalis* (CMO., Hou-po) and *Fructus Aurantii* (FA., Zhi-qiao). Previous research has shown that the effective rate of HQZZ for cow diarrhea is 95% and cure rate is 75% in vivo (Wang et al., 2010). Meanwhile, HQZZ also plays a vital role in fighting against BVD by directly inhibiting BVDV in vitro (Yang et al., 2009). Although HQZZ has been proven to be dramatically efficient in curing BVD, the fundamental molecular action mechanisms are still not systematically explored. The bioactive compounds, the potential targets and the related pathways of HQZZ remain unknown. The advancement of analytical tools including systems biology (Kitano, 2002), network biology (Barabasi and Oltvai, 2004) and network pharmacology (Hopkins, 2007, 2008) potentially offer an attractive way to elucidate the intricate and holistic mechanisms of Chinese herbal formula in treating BVD.

Herein, combining with pharmacokinetic (the absorption, distribution, metabolism, excretion (ADME) properties of drugs) evaluation, as well as pathway and network analysis (Huang et al., 2013), systems pharmacology offers a platform for identifying multiple mechanisms of action of herbal veterinary medicines. In our previous work, we have constructed a systems-pharmacology-based method which is specially designed for drug discovery and therapeutic insight for herbal medicines (Li et al., 2014; Wang et al., 2013). Thus, in order to resolve the underlying action mechanisms of herbal medicines in the treatment of livestock diseases, we urgently need to introduce the method of systems pharmacology.

In this study, we employed a modified systems-pharmacology method to probe the anti-BVDV mechanisms of HQZZ. Firstly, we filtered active compounds from the constructed HQZZ molecular database by calculating pharmacokinetic properties and evaluating their drug-likeness. Then, the potential targets were predicted by our newly constructed cross-species drug–target interaction assessment model (CSDT). And the obtained targets were validated by Gene Ontology enrichment analysis and target-disease interactions analysis. Finally, the acquired pharmacological data were further integrated into compound-target and target–pathway network. The systems pharmacology approach framework for the present work is shown in Fig. 1.

2. Materials and methods

2.1. Molecular database construction

We manually collected all molecules of HQZZ from our in house database TCMSP: Traditional Chinese Medicines for Systems Pharmacology Database and Analysis Platform (http://lsp.nwsuaf.edu.cn/tcmsp.php) (Ru et al., 2014). Finally, a total of 237 molecules with 32 in RS., 7 in CF., 35 in CJ., 22 in RO., 34 in CM., 24 in RRR., 21 in TCR., 20 in PR., 41 in CMO., and 57 in FA. were obtained in this study. Besides, because glycosides are usually hydrolyzed to liberate aglycone which is then absorbed at intestinal mucosa (Németh et al., 2003), the corresponding 74 aglycone chemicals of glycosides in herbs were also added into the molecular database for HQZZ. The information of the total 311 compounds are shown in Table S1.

2.2. Drug-likeness evaluation

Drug-likeness (DL) is a qualitative concept used in drug design for an estimate on how "drug-like" a prospective compound is, which helps to optimize pharmacokinetic and pharmaceutical properties, such as solubility and chemical stability (Vistoli et al., 2008). In this work, Tanimoto Similarity (TS) is used to select out compounds which are considered to be chemically suitable for drugs (Yamanishi et al., 2010) between herbal ingredients and the average molecular properties of all veterinary drugs in FDA (Wishart et al., 2006). The TS index is defined as following:

$$T(A,B) = \frac{A \cdot B}{\|A\|^2 + \|B\|^2 - A \cdot B} \quad (1)$$

where A represents the molecular descriptors of herbal compounds, B represents the average drug-likeness index of all veterinary drugs in FDA. In this study, compounds with DL $\geq$ 0.15 were selected as the candidate bioactive compounds, because the mean DL value for all veterinary drugs in FDA is 0.15.

2.3. Comparison of active compounds and randomly selected compounds based on chemicals

The "Lipinski's rule" for DL defines five simple physicochemical parameters: molecular weight (MW), octanol–water partition coefficient (Mlog P), H-bond donors (nHDon), H-bond acceptors (nHAcc), and number of rotatable bonds (RBN), which serves as a very efficient guideline for orally bioavailable small-molecule drug discovery. In order to validate the efficiency of the potential active compounds, herbal ingredients comparison based on the above chemical properties between the screened active compounds in Section 2.2 and the randomly selected equal number TCMSP compounds (Table S2) that do not overlap with the active compounds were performed. These five important pharmacology-related parameters were calculated by the DRAGON software (version 5.6) (Todeschini et al., 2003).

2.4. Target fishing and analysis

2.4.1. Drug-targeting

Firstly, an in silico CSDT model was applied in this work to derive the target information of active ingredients (Zheng et al., 2016). The details of the CSDT model were provided in Supplementary methods. Then, taking the obtained candidate targets as queries, targets from bovine were preserved by searching Uniprot (http://www.uniprot.org) database. Finally, the targets were mapped to Therapeutic Target Database (TTD, http://database.idrb.cqu.edu.cn/TTD/), Comparative Toxicogenomics Database (CTD, http://ctdbase.org/), Pharmacogenomics Knowledgebase (PharmGKB, https://www.pharmgkb.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.kegg.jp/) to obtain their corresponding diseases, providing a well-defined target-disease network.

2.4.2. GOBP enrichment analysis for targets

In order to further probe the meaningful functional annotation of our achieved targets, in this work, Gene Ontology (GO) enrichment analysis was performed by linking the targets to DAVID (The Database for Annotation, Visualization and Integrated Discovery, http://david.abcc.ncifcrf.gov). The controlled vocabularies with GO can describe genes and gene products in living organisms (Shah et al., 2003). The terms from "Biological Process" (GOBP), which is one of the three broad GO categories (the other two being "Molecular Function" and "Cellular Component"), were utilized to symbol gene function. Only GO terms with p-value $\leq$ 0.05 were selected. FDR (the false discovery rate) was introduced to perform a multiple-hypothesis testing error measure of p-values by using the web tool DAVID, we used a 0.05 FDR criterion as a significance cutoff in our analysis.

2.4.3. Experimental validation

In order to validate the accuracy and efficiency of the CSDT model, we constructed in vitro experiments to further validate the inhibitory effects of compounds on their predicted targets. 2 key and commercially available targets were selected. The inhibitory effects of targets PDE (Cyclic Nucleotide Phosphodiesterase), and SDHA (flavoprotein subunit of complex II) were assayed using Cyclic Nucleotide Phosphodiesterase

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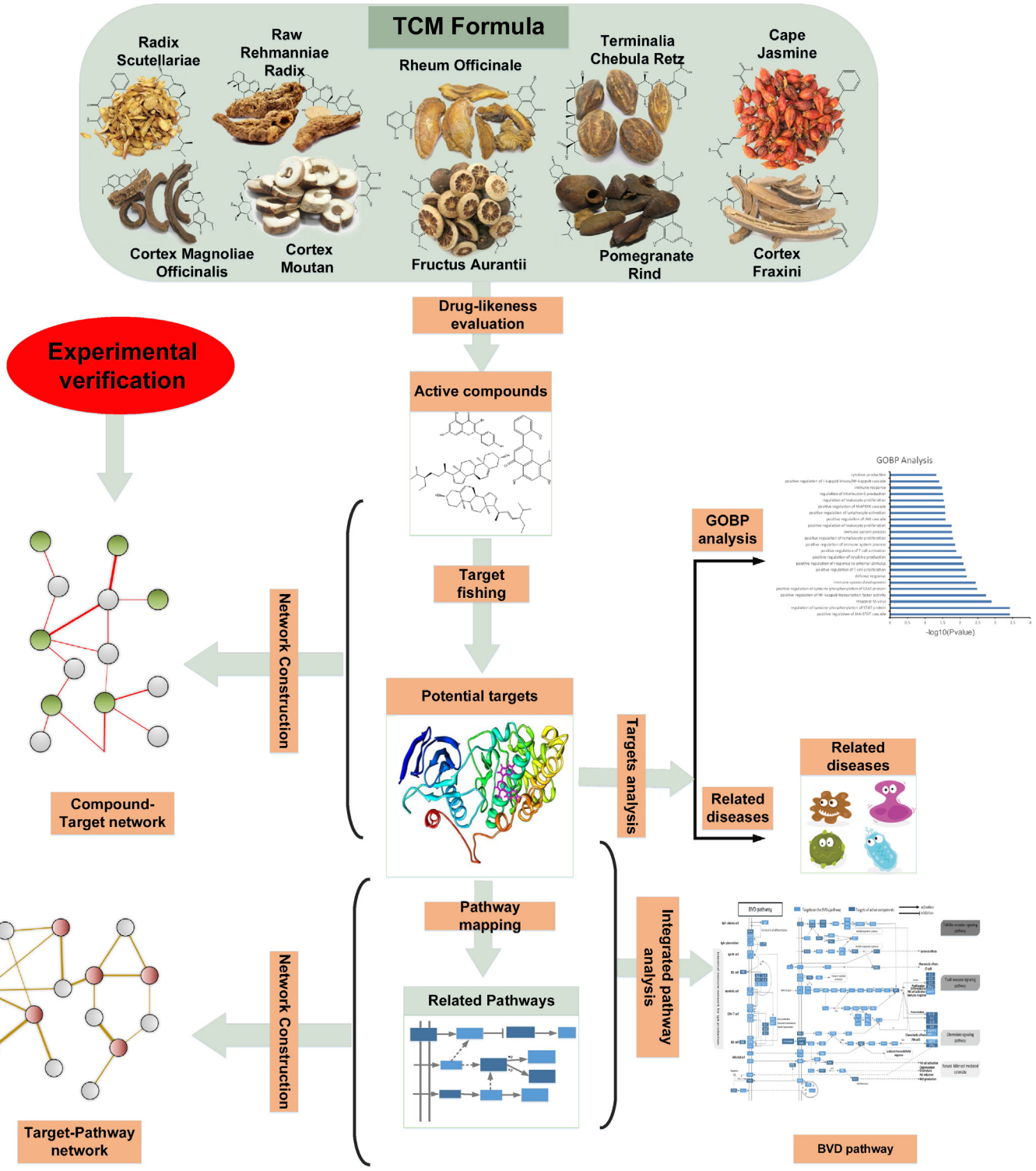

Fig. 1. The detailed flowchart of the systems pharmacology method.

(PDE) assay kit (Enzo Life Sciences, NY, USA) and MitoTox™ OXPHOS Complex III Activity Kit (Abcam, Cam, UK) respectively. MitoTox™ OXPHOS Complex III Activity Kit can be used to screen the direct inhibitory effect of compounds on Complex II and III activity. Magnoflorine, acacetin and isoimperatorin were purchased from Chroma-Biotechnology Co., Ltd. (Chengdu, China). Poncirin was purchased from ChemFaces Co., Ltd. (Wuhan, China). The purity of all the compounds is ≥98%. All drugs were dissolved in DMSO and freshly prepared due to loss of activity under long-term storage.

2.5. Network construction

To comprehensively dissect the interrelationships among compounds, targets and pathways and then to investigate the mechanism of action of herbal medicines, compound–target network (C–T network) and target–pathway (T–P network) were separately constructed in this study. 1) C–T network. The compound-target interactions are visualized by the C–T network. 2) T–P network. Firstly, the potential targets were mapped onto DAVID database to perform pathway

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Table 1
Chemical information of the reported bioactive compounds from HQZZ and their network parameters.

MOL_ID	Compounds	CID	Degree	DL	Literatures (PubMed_ID)	Herbs
MOL052	Chlorogenic acid	1794427	1	0.33	26712785	CJ.
MOL067	Crocetin	5281232	33	0.26	26119275	CJ.
MOL105	Geniposidic acid	443354	2	0.41	26723467	CJ.
MOL119	Imperatorin	10212	1	0.22	26631504	CJ.
MOL127	Isoimperatorin	68081	1	0.23	20937376	CJ.
MOL204	Nicotiflorin	5318767	6	0.24[a]	17448528	CJ.
MOL299	Umuhengerin	73648	2	0.49	3204384	CJ.
MOL300	Ursolic acid	64945	1	0.75	26347199	CJ.
MOL290	Syringaresinol	443023	1	0.72	25415049	CJ.|CMO.
MOL103	Geniposide	107848	2	0.44	26371859	CJ.|CM.
MOL139	Kaempferol	5280863	6	0.24	26694364	CJ.|CM.|FA.|PR.
MOL211	Oleanolic acid	10494	1	0.76	26570984	CJ.|CM.|FA.|PR.
MOL311	β-Sitosterol	222284	6	0.75	26631364	CJ.|CM.|FA.|TCR.|PR.|RO.|CRS.|RRR.
MOL054	Chrysin	5281607	3	0.18	26531689	CJ.|CRS.
MOL284	Stigmasterol	5280794	4	0.76	26067873	CJ.|CRS.
MOL254	Rutin	5280805	3	0.28[a]	26707720	CJ.|FA.|PR.
MOL231	Quercetin	5280343	3	0.28	26712783	CJ.|FA.|PR.|RO.
MOL016	Aesculin	5281417	2	0.36	26567597	CF.
MOL288	Syrigin	5316860	1	0.33	23620140	CF.|FA.
MOL156	Magnoflorine	73337	1	0.55	25840917	CMO.
MOL161	Magnolin	169234	4	0.72	25400863	CMO.
MOL162	Magnolol	72300	1	0.15	26547789	CMO.
MOL210	Obovatol	100771	1	0.18	21512279	CMO.
MOL167	Martynoside	5319292	2	0.58	16198557	CM.
MOL185	Mudanpioside E	21631104	1	0.75	24704488	CM.
MOL213	Oxypaeoniflorin	429559	1	0.78	23881455	CM.
MOL216	Paeoniflorin	442534	2	0.79	26577108	CM.
MOL044	Catechin	9064	2	0.24	26589582	CM.|FA.|PR.|RO.
MOL085	Eriocitrin	83489	4	0.24[a]	25625199	FA.
MOL114	Hesperetin	72281	3	0.27	26610718	FA.
MOL116	Hesperidin	10621	3	0.27[a]	4033591	FA.
MOL130	Isonaringin	85704	1	0.75	24793084	FA.
MOL147	Limonin	179651	3	0.57	26690397	FA.
MOL149	Lonicerin	5282152	1	0.73	21656372	FA.
MOL165	Marmin	6450230	1	0.31	20099458	FA.
MOL191	Narcotine	4544	4	0.88	26690027	FA.
MOL192	Naringenin	439246	1	0.21	26655880	FA.
MOL195	Narirutin	442431	1	0.75	26526495	FA.
MOL205	Nobiletin	72344	1	0.52	26664016	FA.
MOL206	Nomilin	326240	1	0.67	16719503	FA.
MOL209	Obacunone	119041	1	0.77	24927687	FA.
MOL280	Sinensetin	145659	2	0.45	25735898	FA.
MOL291	Tangeretin	68077	2	0.43	26468759	FA.
MOL199	Neohesperidin	442439	1	0.7	26453923	FA.|CMO.
MOL025	Astragalin	5282102	3	0.74	26059910	FA.|PR.
MOL151	Luteolin	5280445	8	0.25	26656210	FA.|PR.
MOL082	Epicatechin	72276	2	0.24	26711450	FA.|PR.|RO.
MOL065	Corilagin	73568	2	0.44	24752860	FA.|TCR.|PR.
MOL011	Acacetin	5280442	2	0.24	26677081	RS.
MOL020	Apigenin	5280443	5	0.21	26722444	RS.
MOL029	Baicalein	5281605	2	0.21	26706290	RS.
MOL030	Baicalin	64982	3	0.75	26648289	RS.
MOL039	Campesterol	173183	7	0.71	26396375	RS.
MOL040	Carthamidin	188308	1	0.24	22805963	RS.
MOL074	Dihydrooroxylin A	177032	1	0.23	9621415	RS.
MOL086	Eriodictyol	440735	3	0.24	26723515	RS.
MOL208	Norwogonin	5281674	1	0.21	20091262	RS.
MOL212	Oroxylin A	5320315	1	0.23	25818981	RS.
MOL252	Rivularin	5320315	1	0.37	26259145	RS.
MOL256	Salidroside	159278	1	0.2	26690894	RS.
MOL258	Salvigenin	161271	1	0.33	24270218	RS.
MOL261	Scutellarein	5281697	4	0.24	26330757	RS.
MOL262	Scutellarin	185617	4	0.79	26608466	RS.
MOL281	Skullcapflavone I	5320399	1	0.29	16206047	RS.
MOL282	Skullcapflavone II	124211	2	0.44	22314230	RS.
MOL306	Wogonoside	29927693	2	0.63	2662276	RS.
MOL075	Diincarvilone A	60155322	1	0.84	22620677	RRR.
MOL077	Echinacoside	5281771	2	0.38	26677709	RRR.
MOL120	Isoacteoside	6476333	1	0.61	25975581	RRR.
MOL128	Isomartynoside	91895373	1	0.56	14522447	RRR.
MOL140	Kankanoside G	44205526	1	0.61	16908167	RRR.
MOL145	Leucosceptoside A	10394343	3	0.7[a]	12132670	RRR.
MOL239	Rehmannioside C	101654197	3	0.86	17490493	RRR.

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Table 1 (*continued*)

MOL_ID	Compounds	CID	Degree	DL	Literatures (PubMed_ID)	Herbs
MOL019	Aloe-emodin	10207	2	0.24	26686868	RO.
MOL055	Chrysophanol	10208	1	0.21	26064235	RO.
MOL241	Rehmannioside D	6325884	2	0.59	17490493	RRR.
MOL081	Emodin	3220	1	0.24	26722474	RO.
MOL126	Isoemodin	102318111	4	0.34	1667410	RO.
MOL246	Rheinoside A	13888125	1	0.68	26434123	RO.
MOL268	Sennoside B	91440	3	0.57[a]	26336586	RO.
MOL024	Arjunic acid	15385516	1	0.72	18306462	TCR.
MOL049	Chebulic acid	12302892	1	0.32	16932919	TCR.
MOL079	Ellagic acid	5281855	3	0.43	25640983	TCR.
MOL108	Glucogallin	124375	8	0.25	23247009	TCR.
MOL169	Maslinic acid	73659	1	0.74	26491566	TCR.
MOL296	Triglochinin	5281124	24	0.26	17340339	TCR.

[a] Represent the predicted DL after deglycosylation.

enrichment analysis. Then, based on the pathogenesis of BVD, we further elucidated the relationship between these significantly enriched pathways and BVD by text mining. Visualization of all networks were implemented by Cytoscape 2.8.1 (Smoot et al., 2011), and the quantitative property "degree" of these networks were analyzed by plugin NetworkAalyzer of Cytoscape (Shannon et al., 2003). In the constructed networks, compounds, targets and pathways were represented by nodes while edges indicate the interactions among them.

2.6. BVD pathway construction

Pathway-level analysis is a powerful approach enabling interpretation of target genes at a high level (Kamburov et al., 2011). To better recognize the integrative mechanisms of HQZZ for BVD therapy, an integrated "BVD pathway" was manually synthesized based on the current knowledge of BVD pathology.

3. Results and discussion

3.1. Identification active compounds through DL evaluation

Due to compounds with satisfactory DL indexes have substantially high probabilities of being drugs, the analysis of the DL indexes via the Tanimoto coefficient, are important with regard to those active compounds in herbs. As a result, 212 active compounds that satisfy the query criteria (DL ≥ 0.15) were obtained (as shown in Table S3), in which 32 are from RS., 4 in CF., 31 in CJ., 24 in RO., 36 in CM., 26 in RRR., 14 in TCR., 15 in PR., 18 in CMO., and 63 in FA. These ingredients with good DL in this work could offer a significant research clue for probing the potential targets in the future. Interestingly, some of the screened active ingredients are quite consistent with the reported pharmacological data, which verify the validity of the DL evaluation model. Among the 212 active compounds, 86 of them were reported in literatures. And the detailed information was shown in Table 1.

The active compounds exert multiple pharmacological actions including anti-inflammation, antiviral and immune regulation. For instance, baicalin (MOL030, DL = 0.75) and wogonin (MOL208, DL = 0.21), extracted from RS. (Ikemoto et al., 2000), were reported to modulate cytokine secretion and stimulate human leukocyte resistance, which are essential for the regulation of innate antiviral immunity (Blach-Olszewska et al., 2008). Aloe-emodin (MOL019, DL = 0.24), which belongs to RO., acts as the anti-inflammatory agent by inhibiting inducible nitric oxide synthase (iNOS) mRNA expression and the production of nitric oxide (NO), cyclooxygenase-2 (COX-2) mRNA and prostaglandin E2 (PGE2) (Park et al., 2009).

In addition, we found that some active ingredients are shared by herbs in HQZZ, demonstrating that HQZZ has a potential additional effect in the treatment of BVD. For example, kaempferol (MOL139, DL = 0.24), found in CJ., CM., FA., and PR., can inhibit TNF-α expression in J774.2 macrophages (Kowalski et al., 2004). Similarly, quercetin (MOL231, DL = 0.28), found in CJ., FA., PR., and RO., was proved to inhibit the production of TNF-α in normal PBMCs (Nair et al., 2006). And these two compounds possess anti-virus activity through enhancing immunity function (Lyu et al., 2005). Luteolin (MOL151, DL = 0.25) that occurs in FA. and in PR. displays specific anti-inflammatory effects at micromolar concentrations through activation of anti-oxidative enzymes, suppression of the NFκB pathway and inhibition of pro-inflammatory substances (Seelinger et al., 2008).

3.2. Chemical space comparison of compounds

The results display that the five drug-associated descriptors (MW, nHAcc, nHDon, RBN and MlogP) of active compounds in HQZZ were all in accord with the standards of Lipinski's rule of five. Further, these active ingredients were compared with compounds randomly selected from TCMSP. As listed in Table 2, from the average value of MWs, it is seen that the MWs values of active compounds (416.62 ± 123.78) are higher than that of the randomly selected compounds (388.80 ± 265.38). The average MlogP value is also higher for the active compounds (0.8 ± 2.46), indicating that the active compounds are more soluble in neutral solvents than the randomly selected compounds (−2.69 ± 69.15). Considering the flexibility of the molecule determines the binding state with target, therefore, the RBN of active compounds (4.77 ± 3.31) show more advantages in achieving better binding properties than that of randomly selected compounds (0.82 ± 69.61). Besides, the average value of nHDon (4.29 ± 2.67) and nHAcc (8.23 ± 4.10) of the active compounds are much larger than randomly selected compounds, demonstrating that the active compounds possess more polar functional groups.

In summary, the above results indicate that the active compounds from HQZZ have obviously better pharmacological profiles than the randomly selected compounds from TCMSP.

3.3. Target prediction and analysis

In this study, by exploiting the CSDT model, a total of 122 targets (as shown in Table 3) were predicted for 167 active compounds, while

Table 2
Comparison of molecular properties between active compounds and randomly selected compounds.

Index	Active compounds (mean ± SD)	Randomly selected compounds (mean ± SD)
MW	416.62(123.78)	388.80(265.38)
MLOGP	0.80(2.46)	−2.69(69.15)
nHDon	4.29(2.67)	−1.77(69.25)
nHAcc	8.23(4.10)	1.20(69.63)
RBN	4.77(3.31)	0.82(69.61)

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Table 3
The information of BVD-related targets of HQZZ and their degree parameters in C–T network.

UniProt ID	Protein_Name	Gene name	Degree	Species
P10894	1-Phosphatidylinositol 4.5-bisphosphate phosphodiesterase beta-1	PLCB1	86	Bovine
O77627	Transcription factor AP-1	JUN	41	Bovine
Q1RMT8	Interleukin-1 receptor-associated kinase 4	IRAK4	22	Bovine
Q2KJH1	Bone morphogenetic protein 4	BMP4	19	Bovine
A6QQZ0	Ganglioside-induced differentiation-associated protein 1	GDAP1	12	Bovine
Q9XTA5	Hypoxia-inducible factor 1-alpha	HIF1A	12	Bovine
P79135	Interferon-induced GTP-binding protein Mx1	MX1	12	Bovine
Q5E9F2	Heme oxygenase 1	HMOX1	10	Bovine
Q2T9M1	PRKCA-binding protein	PICK1	10	Bovine
A6QPT7	Endoplasmic reticulum aminopeptidase 2	ERAP2	9	Bovine
A6QLE5	NACHT, LRR and PYD domains-containing protein 3	NLRP3	8	Bovine
Q3ZC98	Nuclear pore complex protein Nup85	NUP85	8	Bovine
A0JNB0	Tyrosine-protein kinase Fyn	FYN	7	Bovine
Q0P5I0	Menin	MEN1	7	Bovine
F1N3B8	2′-5′-Oligoadenylate synthase 2	OAS2	7	Bovine
Q2LGB3	Interleukin-1 receptor-associated kinase 1	IRAK1	6	Bovine
Q9TTY5	Platelet-activating factor receptor	PTAFR	6	Bovine
P31039	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	SDHA	5	Bovine
Q8SQA6	C–C motif chemokine 3	CCL3	5	Bovine
O97919	C–C motif chemokine 5	CCL5	5	Bovine
Q2HJ57	Coactosin-like protein	COTL1	5	Bovine
Q2KIC2	ETS translocation variant 1	ETV1	5	Bovine
Q09139	Fatty acid-binding protein, brain	FABP7	5	Bovine
Q3MHJ2	Macrophage erythroblast attacher	MAEA	5	Bovine
O77774	Neutrophil cytosol factor 1	NCF1	5	Bovine
P13272	Cytochrome b-c1 complex subunit Rieske, mitochondrial	UQCRFS1	5	Bovine
P14099	cGMP-dependent 3′,5′-cyclic phosphodiesterase	PDE	4	Bovine
Q3T0B9	CCAAT/enhancer-binding protein gamma	CEBPG	4	Bovine
P48617	Erythropoietin	EPO	4	Bovine
O19104	Fanconi anemia group C protein homolog	FANCC	4	Bovine
P07353	Interferon gamma	IFNG	4	Bovine
Q3SWZ6	Ribosome maturation protein SBDS	SBDS	4	Bovine
A0JND4	Protein strawberry notch homolog 2	SBNO2	4	Bovine
P25068	Tracheal antimicrobial peptide	TAP	4	Bovine
Q9GL65	Toll-like receptor 4	TLR4	4	Bovine
P19238	T-cell surface glycoprotein CD5	CD5	3	Bovine
P84088	Complexin-2	CPLX2	3	Bovine
P48035	Fatty acid-binding protein, adipocyte	FABP4	3	Bovine
P01578	Interferon beta-1	IFNB1	3	Bovine
P30367	Interleukin-4	IL4	3	Bovine
Q0P5I2	Interleukin-1 receptor-associated kinase-like 2	IRAK2	3	Bovine
Q4JF28	Interferon regulatory factor 3	IRF3	3	Bovine
Q0VBZ5	Transcription factor jun-B	JUNB	3	Bovine
Q05B92	Transcription factor E3	TFE3	3	Bovine
Q32PJ2	Apolipoprotein A-IV	APOA4	2	Bovine
Q08DE6	Aquaporin-3	AQP3	2	Bovine
Q2KIG1	Histone chaperone ASF1A	ASF1A	2	Bovine
Q3ZCH6	Cyclic AMP-dependent transcription factor ATF-4	ATF4	2	Bovine
P79132	Caveolin-1	CAV1	2	Bovine
Q5MD62	C–C chemokine receptor type 7	CCR7	2	Bovine
Q0P5N1	Protein canopy homolog 3	CNPY3	2	Bovine
P11052	Granulocyte-macrophage colony-stimulating factor	CSF2	2	Bovine
Q3SX05	Evolutionarily conserved signaling intermediate in Toll pathway, mitochondrial	ECSIT	2	Bovine
Q867A9	Endothelin-2	EDN2	2	Bovine
P15396	Ectonucleotide pyrophosphatase/phosphodiesterase family member 3	ENPP3	2	Bovine
Q2KI95	Four and a half LIM domains protein 2	FHL2	2	Bovine
P55106	Growth/differentiation factor 6	GDF6	2	Bovine
Q9BDJ6	Appetite-regulating hormone	GHRL	2	Bovine
P37141	Glutathione peroxidase 3	GPX3	2	Bovine
Q0V8S0	Hepatocyte growth factor-regulated tyrosine kinase substrate	HGS	2	Bovine
P54349	Interleukin-12 subunit alpha	IL12A	2	Bovine
Q28028	Interleukin-15	IL15	2	Bovine
P05016	Interleukin-2	IL2	2	Bovine
A6QL48	Interleukin-34	IL34	2	Bovine
P52173	Interleukin-5	IL5	2	Bovine
P26892	Interleukin-6	IL6	2	Bovine
P79255	Interleukin-8	IL8	2	Bovine
Q28880	Lingual antimicrobial peptide	LAP	2	Bovine
Q27956	Leukemia inhibitory factor	LIF	2	Bovine
Q32KY3	Myeloid leukemia factor 1	MLF1	2	Bovine
Q6RUW3	Pro-neuropeptide Y	NPY	2	Bovine
P01211	Proenkephalin-A	PENK	2	Bovine
Q3SZV5	Proteasome maturation protein	POMP	2	Bovine
P45478	Palmitoyl-protein thioesterase 1	PPT1	2	Bovine
P01239	Prolactin	PRL	2	Bovine

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Table 3 (*continued*)

UniProt ID	Protein_Name	Gene name	Degree	Species
Q4U5R3	Proteasome activator complex subunit 1	PSME1	2	Bovine
Q148F6	Protein rogdi homolog	ROGDI	2	Bovine
Q32LP7	Src kinase-associated phosphoprotein 2	SKAP2	2	Bovine
Q29RL9	Transcription elongation factor A protein 1	TCEA1	2	Bovine
Q06805	Tyrosine-protein kinase receptor Tie-1	TIE1	2	Bovine
Q95LA9	Toll-like receptor 2	TLR2	2	Bovine
Q5TJ59	Toll-like receptor 3	TLR3	2	Bovine
Q95L54	Annexin A8	ANXA8	1	Bovine
Q03247	Apolipoprotein E	APOE	1	Bovine
Q3T904	Autophagy-related protein 9A	ATG9A	1	Bovine
Q8HZJ4	Calcium-binding protein 4	CABP4	1	Bovine
Q9TTS6	Eotaxin	CCL11	1	Bovine
Q8SQB1	C–C motif chemokine 20	CCL20	1	Bovine
Q3MHH5	G1/S-specific cyclin-D3	CCND3	1	Bovine
A9QWP9	C–X–C motif chemokine 9	CXCL9	1	Bovine
P25930	C–X–C chemokine receptor type 4	CXCR4	1	Bovine
Q0IIB6	DNA damage-inducible transcript 3 protein	DDIT3	1	Bovine
A0JN51	ETS-related transcription factor Elf-1	ELF1	1	Bovine
Q71SP7	Fatty acid synthase	FAS	1	Bovine
A7MB54	H2.0-like homeobox protein	HLX	1	Bovine
Q2KHU9	Heat shock protein beta-3	HSPB3	1	Bovine
P56830	Interferon tau-2	IFNT2	1	Bovine
Q0V8R5	Pro-interleukin-16	IL16	1	Bovine
P09428	Interleukin-1 beta	IL1B	1	Bovine
P07995	Inhibin beta A chain	INHBA	1	Bovine
Q58DS6	Bifunctional arginine demethylase and lysyl-hydroxylase	JMJD6	1	Bovine
O62644	Leukocyte cell-derived chemotaxin-2	LECT2	1	Bovine
P80513	Mesencephalic astrocyte-derived neurotrophic factor	MANF	1	Bovine
Q599T9	Myeloid differentiation primary response protein	MYD88	1	Bovine
Q3MHN7	Endonuclease 8-like 3	NEIL3	1	Bovine
Q58CW4	Neuromedin-U receptor 2	NMUR2	1	Bovine
P29473	Nitric oxide synthase, endothelial	NOS3	1	Bovine
Q9BGI3	Peroxiredoxin-2	PRDX2	1	Bovine
P11023	Ras-related protein Rab-3A	RAB3A	1	Bovine
Q9TU25	Ras-related C3 botulinum toxin substrate 2	RAC2	1	Bovine
Q3SZJ2	Receptor-interacting serine/threonine-protein kinase 2	RIPK2	1	Bovine
Q28175	Retinoid isomerohydrolase	RPE65	1	Bovine
Q2HJF9	Radical S-adenosyl methionine domain-containing protein 2	RSAD2	1	Bovine
A0JN71	SAM and SH3 domain-containing protein 3	SASH3	1	Bovine
Q29RN6	Suppressor of cytokine signaling 5	SOCS5	1	Bovine
P20414	Metalloproteinase inhibitor 1	TIMP1	1	Bovine
Q3SZI1	Tubulointerstitial nephritis antigen	TINAG	1	Bovine
Q27987	Thyrotropin receptor	TSHR	1	Bovine
Q0P5K3	Ubiquitin-conjugating enzyme E2 N	UBE2N	1	Bovine
P45879	Voltage-dependent anion-selective channel protein 1	VDAC1	1	Bovine
Q9GMA3	Visual system homeobox 1	VSX1	1	Bovine
Q08DH8	DNA repair protein	XRCC3	1	Bovine

other 45 compounds have no related targets. Table S4 displays the correlations between these targets and their relevant diseases, which demonstrates that these herbs reliably provide protection against BVD by regulating immune response and treating BVD associated symptoms.

For instance, the first protector against the virus infection is the interferon system, which plays an important role in connecting the innate and adaptive immune responses (Mohty et al., 2003). BVDV was reported to trigger IFN-β very inefficiently (Gil et al., 2006). Our results show that IFN-β can be hit by crocetin from CJ. and glucogallin from TCR., demonstrating HQZZ might contribute to treating BVD through regulating interferon system. In addition, BVDV infection is usually inconspicuous but can result in abortion or congenital anomalies, mucosal disease marked by diarrhea, necrosis, and erosions of the alimentary tract (Baker, 1987). Fortunately, as shown in Table S4, there are other targets referring to BVD-related symptoms. Proteins like IFNG (Interferon gamma), MYD88 (Myeloid differentiation primary response protein), TLR2 (Toll-like receptor 2), TLR4 (Toll-like receptor 2) and FAS (Fatty acid synthase) addressed by HQZZ, may take part in abortion in veterinary practice. Targets related to fever such as TLR4, TLR2, MYD88 can be regulated by HQZZ. Over analyzing the relationships between target proteins and their relevant diseases, the results show that anti-BVD action of HQZZ is mainly through adjusting immunity function effectively and curing BVD associated symptoms.

3.3.1. GOBP enrichment analysis for targets

In order to validate whether the potential targets are indeed match for BVD, GOBP analysis was performed. Fig. 2 lists the top 22 significantly enriched GOBP terms (p-value ≤ 0.05) of these targets. p-Values and FDR are shown in Table S5. The results suggest that a number of targets involve in inflammatory and immunity associated biological processes such as positive regulation of NF-κB transcription factor activity, positive regulation of T cell proliferation, response to virus and leukocyte activation, which are closely related to the pathogenesis of BVD. For instance, IL16 shows to associate with the progression of leukocyte chemotaxis in the GOBP analysis results, and its lymphocyte chemotactic activity has been validated by a clinical research (Mashikian et al., 1998). Previous study has confirmed the antiviral and immunoregulatory activities of IFNG, mainly through JAK-STAT cascade depending on constitutively expressed

Table 4
IC50 values for the selected key drug–target interactions.

No.	Target gene name	Compound	Targets inhibition assay (IC50 μM)
1	PDE	Magnoflorine	0.24
2	PDE	Isoimperatorin	13.67
3	SDHA	Acacetin	53.5

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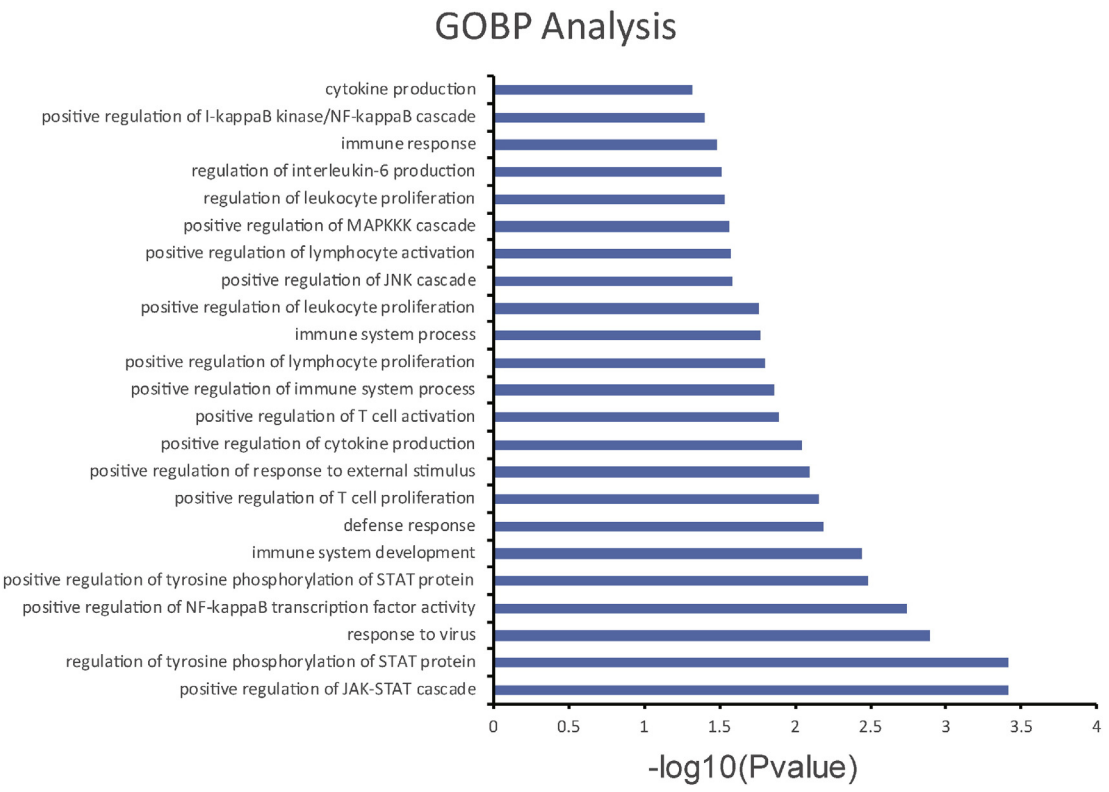

Fig. 2. Gene Ontology (GO) analysis of therapy target genes. The y-axis shows significantly enriched 'Biological Process' (BP) categories in GO relative to the target genes, and the x-axis shows the enrichment scores of these terms (p-value ≤ 0.05).

IL-1α (Hurgin et al., 2007). GOBP analysis also demonstrates that FAS involves in the regulation of immune system process (Strasser et al., 2009).

3.3.2. Experimental validation

The experiment results were provided in Table 4. As shown in Table 4, magnoflorine (MOL156) and isoimperatorin (MOL127) are potent compounds able to inhibit PDE with IC50 values of 0.24 μM and 13.67 μM respectively. Actually, the statistical analysis indicates that magnoflorine shows higher inhibition effect on PDE than isoimperatorin, indicating PDE is more sensitive to magnoflorine. In addition, acacetin (MOL011) exerts inhibitory activity against SDHA with an IC50 value of 53.3 μM. The experimental results are in good agreement with our theoretical predictions. The results demonstrate that the drug-target interactions predicted by the CSDT model are reliable.

3.4. Network construction and analysis

3.4.1. Compound–target network and analysis

As shown in Fig. 3, the resulting C–T network is comprised of 289 nodes (167 molecules and 122 potential targets) and 506 edges. Subsequently, network analysis was performed by evaluating the degree of the nodes, resulting in an average degree of 3.03 per compound and 4.15 per target respectively, which validate the multi-components multi-targets characteristics of the complex mechanism of TCMs. Among the 167 active compounds, 67 of them show high degree, which may play hub roles in the network. Meanwhile, each herb is associated to multiple targets, manifesting the potential synergistic effects among them.

Studies have proven that persistent and primary postnatal infections with BVDV lead to immunosuppression in cattle (Potgieter, 1995). Thus, the intensification of the immunological response is a vital treatment strategy against BVD (Braun et al., 1973). Fortunately, further analysis of the C–T network shows that a certain amount of targets with high degrees implicate the process of immune response. For example, a growing amount of evidence suggests that HIF1A (Hypoxia-inducible factor 1-alpha) (degree = 12) is in control of the immune responses (Acosta-Iborra et al., 2009; Cramer et al., 2003). BMP4 (Bone morphogenetic protein 4) (degree = 19) participates in lymphocyte maturation and thymopoiesis (Tsai et al., 2003). Additionally, it is verified that BMP4 may play a crucial role in controlling adaptive immune system (Kaplan et al., 2005). PLCB1 (1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-1) (degree = 86) with the highest degree can significantly suppress the expression of proinflammatory cytokine including IL-1 beta, IL-6, and IL-8 in an LPS-induced endothelial cell inflammation model (Lin et al., 2015). Thus, these targets with high degrees may play important roles in combating BVD by intensifying the immunological responses in hosts.

In addition, the HQZZ formula also has been proved to be very effective at removing BVDV from host cells via strengthening the immune system. For instance, CCL3 (C–C motif chemokine 3) (degree = 5) can eliminate hepatitis virus by enhancing virus-specific $CD8^+$ T cell differentiation and migration (Trifilo et al., 2003). As we know, BVDV is one of the classical examples of Flaviviridae, which also includes the genera *Flavivirus*, *Pestivirus* and *Hepacivirus* (HCV) (Collett et al., 1988). Interestingly, BVD and HCV share a significant degree of local protein region homology, common replication strategies, and probably the same subcellular location for viral envelopment (Zitzmann et al., 1999). Thus, it is considered that CCL3 is probably a vital target, which potentially aids in BVDV elimination and facilitates anti-BVDV effect of HQZZ.

In summary, based on the comprehensive analyses of the C–T network, we conclude that the clinical efficacy of HQZZ formula is primarily dependent upon the intensification of the immunological responses. And different ingredients in HQZZ formula might target the same protein, exhibiting synergistic effects for BVD therapy.

3.5. T–P network

As a result, the 18 significantly enriched pathways (p-value ≤ 0.05, please see in Table S6) might be the major pathways in which HQZZ

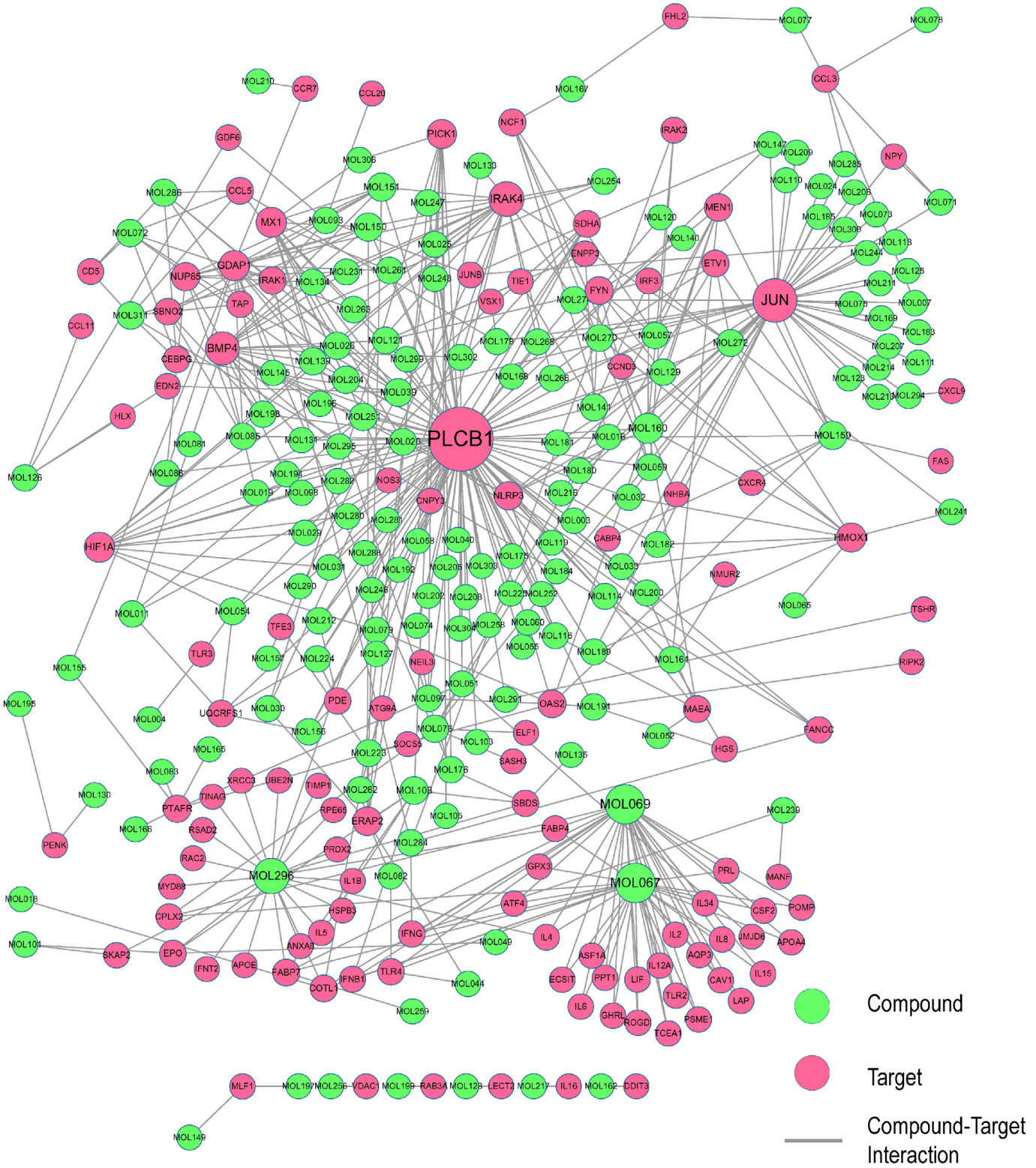

Fig. 3. Compound–target network. Red nodes represent potential drug targets, green nodes remark drug ingredients and each edge represents the interaction between them. Node size is proportional to its degree. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

acts and plays an important role in the treatment of BVD. 51 out of 122 targets were mapped to these 18 pathways, resulting in a bipartite T–P network (shown in Fig. 4). The T–P network contains 69 nodes (51 targets and 18 pathways) and 146 edges, which shows an average degree of 3 per target and 8 per pathway, respectively. The results demonstrate that more than half of the targets (25/51) locate in multiple pathways (≥ 3), which may play a crucial role in the treatment of BVD. Meanwhile, 8 out of 18 pathways enriched within multiple targets (≥ 8) of HQZZ could be the key factors contributing to the anti-BVDV effect of the herbal formula, such as Toll-like receptor signaling pathway (degree = 16), Jak-STAT signaling pathway (degree = 14), Chemokine signaling pathway (degree = 11) and Natural killer cell mediated cytotoxicity

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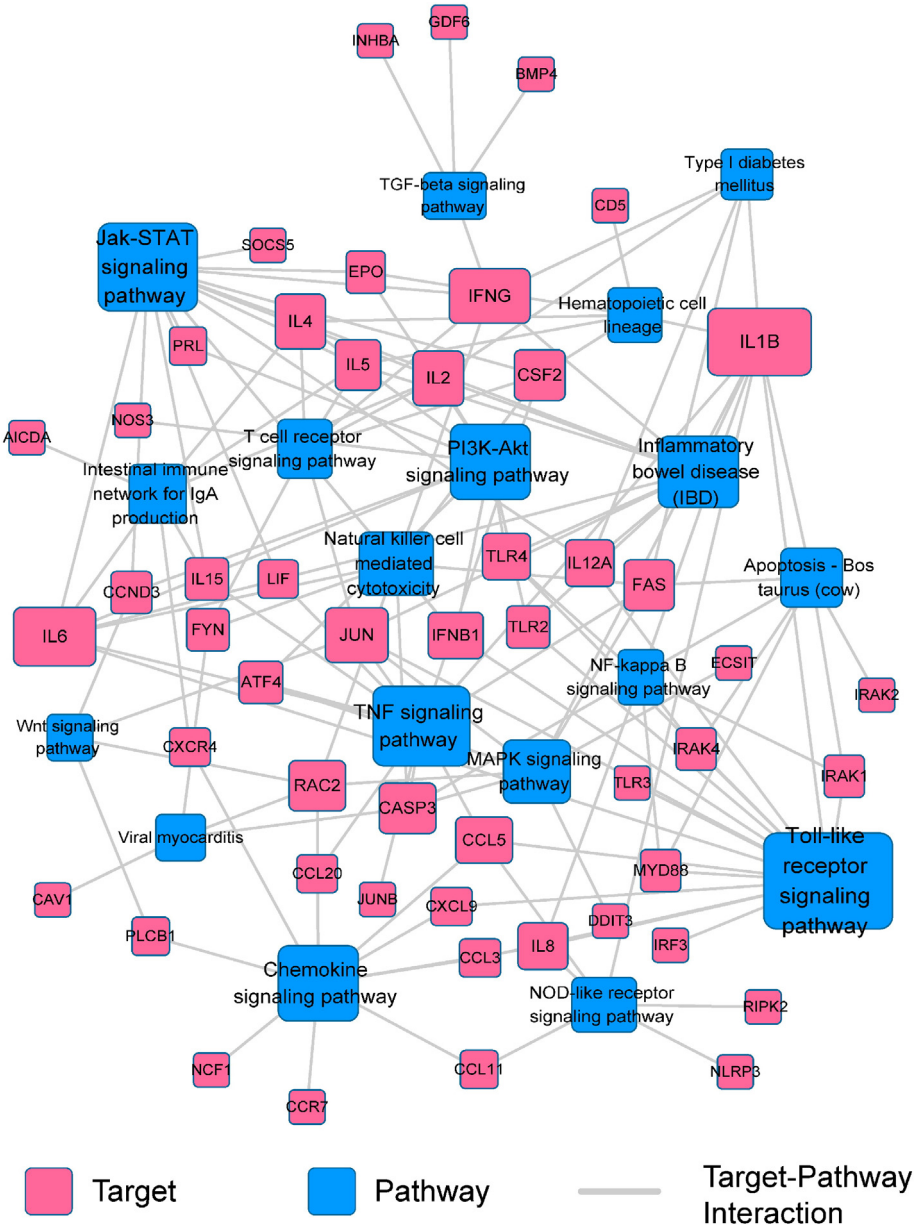

Fig. 4. Target–pathway network. The blue nodes represent the diseases and the yellow nodes represent the targets. Node size is proportional to its degree. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(degree = 8). Actually, these pathways enriched within a number of targets may provide scientific basis for the therapeutic activity of HQAA.

The results display that the herbal medicines reduce inflammatory reactions and evoke immune response via mediating multiple pathways. For instance, Toll-like receptors (TLRs) have been identified as key host molecules in innate immune (Schröder et al., 2003), which can be targeted by numerous active ingredients like epicatechin (MOL082), crocetin (MOL067), chrysin (MOL054) and so forth. Hence, Toll-like receptor signaling pathway with the highest degree may be a key pathway involved in the immune surveillance against virus infection. Moreover, numerous immunological and inflammatory response related pathways were also enriched, like Natural killer cell mediate cytoxicity and Intestinal immune network for IgA production. They might also be crucial pathways that protect bovine from virulent BVDV challenge. In addition, Jak-STAT signaling pathway also stands out in the enriched pathway list, which can mediate a variety of specific IFN-dependent antiviral responses. (Lin et al., 2004). This further verifies that as anti-BVDV agents, HQZZ probably works through several pathways which enable us to efficiently treat BVD. Consequently, we deduce that HQZZ primarily regulates these pathways to exert inhibition of inflammation, intensification of the immunological response, defense of virus infection and thereby has the potential to prevent persistent and primary postnatal infections with BVDV (Potgieter, 1995).

3.6. BVD pathway

Considering the way BVDV invades the host and the pathological state, in this section, an integrated BVD pathway that comprises of 5 signaling pathways such as Toll-like receptor signaling pathway, T cell receptor signaling pathway, Natural killer cell mediated cytoxicity and Intestinal immune network for IgA production were assembled. As can be seen from Fig. 5, 30 proteins (dark blue rectangles) located from upstream to downstream on the BVD pathway can be linked with active ingredients in our work. Target proteins in this integrated pathway

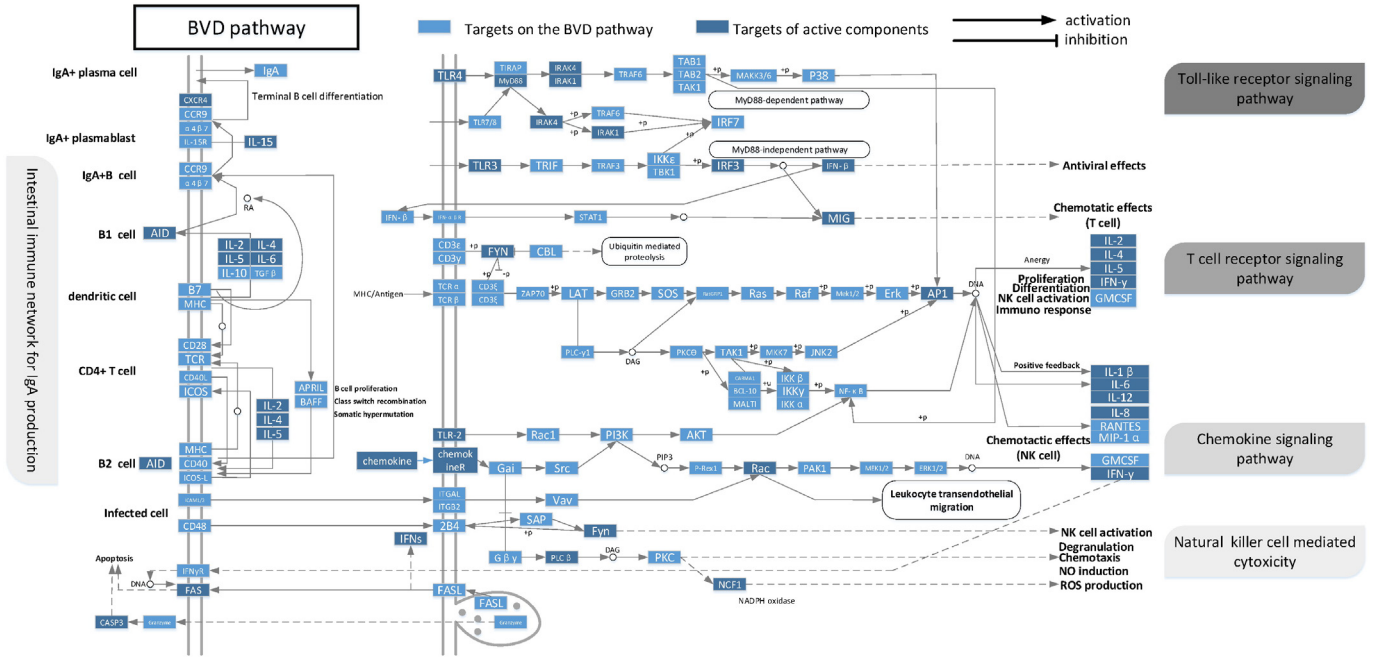

Fig. 5. BVD pathway. Distribution of target proteins of herbs on the compressed 'BVD pathway'. Five pathways (gray rectangle) form the compressed BVD pathway. Light blue rectangle remark targets on the BVD pathway, dark blue rectangle represent targets of active compounds. Arrows indicate activation, T-arrows indicate inhibition. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

take control of these three therapeutic modules, including antiviral module, immunoregulatory module, and inflammation module.

3.6.1. Antiviral module

As seen from Fig. 5, TLRs and IRF3 trigger activation of a common MyD88-dependent signaling pathway, leading to the induction of IFNβ (Yamamoto et al., 2003). The induction of IFNβ can limit virus infection by suppressing viral replication and modulating adaptive immunity (Meylan et al., 2005). Thus, chrysin (MOL054) and magnolignan I (MOL160) binding to TLR3, IRF3 respectively can provide more positive regulatory effects in the activation of this MyD88-independent pathway and IFNβ, indicating that HQZZ has the potential value to inactivate virus in the therapy process of BVD.

3.6.2. Immunoregulatory module

As shown in Fig. 5, targets are mapped to Toll-like receptor signaling pathway, T cell receptor signaling pathway, Natural killer cell mediated cytoxicity and Intestinal immune network for IgA production these four key immunoregulatory pathways, which indicate the pivotal role of immunoregulatory in the treatment of BVD. For instance, paeoniflorin (MOL216), oleanolic acid (MOL211) and magnolignan I (MOL160) acting on AP1 (transcription factor AP-1), ultimately interfering with the Toll-like receptor signaling pathway, can trigger innate immune responses (Barton and Medzhitov, 2003). Intestinal immune network for IgA production pathway (Fig. 5) is able to produce a sequence of interleukins including IL-2, IL4, IL5 and IL6. These interleukins can stimulate the expression of AID (Single-stranded DNA cytosine deaminase) in B cell and further activate CXCR4 and IL-15 in IgA + plasmablast. The above phenomenon can accelerate the formation of IgA + plasma cell, and terminally lead to the release of noninflammatory immunoglobulin A (IgA), which is the first line of defense in immune system (Lemaitre-Coelho et al., 1978). Thereby, crocetin (MOL067) interacting with IL-2, IL-4, IL-6 and AID, and magnolignan H (MOL159) targeting at CXCR4 might be the potential regulatory factors in IgA production in the intestinal immunity system. In addition, Natural killer cell mediated cytoxicity pathway displays that FYN (Tyrosine-protein kinase) can trigger Natural killer (NK) cells activation (Lowin-Kropf et al., 2002). NK cells have been proven to be a central defense against viral infection. Thus, active compounds like citrusin A (MOL057) and isoacteoside (MOL120) controlling over FYN may have potential anti-BVDV effects by interfering in the Natural killer cell mediated cytoxicity pathway. In summary, all these suggest that immunization enhancement is of benefit to treat BVD.

3.6.3. Inflammation module

Inflammatory chemokine controls the recruitment of effector leukocytes in infection (Baggiolini, 1998). As displayed in Fig. 5, isoemodin (MOL126), fritillaziebinol (MOL093) and mudanpinoic acid A (MOL179) activate the interaction of chemokines with their receptors, which raises the expression of the downstream proteins Gai and Gβγ. The signaling linkage for Gai receptor will activate Rac through coupling with Src, PI3K and P-Rex-1. The activation of Gβγ stimulates the expressions of PLCβ and PKC, which finally increase the activity of NCF1. Both Rac and NCF1 are the targets that related to the production of ROS, which is the brave soldier in killing bacterial vegetative form, virus and fungi depending on its strong oxidation.

3.6.4. Cross-talk effects

It can be seen from Fig. 5 that there exists several mutual targets among these pathways which link them together to achieve immune regulatory effects. The most typical representative is the joint effects between the Toll-like receptor signaling pathway and the T cell receptor signaling pathway. These two pathways are bonded together to regulate NF-κB activity through mediating the intracellular signaling cascades. NF-κB takes control of the activity of many agents in early immune response and all phases of the inflammatory reaction, such as IL-2, IL-4, IL-5 and IFN-y. Thus, compounds like crocetin (MOL067) and epicatechin (MOL082) might exert their immune regulatory effects by acting on the upstream targets TLR2 and TLR4.

4. Conclusion

BVD has caused significant economic losses in the dairy industry worldwide. In China, herbs have historically been developed as a source of therapeutic agents for the treatment of BVD. In this study, we

presented a system pharmacology-based approach that integrates active constituents screening, targets prediction and validation, network analysis and pathway analysis to probe the multi-compound, multi-target, and multi-pathway properties of Chinese herbal formula for BVD therapy. The main contributions of this work are listed as follows.

(1) 212 active compounds were filtered out from the HQZZ formula through DL evaluation, which will offer valuable clues in our further studies. And 41% (86/212) of them were reported by literatures, verifying the reasonability of our evaluation model.
(2) 122 targets were predicted by the CSDT model, demonstrating the multi-target characteristic of the HQZZ formula. Results of the drug–target interactions experiments indicate the reasonability of our systems-based method. GOBP enrichment and C–T network analysis display that HQZZ contributes to preventing BVDV persistent infection and removing BVDV from host cells via intensification of the immunological response and suppression of inflammation.
(3) The T–P network and the integrated BVD pathway indicate that the major ingredients of HQZZ might exert anti-BVDV effect by modulating numerous different pathways including Toll-like receptor signaling pathway, T cell signaling pathway, Natural killer cell mediate cytoxicity, and Jak-STAT signaling pathway, which are involved in antiviral, immunoregulatory, and anti-inflammatory processes.
(4) Taken together, by dissection of mechanisms of HQZZ as an effective treatment for BVD, a novel systems pharmacology-based natural compounds in this work will promote the development of phytomedicines as a source of new therapeutics for livestock diseases, which would be of great value.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ejps.2016.05.018.

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