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Original article

Potential molecular targets and pathways of a traditional Chinese medicine formula for bovine endometritis identified by network pharmacology

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Abstract

Bovine endometritis has become a persistent issue in the global dairy business, resulting in huge economic losses. Due to their numerous positive benefits, Chinese herbal medicines (CHMs) have recently demonstrated remarkable pharmacological potential against endometritis. The objective of this study was to investigate the effects and elucidate the underlying mechanisms of the *Yimucao formula* (YMF) that involves five herbs in lactation cows under endometritis conditions. Initially, the possible impacts of YMF on cows with endometritis were assessed. Then, using network pharmacology, potential molecular processes by which the YMF prevents endometritis were suggested. The findings demonstrated a considerable improvement in endometritis-related clinical complaints following YMF treatment. Mechanically, 150 active compounds were identified from the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP); of these, quercetin, kaempferol, beta-sitosterol, apigenin, isorhamnetin, and sitogluside were the most prevalent active substances. The NCBI gene, GeneCard, and OMIM databases had 110 genes linked to endometritis. The intersection of these targets with the 213 active ingredient targets produced 17 common targets, of which BCL2, IL-6, MMP9, HIF1α, TNF, IL-1β, and ICAM1 were the top 7 core targets. According to the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment data, atherosclerosis, fluid shear stress, and the AGE-RAGE signaling pathway are the primary causes of YMF's anti-endometritis action. Finally, our results indicate that the YMF works on endometritis through various and multi-targeted signaling pathways, which provide reference for clinical practice, based on network pharmacology and molecular docking.

Keywords: endometritis, Chinese herbal medicines, *Yimucao formula* (YMF), network pharmacology

Introduction

Bovine endometritis is one of the major dangerous disorders affecting milk production and reproductive performance in dairy cows (LeBlanc et al. 2002). Endometritis causes more than one-third of all dairy cow culls annually, which continues to be a significant health and financial burden on the worldwide dairy sector (Sheldon et al. 2019, L et al. 2020, Wang et al. 2020). Generally, endometritis is caused by bacterial infections in the uterine lumen during the early postpartum period (Sheldon et al. 2006, Jiang et al. 2017). Accordingly, the main clinical approach for endometritis is antibiotic therapy (McDougall et al. 2013). However, the widespread presence of drug residues in milk following antibiotic usage poses a serious risk to public health. Furthermore, incorrect and extended use of antibiotics may accelerate the development of bacterial resistance (Haimerl and Heuwieser 2014). Thus, safe and effective treatments are urgently needed for endometritis.

Traditional Chinese medicine (TCM) has various benefits for treating endometritis, including a lower rate of recurrence and no adverse effects (Xu et al. 2017). With a long history of using multi-component formulae to combat infections and inflammation, the *Yimucao formula* (YMF) is a well-known TCM formula that offers a possibly sustainable solution (Jia et al. 2017). It involves six common Chinese herbal medicines: Yi-mu-cao (Leonurus japonicus), Danggui (angelica), Chuanxiong (ligusticum), Taoren (peach kernel), Paojiang (prepared ginger), and Gancao (processed licorice), which have previously been demonstrated the anti-inflammation activity (Huang et al. 2013, Song et al. 2015, Ji et al. 2016, Mao et al. 2019). According to pharmacological research, the YMF is good for pyometra, cervicitis, vaginitis, postpartum inflammation, pus, acute and chronic endometritis, and reproductive tract infections (Zhu et al. 2018, Miao et al. 2019). YMF is a type of herbal medicine having a variety of constituents and intended uses, thus further research is required to fully understand its therapeutic processes as they are currently unclear.

Through drug-target-gene-disease interaction network analysis, network pharmacology is a novel research strategy that combines system biology, multi-direction pharmacology, network analysis, and computer technology to investigate the relationship between medications and disorders (Han et al. 2022, Choi et al. 2023, Li et al. 2023, Liao et al. 2023, Liu et al. 2023, Zhao et al. 2023). Because much of TCM's underlying mechanism is still unclear, this study approach has the potential to greatly advance TCM (Li and Zhang 2013). Pharmacology and pharmaco dynamics together may provide light on the mechanisms and synergistic effects of many medications by examining the various networks engaged in the intricate and multi-level interactions (Hong et al. 2017). With network analysis and sketching, it offers a system-level knowledge of the intricacy of sickness and drug action.

In the present study, we first evaluated the pharmacological effects of the YMF on bovine endometritis. Then, we applied a network pharmacology approach to achieve a multilevel study to determine the interaction between YMF and bovine endometritis. Our findings help researchers and pharmacologists understand the mechanisms of YMF.

Materials and Methods

Sample preparation and ingredient identification

For preparing the YMF, 480 g Yi-mu-cao (Leonuri Herba), 300 g danggui (angelica), 120 g chuanxiong (ligusticum), 120 g taoren (peach kernel), 60 g paojiang (prepared ginger), and 60 g gancao (processed licorice) were first ground to make the herb powder. Then, the Yi-mu-cao and gancao were decocted with water (1:10, w/v) for 2 h after 0.5 h of soaking. The extract was filtered and the residues were further decocted (1:5, w/v) for 1 h. The two-time filtrates were combined and concentrated to 500 ml. Then 90% ethanol was added and made 85%, combined with the above standby extract of the other four flavors, stood for 24 hours, filtered, and the filtrate was recovered from ethanol until no Alcohol, added an appropriate amount of sodium benzoate, adjusted the pH value to 6.5~7.0, added water to 1000ml, filtered, filled and sterilized.

Animals

This experiment was conducted at a commercial dairy farm in Northwest China, from June to August 2023. At the time of the experiment, this farm had 1,826 Holstein cows, of which 508 were lactating. Cows were managed in a free-stall barn, bedded with wood shavings. Postpartum cows were randomly divided into four groups (n=10 cows/group), YMF group, control group (*Rifaximin*), positive control (untreated), and negative control (healthy) group, respectively. In the YMF group, the cows with clinical endometritis received YMF (200 ml) through uterine perfusion for 3 consecutive days (once per one day), while in the *Rifaximin* group, the cows with clinical endometritis received *Rifaximin* (200 ml) through uterine perfusion two times (once per two days). The cows in the disease and healthy groups received the same volume of distilled water. To the experiment end, endometrial tissue biopsy were collec-

ted from each group (n=3) using uterine biopsy forceps for qRT-PCR. All animal handling procedures were approved by the Committee for the Ethics on Animal Care and Experiments at Shanxi Agricultural University (approval number SX-2022-MI-23)**.** Animals were diagnosed as having postpartum endometritis based on the presence of purulent mucus, i.e. Vaginal mucus assessment (VMS) of 3 at 21 days postpartum (DPP) or VMS ≥2 at 50 DPP (Williams et al. 2005, Miranda-CasoLuengo et al. 2019).

Screening of active ingredients and targets

The active ingredients and corresponding targets of Angelica sinensis, Ligusticum, Prunus armeniaca, Zingiber officinale, and Glycyrrhiza uralensis were retrieved from the Traditional Chinese Medicine Systems Pharmacology database (TCMSP, https://old.tcmsp-e.com/tcmsp.php) (Wei et al. 2020). Oral bioavailability (OB) ≥30% and drug-likeness (DL)>0.18 were set as the thresholds to filter potential active ingredients.

Conversion of drug targets to gene symbols

To convert the target proteins of active ingredients to gene symbols, the Bostaurus (cattle) proteome was searched against the UniProt Knowledgebase (UniProtKB, https://www.uniprot.org/). Validated bovine proteins matched with drug targets were converted to the corresponding gene symbols for subsequent analyses.

Screening of endometritis-related targets

Bos taurus and endometritis were used as search items against databases including NCBI gene, GeneCard (https://www.genecards.org), and OMIM (https://omim.org) to retrieve genes related to bovine endometritis.

Construction of the protein-protein interaction network

The disease genes of endometritis were intersected with the drug targets. The common targets were uploaded to the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, https://www.string-db.org/) to construct a protein-protein interaction (PPI) network for elucidating the mechanism of the herbal formula against endometritis (Chen et al. 2021). Cytoscape 3.10.2 software was used to optimize the PPI network based on the degrees of the nodes. In the constructed PPI network, the node size and color change reflect the degree value, and the larger the degree value, the larger the node size.

Enrichment analysis of core targets

The Gene Ontology (GO) enrichment analysis was conducted on the intersecting targets using R software (version 4.2.1), specifically employing the Bioconductor package ClusterProfiler. This comprehensive analysis encompassed three distinct modules: cell composition (CC), biological process (BP), and molecular function (MF). The threshold for significance was set at a p-value of less than 0.05, and the top 30 GO terms were selected based on the abundance of targets implicated in each category. In parallel with the GO enrichment, a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was also performed on the over-lapping targets. Utilizing the same stringent p-value cutoff of less than 0.05, the analysis identified the top 30 pathways with the most significant enrichment scores, and the enrichment analysis results were visualized.

Molecular docking validation

The binding activity of active compounds and key targets is confirmed by molecular docking. The key genes were searched in the Protein Data Bank (PDB) database (http://www.rcsb.org/) to obtain structural proteins for molecular docking with active ingredients using Discovery Studio software. AutoDock Tools was used to separate ligands from protein structures, hydrogenate protein structures, remove water molecules, and convert them into PDBQT format for docking. Acquire small molecule ligand 3D structures using the RCSB PDB online database (https://www.rcsb.org).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The total RNA of the endometrial tissues from each group was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), and total RNA was reverse-transcribed to cDNA using the iScript advanced cDNA Synthesis Kit (Bio-Rad Laboratories) according to the manufacturer's instructions. qRT-PCR was performed using SYBR®PrimeScript™ RT-PCR kit (Takara Bio, Inc.) on the Applied Biosystems Quantstudio6 flex (Applied Biosystems, Thermo Fisher Scientific, Inc.). The reaction conditions were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. The fold changes were calculated using the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen 2001). The primer sets for BCL2, IL-6, MMP9, HIF1α, TNF, IL-1β, and ICAM1, and GAPDH genes are listed in Table 1.

Table 1. Primer sequences.

Genes		Primer Sequence (3'-5')
BCL2	Forward	TGGAGAGCGTCAACAGGGAGA
	Reverse	CCCAGCCTCCGTTATCCTGG
IL-6	Forward	GGTACATCCTCGACGGCATCT
	Reverse	GGTACATCCTCGACGGCATCT
MMP9	Forward	GGCGTGTCTGGAGATTCG
	Reverse	TACTGGAAGATGTCGTGTGAG
HIF1α	Forward	GAACGTCGAAAAGAAAAGTCTCG
	Reverse	CCTTATCAAGATGCGAACTCACA
TNF	Forward	CCCAGGCAGTCAGATCATCTTC
	Reverse	AGCTGCCCCTCAGCTTGA
ICAM1	Forward	ACGTTGGATGAGCACTCAAGGGGAGGTCAC
	Reverse	ACGTTGGATGGCTACCACAGTGATGATGAC
IL-1β	Forward	GAAATGCCACCTTTTGACAGTG
	Reverse	TGGATGCTCTCATCAGGACAG
GAPDH	Forward	TCAACGACCCCTTCATTGACC
	Reverse	CTTCCCGTTGATGACAAGCTTC

Table 2. The effects of *Yimucao formula* (YMF) treatment on the cows.

Group	Number of cases	Recovery number	Recovery rate (%)	Significance of number	Significance rate (%)	Effective number	Effective rate (%)
YMF	10	6	60	8	80	9	90
Rifaximin	10	4	40	6	60	7	70
Positive	10	0	0	1	10	2	20
Negative	10	-	-	-	-	-	-

Statistical analysis

All statistical analyses were performed using GraphPad Prism Software (version 6.0, La Jolla, CA, USA). The data are expressed as the means and standard errors of the mean (SEM). Multiple comparisons were performed using one-way analysis followed by Dunnett's test. A p-value of <0.05 was considered significant.

Results

YMF treatment improved the clinical symptoms caused by bovine endometritis

We performed an experimental clinical trial based on the principles of natural product clinical trial techniques and veterinary traditional Chinese medicine in order to verify the impact of YMF uterine perfusate on bovine endometritis. When comparing the YMF group's clinical symptoms to those of the negative and positive groups, the results demonstrated a considerable improvement in the symptoms of endometritis in cows. The cervical mucus was clean and odorless, and the majority of the uterus had restored to normal. In comparison to the negative and positive groups, the cure and effective rates were substantially higher at 60% and 90%, respectively (Table 2). The results showed that YMF significantly improved endometritis in dairy cows.

Screening of active ingredients and targets

The drugs consisted of five herbal medicines, namely Angelica sinensis, Ligusticum, Prunus armeniaca, Zingiber officinale, and Glycyrrhiza uralensis. After eliminating duplicate ingredients, 150 active ingredients from medications were removed. These included 11 active ingredients from Curcuma longa, 4 from Angelica sinensis, 11 from Citrus reticulata, 28 from Prunus persica, 12 from Leonurus heterophyllus, and 92 from Glycyrrhiza uralensis.

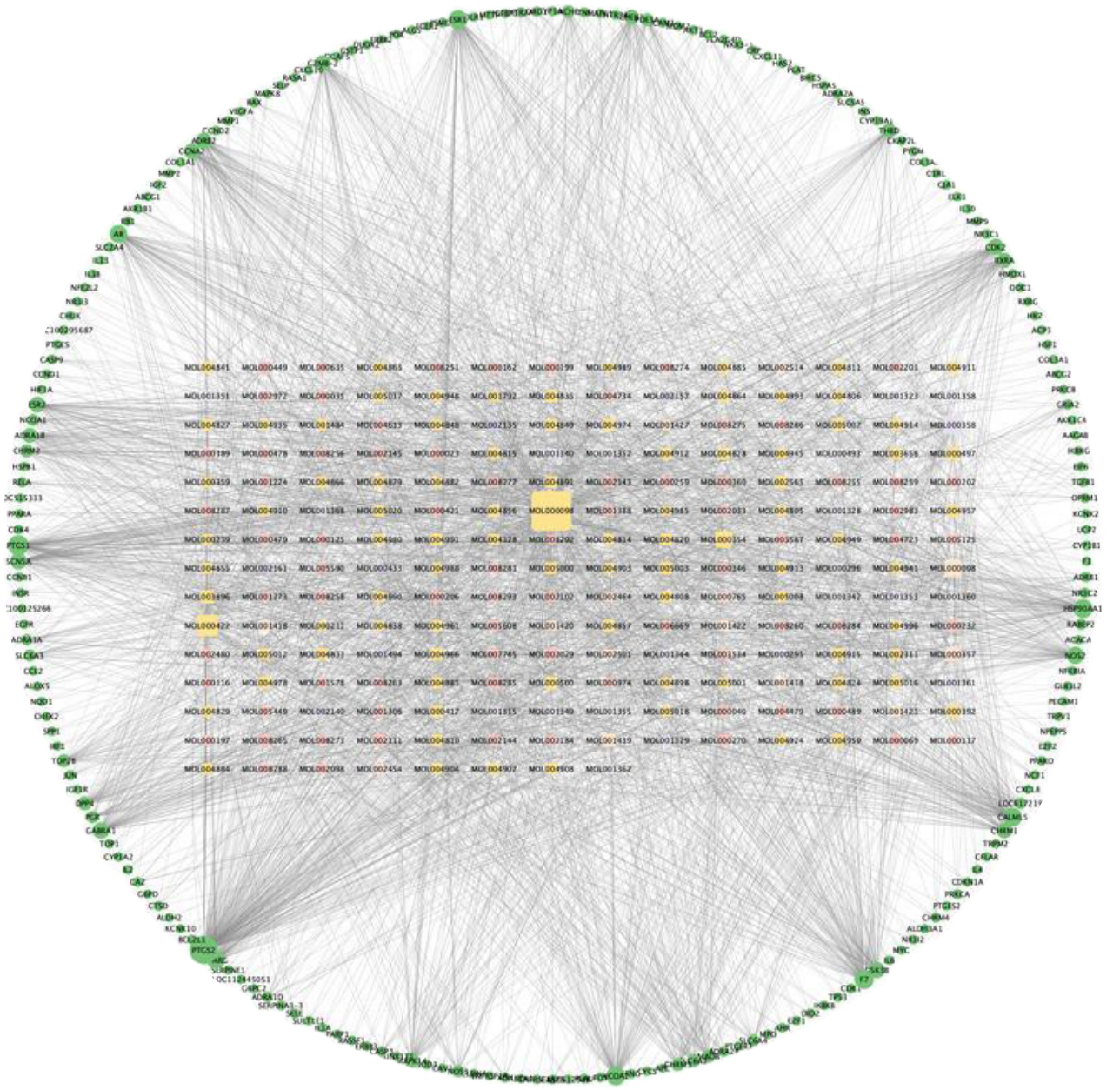

Fig. 1. Compound-target network.

Ingredient analysis of YMF based on the ingredient-target network

All active ingredients of drugs and their targets were matched with the UniProt protein database, obtaining 212 genetic targets. A compound-target network diagram was shown using Cytoscape 3.10.1 software (Fig. 1). Among them, the connection degrees (Degree) of the drug components quercetin, kaempferol, beta-sitosterol, apigenin, isorhamnetin, and Sitogluside were all >40 (Table 3), which were the core target compounds of this Chinese herbal formula. Among all the genetic targets, the degree values of PTGS2, PTGS1, F7, NCOA2, HSP90AA1, ESR1, CALML5, AR, PPARG, NOS2, GABRA1, SCN5A, CDK2, and GZMB-2 were all >60 (Table 4), which were the main targets of the active ingredients of the formula.

Construction of the PPI network for the formula targets against bovine endometritis

In addition, a total of 110 genes associated with bovine endometritis were obtained from GeneCard and OMIM databases, and the genes were entered into the STRING database for obtaining a protein interaction, and finally 17 common targets of bovine endometritis and drugs were obtained (Fig. 2A). Uploading these targets to STRING generated the PPI network comprising 17 nodes, 102 interactions, and an average degree of 12 with a local clustering coefficient of 0.882 (Fig. 2B).

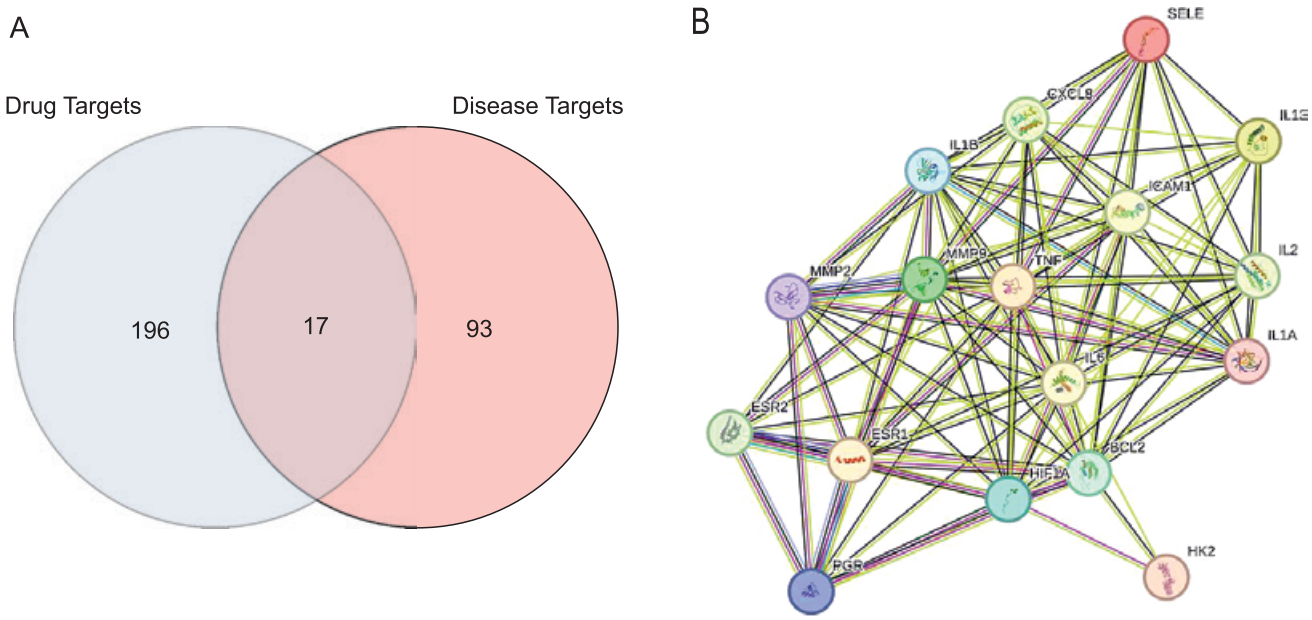

Fig. 2. Construction of the protein-protein interaction (PPI) network for the formula targets against bovine endometritis. (A) Venn diagram of drug targets and disease targets. (B) PPI network for predicting drug-disease targets.

Table 3. The main active ingredients of YMF.

Molecule	MOLID	Degree
quercetin	MOL000098	264
kaempferol	MOL000422	106
beta-sitosterol	MOL000358	84
apigenin	MOL000008	67
isorhamnetin	MOL000354	64
Sitogluside	MOL000357	48

Table 4. The main targets of the active ingredients of YMF.

Target	uniprot_ID	Degree
PTGS2	Prostaglandin G/H synthase 2	164
PTGS1	Prostaglandin G/H synthase 1	95
F7	Coagulation factor VII	86
NCOA2	Nuclear receptor coactivator	84
HSP90AA1	Heat shock protein HSP 90-alpha	84
ESR1	Estrogen receptor	83
CALML5	CALML5 protein	81
AR	Androgen receptor	73
PPARG	Peroxisome proliferator-activated receptor gamma	72
NOS2	Nitric oxide synthase, inducible	70
GABRA1	Gamma-aminobutyric acid receptor subunit alpha-1	62
SCN5A	Sodium channel protein	61
CDK2	Cyclin-dependent kinase 2	61
GZMB-2	Granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	60

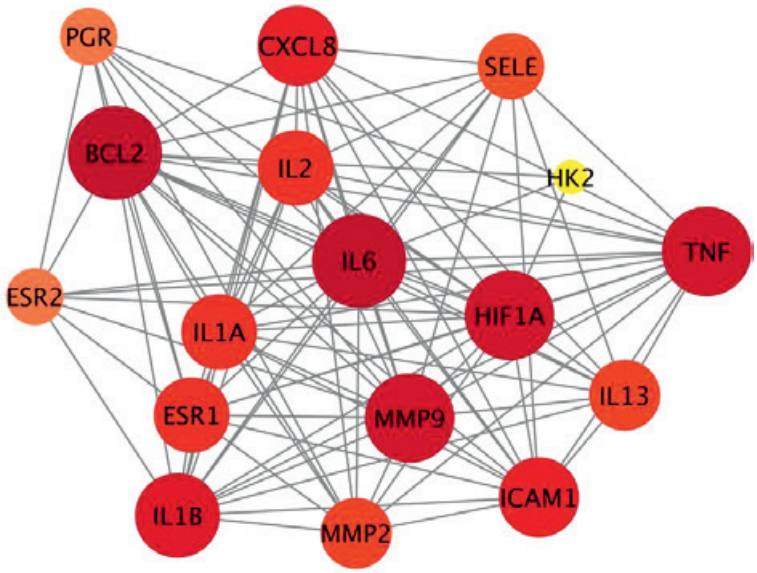

Fig. 3. PPI network diagram based on degree value. The more a target is connected to other targets, the more important the target is.

Screening of core targets

The 17 interaction targets of the formula against endometritis were imported into the Cytoscape software for analysis. As shown in Fig. 3, the size and color of the node were positively correlated with the Degree value; the larger the node and darker colors, the larger the Degree value in this network. In the interaction network, BCL2, IL-6, MMP9, HIF1α, TNF, IL-1β, and ICAM1 were identified as candidate core targets for YMF treatment of bovine endometritis, and may be involved in the YMF against endometritis.

KEGG pathway and GO enrichment analysis of the target network

GO enrichment analysis

Gene Ontology (GO) is the *de facto* standard for gene function description and was widely used in functional annotation and enrichment analysis (Du et al. 2010). To elucidate the biological functions of these major hubs, we analyzed the candidate targets by performing a GO enrichment analysis. As shown in Fig. 4A, 17 targets were significantly enriched ($p<0.01$) in 763 GO terms, including 746 biological processes, 4 cellular components, and 13 molecular functions. The top enriched processes involved leukocyte migration, regulation of inflammatory response, gland development, epithelial cell development, negative regulation of apoptotic signaling pathway, etc. Cellular components indicated the involvement of the mitochondrial outer membrane, organelle outer membrane, and outer membrane. Enriched molecular functions included cytokine activity, cytokine receptor binding, receptor--ligand activity, nuclear steroid receptor activity, etc.

KEGG pathway enrichment analysis

The 17 core targets were subjected to KEGG pathway enrichment analysis, which identified 66 significantly enriched pathways ($p<0.01$). The results showed that the targets were mainly involved in the AGE-RAGE signaling pathway in diabetic complications, fluid shear stress and atherosclerosis, lipid and atherosclerosis, cytokine-cytokine receptor interaction, malaria, inflammatory bowel disease, rheumatoid arthritis, IL-17 signaling pathway, TNF signaling pathway, estrogen signaling pathway, and influenza A (Fig. 4B). Based on the pathway-target network diagram, the AGE-RAGE signaling pathway in diabetic complications, fluid shear stress and atherosclerosis, lipid and atherosclerosis, and cytokine-cytokine receptor interaction pathways were found to involve more gene targets (Fig. 4C).

Molecular docking

To further validate the interactions between drug molecules and targets, molecular docking was performed between representative gene BCL2 protein and related drug molecules. The results demonstrated favorable docking of BCL2 protein with apigenin (LibDockScore 97.4576), β-sitosterol (101.298), kaempferol (97.6562), and quercetin (114.916) through hydrogen bonding and van der Waals forces (Fig. 5). This provided structural evidence that these active ingredients from the herbal formula could directly bind and modulate the BCL2 protein, one of the potential core targets for the treatment of dairy endometritis identified by the network pharmacology analysis. The molecular docking analysis hereby complemented the network pharmacology predictions, supporting the hypothesis that the herbal formula may exert therapeutic effects

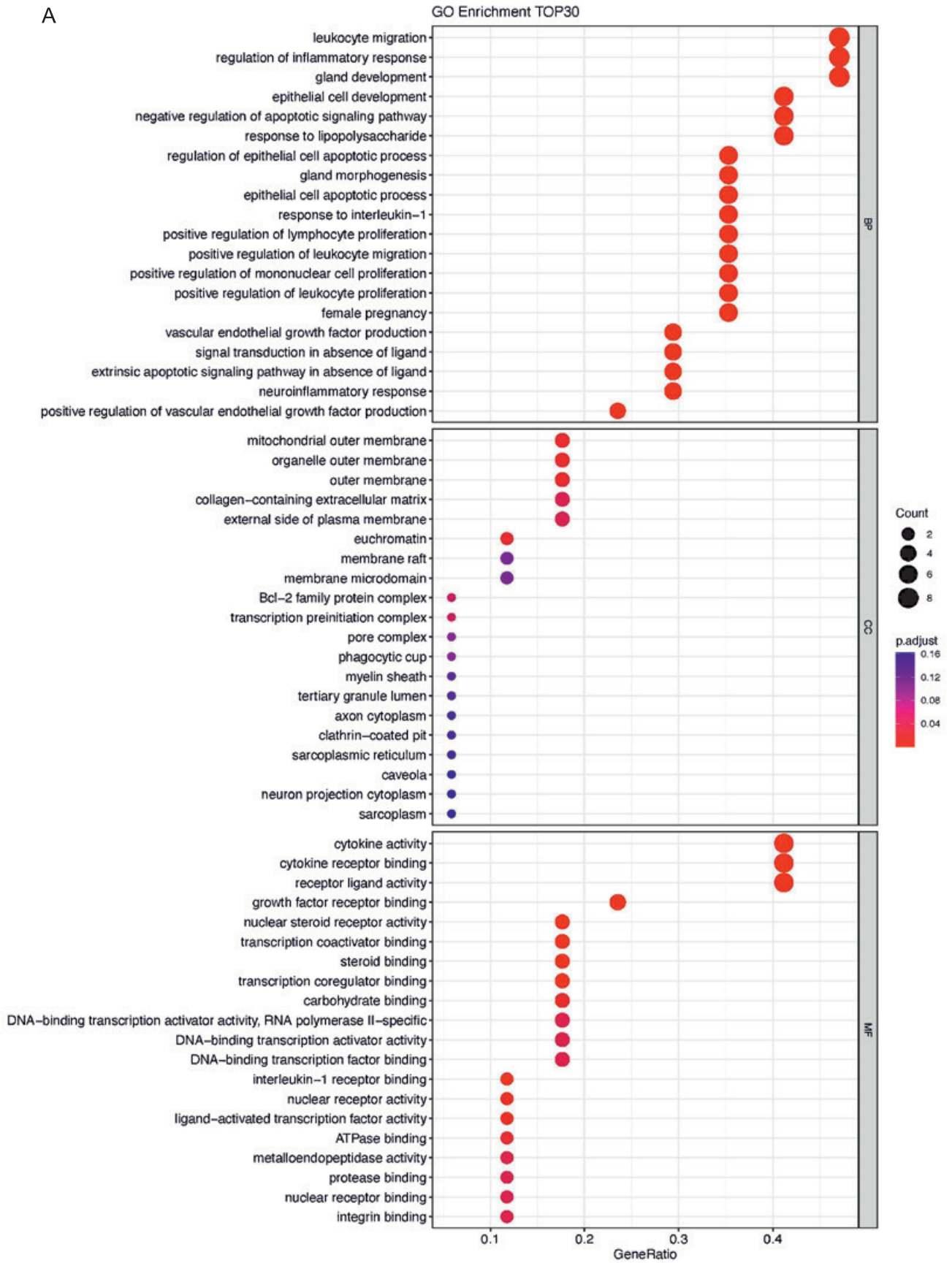

Fig. 4. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) enrichment analysis of the target network. (A) GO function enrichment analysis of core target. Note: Left side of the picture above demonstrates the top 30 significantly enriched BP, CC, and MF categories, while below the picture shows the number of enriched genes for these terms ($P<.05$). BP = biological process, CC = cellular component, MF = molecular function.

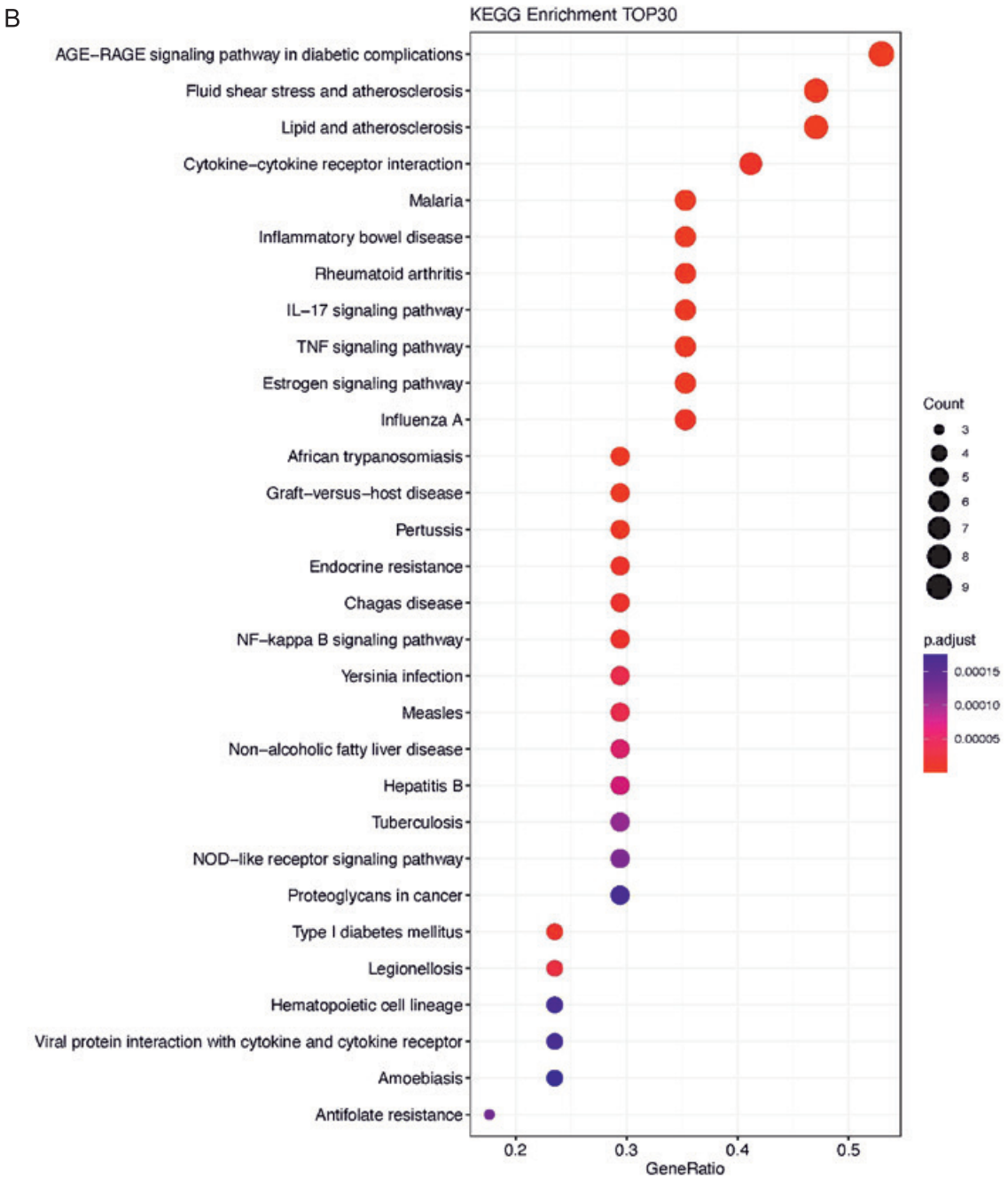

Fig. 4. KEGG pathway and GO enrichment analysis of the target network. (B) Bubble diagram of KEGG pathway enrichment analysis of core target. Note: The y-axis demonstrates the top 30 significantly enriched KEGG pathways, while the x-axis shows the number of enriched genes for these terms (P<.05), the colors and the sizes indicate different P-value ranges; the redder and bigger it is, the more significantly enriched it is.

against the disease through the studied ingredients and targets.

YFM inhibits the inflammatory response and apoptosis in dairy endometritis

On the basis of previous studies and combined with PPI analysis, IL-6, IL-1β, TNF, ICAM1, MMP9, HIF1α and BCL2 selected as biomarkers were quantified to evaluate the therapeutic effects of YMF in the treatment of dairy endometritis. IL-6, IL-1β, TNF, ICAM1, MMP9 and HIF1α were upregulated in the endometritis group compared with the control group. However, the levels of IL-6, IL-1β, TNF, ICAM1, MMP9 and HIF1α were downregulated in YMF + endometritis group, with significant differences compared with the endometritis group, indicating that YMF had greater anti-inflammatory capability (Fig. 6 A-F). Moreover, compared with the endometritis group, BCL2 expression significantly decreased in endometritis group,

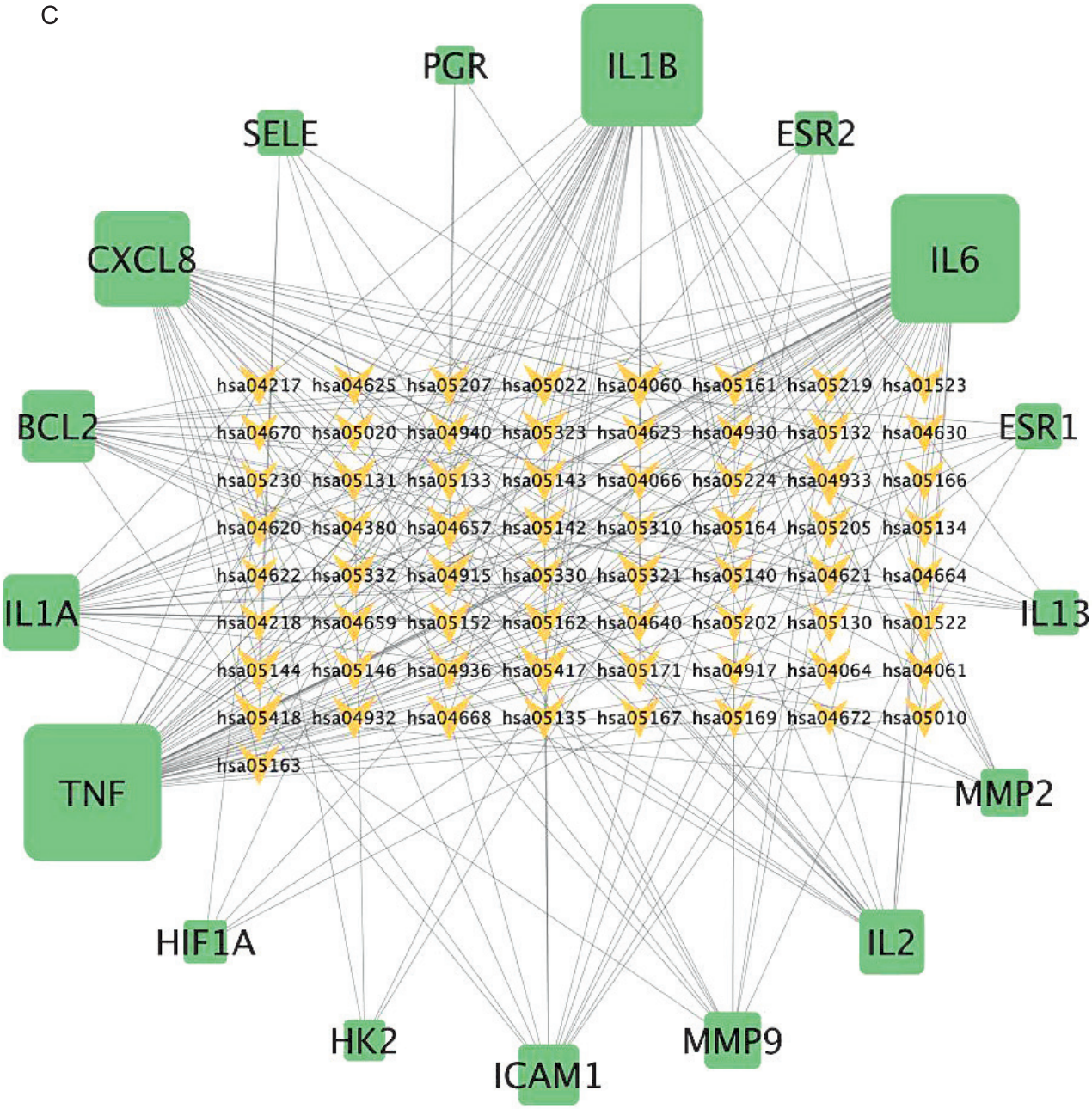

Fig. 4. KEGG pathway and GO enrichment analysis of the target network. (C) Core target screening. Note: Picture above is the core target, where the larger the node, the higher the degree value.

while its expression levels were reversed after YMF in the treatment, as shown in Fig. 6G. Therefore, YMF may exhibit the protective effects on improving dairy endometritis by the anti-inflammatory response and anti-apoptotic effect via regulating the expression of IL-6, IL-1β, TNF, ICAM1, MMP9, HIF1α and BCL2.

Discussion

In this work, we have comprehensively uncovered the molecular mechanism by which YMF efficiently alleviates the clinical symptoms associated with endometritis, based on network pharmacology. According to preliminary findings from network pharmacology, YMF can treat endometritis by controlling key targets such as BCL2, IL-6, MMP9, HIF1α, TNF, IL-1β, and ICAM1, as well as pertinent signaling pathways like AGE-RAGE, fluid shear stress, and atherosclerosis. This paves the way for the unique molecular mechanism of YMF's action against endometritis.

A prevalent ailment among dairy cows, bovine endometritis is thought to be the primary cause of infertility and financial loss for cattle breeding businesses (Raliou et al. 2019). Intrauterine infection may be a trigger of endometritis in dairy cows. When cows are during

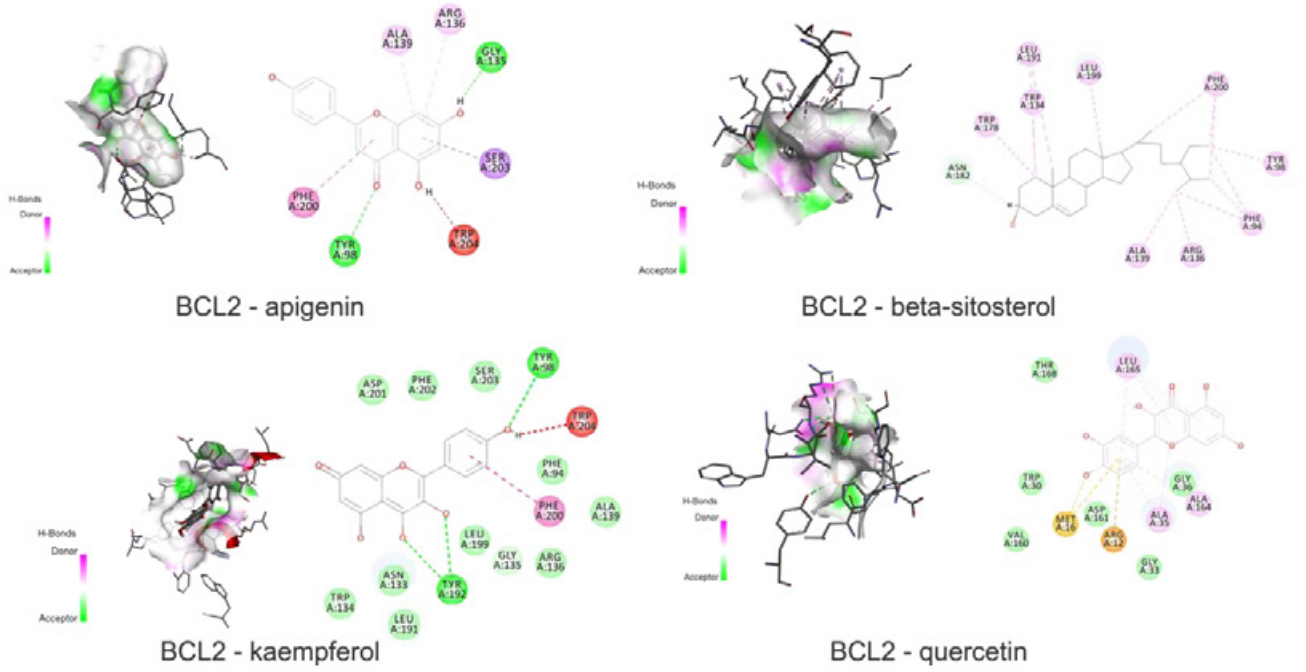

Fig. 5. Visualization result of Molecular docking

or after postbreeding, due to poor hygiene or improper midwifery operation, pathogens may lead to invade the uterus, thus triggering endometritis (Tsuboi et al. 2011, Tsuboi et al. 2013). Causative agents of intrauterine infection include microorganisms, such as bacteria, viruses, protozoa, and fungi, which cause infertility, embryonic death, and abortion in cattle. Among them, bacteria are the most reported agents for bovine endometritis (Williams et al. 2007). Bacterial toxins can harm ovarian function by continuing to affect follicular growth and corpus luteum formation. In severe circumstances, this can even result in anoestrus in cows by extending their estrous cycle. Thus far, a few therapeutic approaches for endometritis in cows have been documented, including intrauterine antibiotic infusions and the introduction of hormones like PGF2α or estradiol (Kasimanickam et al. 2005). Antibiotic-resistant germs, withdrawal periods, and safe and appropriate treatments are required to address these uterine disorders in addition to issues with leftover hormones or antibiotics. On the other hand, due to its potent anti-inflammatory benefits and few side effects, Traditional Chinese medicine (TCM) has drawn the attention of researchers and is currently being used in the treatment of endometritis (Willcox and Bodeker 2004, Buyel 2018). In mice with endometritis caused by *Staphylococcus aureus*, for instance, the pharmacological effects of *Syringa oblata Lindl.* (SOL) and its active ingredients have been confirmed (Wang et al. 2022). Another study discovered that matrine from the traditional Chinese herb *Sophora flavescens* inhibited the TLR2-mediated NF-κB pathway, protecting against Staphylococcus aureus lipoteichoic acid (LTA)-induced endometritis (Jiang et al. 2019). Therefore, multi-component herbal medicines that target important components of this pathogenic mechanism show promise for a safer and more successful resolution of endometritis in cows. The high cure rate of cows in our investigation indicated that YMF has preventive effects against bovine endometritis. More significantly, YMF's effects outperformed those of the clinical medication *Rifaximin*. However, more characterization and identification are required to fully understand the potential molecular mechanism of YMF for the treatment of endometritis in cows.

YMF has the anti-inflammatory properties and the efficacy of this prescription can be explained by the theory of TCM compatibility. However, the molecular mechanisms underlying the effects of YMF are not clear. Hence, in the present study, a set of network pharmacology methods was used for predicting, elucidating, and confirming the potential mechanisms of action of YMF on bovine endometritis by integrating target prediction, network construction, and molecular docking analyses. Following network pharmacology analysis, we discovered that the primary active ingredients exhibiting pharmacological effects were quercetin, beta-sitosterol, apigenin, kaempferol, sitogluside, arachidonic acid, and isorhamnetin, which were all ranked in the top seven based on network topology parameter (Degree). These findings imply that the majority of YMF's components have an impact on several targets. In our study, 212 potential target proteins were identified for the ingredients including Ligusticum, Angelica sinensis, Zingiber officinale, Prunus armeniaca, and

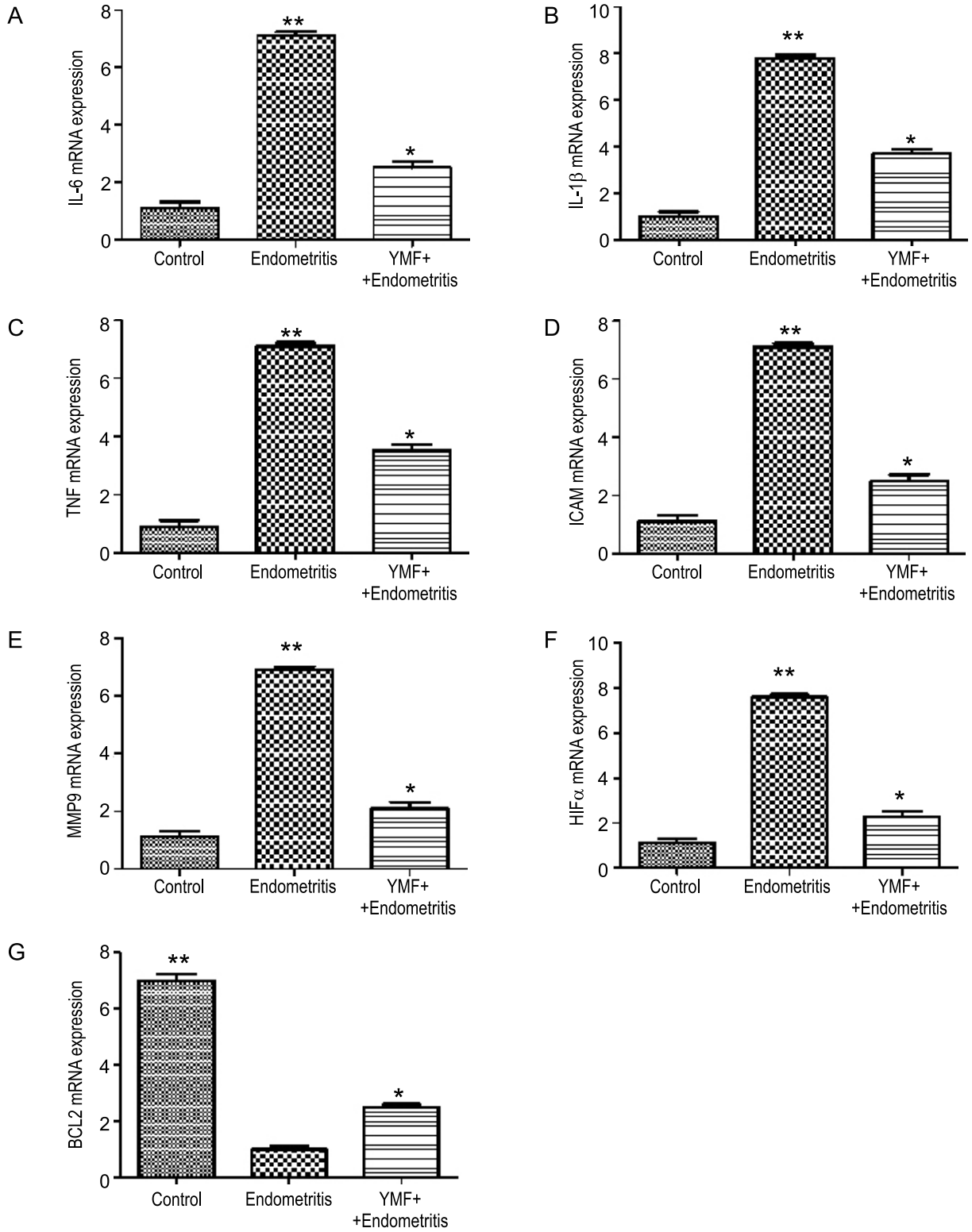

Fig. 6. YMF inhibits inflammatory response in bovine endometritis. The expression levels of BCL2, IL-6, MMP9, HIF1α, TNF, IL-1β, and ICAM1 in endometrial tissues from each group.

Leonurus. Simultaneously, distinct YMF compounds could have the same target, illustrating the synergistic interaction of various active components.

PPI networks are useful tools for comprehending cell functions, disease mechanisms, and medication formulation and repositioning (Vella et al. 2018). Moreover, the biological impact and interaction between YMF-endometritis-linked targets are obtained by the PPI network and GO enrichment analysis. In this work, a PPI network was built to identify the critical targets

from a set of 17 overlapping targets, which may be very promising targets for YMF in its fight against dairy endometritis. These targets include BCL2, IL-6, MMP9, HIF1α, TNF, IL-1β, and ICAM1. As we known, Bovine endometritis is characterized by the activation of Toll-like receptors (TLRs) by *E. coli*, which in turn triggers inflammation and apoptosis. Among them, anti-apoptotic protein Bcl-2 was involved in regulating the apoptotic process in endometrial cells (Chen et al. 2023). MMP9 has been confirmed to be associated with inflammation in endometritis (Jiang et al. 2017). HIF1 α, IL-1β, IL6 and TNF may affect the health of the endometrium by regulating the inflammatory response and tissue repair, while ICAM 1 may participate in the recruitment and activation of immune cells, and can be regulated by the active components of YMF (Wu et al. 2023). Moreover, previous studies indicated that the inhibition of inflammation and apoptosis could attenuate endometritis (Lv et al. 2015, Zhang et al. 2019, Shaukat et al. 2024). In our study, the levels of IL-6, IL-1β, TNF, ICAM1, MMP9 and HIF1α were significantly upregulated, while BCL2 was downregulated in the endometritis group compared with the control group. However, the levels of IL-6, IL-1β, TNF, ICAM1, MMP9 and HIF1α were downregulated, BCL2 was upregulated after YMF treatment, indicating that YMF had greater anti-inflammatory and anti-apoptotic capability.

Endometritis is a local inflammation related to immune recognition disorders. The KEGG enrichment results indicate several pathways related to inflammation and oxidative stress: the AGE-RAGE signaling pathway in diabetic complications, fluid shear stress and atherosclerosis, lipid and atherosclerosis, and cytokine-cytokine receptor interaction pathways. Studies suggest that endometritis may cause increased inflammation and oxidative stress in local tissues, and these changes may increase the formation and accumulation of AGEs. AGEs bind to RAGE to activate transcription factors such as NF-κB and promote the expression and release of inflammatory factors such as IL-1β, IL-6 and TNF-α. The increase in these inflammatory factors will further attract immune cells such as neutrophils and macrophages to the sites of inflammation, exacerbating the inflammatory response (Kay et al. 2016, Asadipooya and Uy 2019). In our study, as suggested by the KEGG enrichment results, YMF may improve the inflammatory response through regulating AGE-RAGE signaling pathway. It has also been confirmed by other studies that YMF exerts anti-inflammatory effect by downregulating the expression of proinflammatory cytokines and inhibiting AGE-RAGE signaling pathway (Wu et al. 2023).

There are some limitations in our studies. Our study initially elucidated the molecular mechanism based on network pharmacology integrating molecular docking and pharmacodynamics, and further studies are needed to validate the biological processes and pathways of YMF against endometritis. Future animal and clinical research aimed at creating potent herbal treatments for dairy endometritis, a serious reproductive condition plaguing the global dairy industry, can be guided by this scientific paradigm.

Taken together, our data indicate that YMF could alleviates the clinical symptoms associated with endometritis. Network pharmacology results preliminarily indicate that YMF can be used for treating endometritis by regulating the core targets, including BCL2, IL-6, MMP9, HIF1α, TNF, IL-1β, ICAM1 and relevant signaling pathways, which lays a foundation for the specific molecular mechanism of YMF against endometritis. In future studies, the individual compounds causing the therapeutic effects should be identified to clarify their mechanism of action, and corroborate the target proteins and the related signaling pathways.

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