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In silico evaluation of kuwanon compounds as antiviral agents targeting H9N2 influenza virus

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Author contributions

Ansari Vikhar Danish Ahmad contributed to conceptualization, investigation, validation, and writing-review and editing. Ansari Altamash was responsible for methodology and software. Subur W. Khan provided resources and handled data curation. Mohd Mukhtar Khan contributed to investigation and validation. Sarfaraz Khan worked on writing the original draft. Yasar Qazi conducted molecular docking, and contributed to software and data curation. Syed Iftequar Ahmed contributed to writing-review and editing. All authors carefully reviewed and approved the final version of the manuscript.

Competing interests

The authors declare no conflicts of interest.

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Peer review information

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Abbreviations

PPI, protein-protein interaction; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomic analysis; IFNB1, human interferon-beta crystal structure; PDB, Protein Data Bank; CCL5, C-C motif chemokine ligand 5; DDX58, DEAD (Asp-Glu-Ala-Asp) Box Polypeptide 58, IL1B, interleukin 1 beta, PTGS2, prostaglandin-endoperoxide synthase 2, IFIH1, interferon induced with helicase C domain 1; H9N2, subtype avian influenza A.

Citation

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Abstract

Background: The threat of avian influenza a subtype avian influenza A (H9N2) virus remains a significant concern, necessitating the exploration of novel antiviral agents. This study employs network pharmacology and computational analysis to investigate the potential of kuwanons, a natural compounds against H9N2 influenza virus. **Methods:** Leveraging comprehensive databases and bioinformatics tools, we elucidate the molecular mechanisms underlying Kuwanons pharmacological effects against H9N2 influenza virus. Network pharmacology identifies H9N2 influenza virus targets and compounds through integrated protein-protein interaction and Kyoto Encyclopedia of Genes and Genomes analyses. Molecular docking studies were performed to assess the binding affinities and structural interactions of Kuwanon analogues with key targets, shedding light on their potential inhibitory effects on viral replication and entry. **Results:** Compound-target network analysis revealed complex interactions (120 nodes, 163 edges), with significant interactions and an average node degree of 2.72. Kyoto Encyclopedia of Genes and Genomes analysis revealed pathways such as Influenza A, Cytokine-cytokine receptor interaction pathway in H9N2 influenza virus. Molecular docking studies revealed that the binding free energy for the docked ligands ranged between −5.2 and −9.4 kcal/mol for the human interferon-beta crystal structure (IFNB1, Protein Data Bank: 1AU1) and −5.4 and −9.6 kcal/mol for Interleukin-6 (IL-6, PDB: 4CNI). **Conclusion:** Our findings suggest that kuwanon exhibits promising antiviral activity against H9N2 influenza virus by targeting specific viral proteins, highlighting its potential as a natural therapeutic agent in combating avian influenza infections.

Keywords: network pharmacology; molecular docking; kuwanons; H9N2 influenza virus; natural compounds

Background

Influenza is a serious infectious disease affecting the respiratory system and has a significant impact on morbidity and mortality worldwide. Annually, it causes an estimated 290,000 to 650,000 deaths, underscoring its severe implications. Among the different types, influenza A viruses are notably adaptable and can infect a wide range of hosts, including humans, pigs, and various bird species [1]. The subtype avian influenza A (H9N2) influenza virus, part of the Orthomyxoviridae family, is particularly noteworthy due to its broad host range and its role in creating new reassortant viruses. First isolated in turkeys in Wisconsin in 1966, H9N2 influenza virus has since been found in regions including Asia, the Middle East, Africa, and Europe, highlighting its global spread and importance in avian populations [2, 3]. Ecologically, H9N2 influenza virus has established itself in various avian hosts, with domestic poultry being primary reservoirs. This wide host range allows for the development of genetic variants and reassortants, which complicates control efforts [4, 5]. The ability of H9N2 influenza virus to transmit between species, especially its role in generating new influenza viruses through reassortment with other influenza A viruses subtypes, emphasizes its importance in the emergence of zoonotic diseases [6].

H9N2 influenza virus possess distinct molecular features that enhance their virulence and adaptability to mammalian hosts. These attributes improve the virus's capacity to bind to human-like receptors and facilitate efficient replication in mammalian cells, underscoring their potential to infect humans [7, 8]. Since the late 1990s, sporadic human infections with H9N2 influenza virus have been reported, typically manifesting as mild upper respiratory tract illnesses. These cases highlight the virus's ability to cross species barriers [3, 9]. The continuous evolution of H9N2 influenza virus, coupled with their ecological and epidemiological significance, calls for ongoing surveillance and research. These efforts are essential to understand their pathogenicity, transmission dynamics, and potential public health impacts. Implementing effective control strategies against H9N2 influenza virus. Viruses is imperative to protect the poultry industry and avert possible public health crises arising from these avian influenza strains.

Recent research underscores the antiviral properties of plant extracts and their constituents, presenting substantial evidence for their effectiveness against a variety of viruses [10, 11]. Significantly, over 40% of modern pharmaceuticals are derived from botanical sources [12]. Plant-derived small molecules have shown particular efficacy against influenza viruses. For instance, catechins from green tea have demonstrated notable inhibitory effects against influenza A and B viruses *in vitro* [13]. The H9N2 influenza virus which has the capability to infect humans, contributes genetic material to other human-infective influenza strains such as H7N9, H5N1, H5N6, and H10N8, posing significant threats [14–16].

Natural compounds not only serve as vital precursors for developing new antiviral agents but also offer safer and more cost-effective methods for virus prevention [17, 18]. *Morus alba L.,* commonly known as mulberry, is used in various forms including food products and traditional medicine. It is recognized for its wide range of therapeutic benefits, such as anti-inflammatory, antioxidative, and cardioprotective effects [19, 20]. The antiviral properties of *M. alba L.* have been validated against pathogens like herpes simplex virus-1, influenza virus, and human norovirus [21, 22]. Kuwanons are selected as the main antiviral candidates because of the potent bioactivity, by targeting viral proteins and pathways that inhibit them. They were found to possess great antiviral potency, especially against diseases like herpes simplex virus and other viral infections [23]. Kuwanons have an edge over others because they are low-toxicity flavonoid compounds with good bioavailability and come from natural sources. Their molecular structure provides them an excellent ability to interface with viral enzymes or receptors; thus, they look promisingly useful therapeutically when compared to other candidates. Network pharmacology is an integrative approach that studies the interactions between drugs, proteins, genes, and diseases within biological networks. It aims to identify the molecular mechanisms underlying drug effects by considering the complexity of multiple targets and pathways. This method enhances drug discovery by focusing on polypharmacology and system-level therapeutic strategies rather than single-target drugs. The node degree refers to the number of connections (or edges) a specific node (representing a target, compound, or gene) has within a biological network. A higher node degree indicates that the node is more highly connected, suggesting that it plays a more central role in the network's structure and potentially has greater biological relevance. This concept is often used to identify key targets or compounds with a broad impact on disease mechanisms or therapeutic outcomes. In essence, nodes with higher degrees can be critical for drug efficacy, as they may influence multiple biological pathways simultaneously. The clustering coefficient measures the degree to which nodes (e.g., genes, proteins, or compounds) in a network tend to cluster together. It reflects the local density of connections around a particular node, indicating how likely its neighbors are to be connected to each other. A high clustering coefficient suggests that a node is part of a tightly connected group, which may represent functional modules or pathways in biological systems. This metric is useful for understanding the organization of complex networks, such as protein-protein interaction (PPI) networks, and can provide insights into the robustness and efficiency of drug-target interactions or disease mechanisms.

Influenza remains a significant global health concern, with the H9N2 strain posing particular challenges due to its potential for zoonotic transmission and pandemic threats. Current therapeutic approaches, including M2 channel blockers and neuraminidase inhibitors, have proven effective in the past but now face considerable limitations. The emergence of drug-resistant viral strains and the occurrence of adverse effects associated with these therapies underscore an urgent need for novel antiviral strategies. In this context, the study explores the potential of Kuwanon compounds as promising antiviral agents against the H9N2 influenza virus. By integrating network pharmacology and molecular docking techniques, the research aims to elucidate the molecular interactions and pathways through which these compounds exert their antiviral effects. This investigation seeks to identify novel therapeutic candidates with enhanced efficacy and reduced likelihood of resistance, offering a valuable contribution to the development of next-generation influenza treatments.

Materials and methods

Protein-protein interaction network

Protein-protein interactions are critical for understanding how proteins work in diverse metabolic pathways. This study improves our understanding of cellular structure, biological processes, and functional mechanisms. We used STRING 11.0 (https://string-db.org/) to map the complex interactions between proteins [24]. Data for key genes were uploaded to the STRING database to better understand the complexity of these interactions. The PPI network was designed exclusively for the human genome, "Homo sapiens", with a strict confidence level set above 0.9 to assure high reliability. The network's graphical representation presents individual proteins as nodes and their interactions as edges, effectively displaying the web of connections between various protein entities. We have identified the disease-overlapped genes between disease-specific targets and compound-related targets using comprehensive analysis with the Gene Cards and Swis Target Prediction databases. These selected genes for STRING analysis are used to explore their PPI network, which can be employed in obtaining more profound insight into the molecular mechanism of the pathology of the H9N2 influenza virus. Overlapping genes have been selected for the reason that they may provide critical nodes in the interaction network, thus representing shared pathways or biological processes relevant to both the disease and the therapeutic compound. These genes were associated with H9N2 influenza based on their involvement in viral replication, modulation

of immune responses, and inflammatory pathways, evidenced from previous literature evidence and database annotations. The selection was based on gene expression levels, functional annotation, and their known or predicted interaction with key proteins implicated in the pathogenesis of H9N2 virus infection.

Selection of compound and disease target genes

The first stage in developing the compound-target network is to identify the genes related with the disease. We methodically gathered information on target genes associated with the H9N2 influenza virus from known databases such as GeneCards (https://www.genecards.org) [25]. Additionally, extensive data on Kuwanon's protein targets were obtained from SwissTargetPrediction (http://www.swisstargetprediction.ch/).

Construction of a compound-target network

Following the protein-protein interaction research, we sought to understand the complicated molecular pathways involved. This was accomplished by creating a thorough compound-target network with the Cytoscape program (version 3.7.1) [26]. This network design is critical for understanding and studying the interactions between bioactive chemicals and their targets, which aids in the identification of routes within this complex biological system.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation

We used the ShinyGo platform to annotate GO and KEGG pathways [27]. The GO analysis assessed the gene clusters inside the network, improving the accuracy of data prediction. GO is a systematically organized collection of standardized words linked to biological processes, molecular activities, and cellular components, which includes both curated and predicted gene annotations from different species. Annotating biological processes with GO is extremely useful for pathway enrichment analysis, allowing the identification of essential biological processes within the study's environment. In addition, KEGG was used to investigate the activities and metabolic pathways of genes and compounds inside the network. This research helps to discover pathways linked with disease characteristics and provides insights into the complicated relationships between molecular entities and disease mechanisms [28].

Molecular docking

Software tools

Target proteins were retrieved using the Protein Data Bank (PDB) (https://www.rcsb.org/). Following recovery, the target structures were refined and ready for docking with Discovery Studio Visualizer 2020. This preparation includes the deletion of unwanted water molecules and associated ligands from the protein structure, followed by the storing of the optimized structures in the PDB file format within the same directory. Docking experiments of selected ligands and authorized medicines (Umifenovir, Favipiravir, and Baloxavir_Marboxil) were performed with AutoDock Vina 1.1.2 integrated into PyRx 0.8. The results were evaluated and shown with Discovery Studio Visualizer 2020 and UCSF Chimera to reveal interactions with the individual amino acids.

Ligand preparation

The ligand and authorized standard drug structures, which are provided in SDF format, were obtained from the official website of the United States National Library of Medicine PubChem (https://pubchem.ncbi.nlm.nih.gov/), as shown in Table 1. These structures were then imported into PyRx 0.8 via the open Babel tool, and energy minimization (optimization) was performed using fundamental parameters such as element, hybridization, and connection based on the Universal Force Field. The ligand structures were then translated into the AutoDock Ligand format (PDBQT).

Target preparation

Molecular docking investigations of chosen natural chemicals and approved standard medicines were carried out with PyRx 0.8 and AutoDock Vina 1.1.2. To find prospective options for treating the avian influenza A (H9N2 influenza virus), we performed molecular docking studies on two critical proteins: interferon-beta 1 (IFNB1: PDB-1AU1) and interleukin-6 (IL-6: PDB-4CNI). The crystal structures of both targets, 1AU1 and 4CNI, were obtained from the RCSB Protein Data Bank, each with a resolution of 2.20 Å, as shown in Figure 1. Discovery Studio Visualizer 2020 was used to optimize, purify, and prepare the viral protein structure for docking. This involved removing unwanted water molecules and associated ligands from the protein structure. The optimized structure was then stored as a PDB file within the same directory.

Table 1 2D structures of compounds with their ID

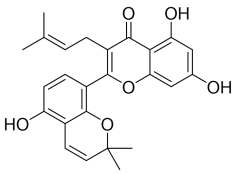
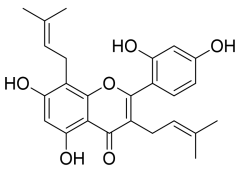
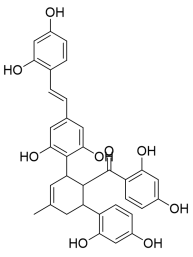
Compound	Drug ID	2D structure
Kuwanon A	44258296	
Kuwanon C	5481958	
Kuwanon X	14334306	

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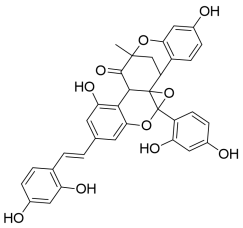
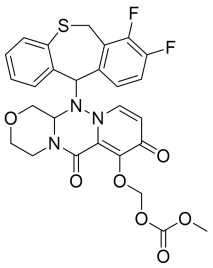
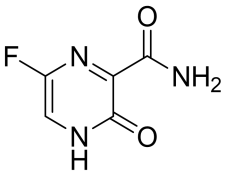
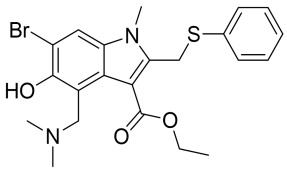
Table 1 2D structures of compounds with their ID (*continued*)

Compound	Drug ID	2D structure
Kuwanon Z	21594954	
Baloxavir marboxil	124081896	
Favipiravir	492405	
Umifenovir	131411	

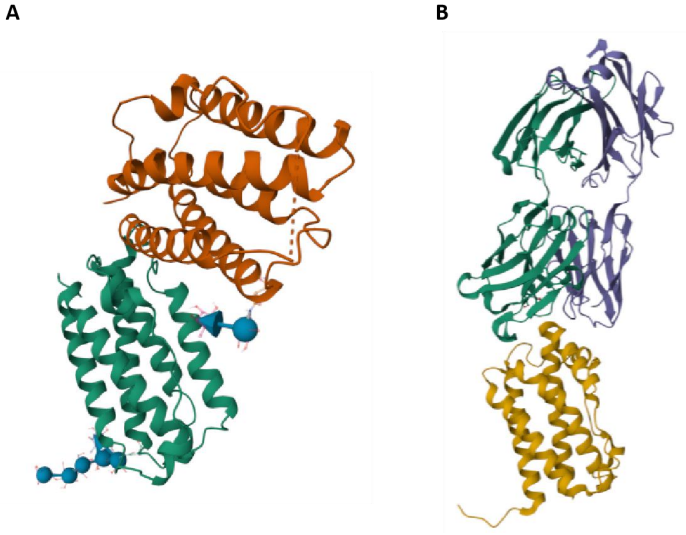


Figure 1 Protein targets. (A) IFNB1 (PDB ID: 1AU1). (B) IL- 6 (PDB ID-4CNI)

Docking procedure

The target proteins' structures were entered into the docking software PyRx 0.8 using the "load molecule" option in the File toolbar. The receptor structure was then translated to AutoDock macromolecule format (PDBQT) using the right-click option. The Vina Wizard Tool from PyRx 0.8 was used to conduct binding affinity tests. The PDBQT files of both ligands and targets (Supplementary File) were used in the docking process. For molecular docking simulation, the three-dimensional grid box (size_x = 5.90Å; size_y = 31.76Å; size_z = 39.21Å) for Interferon-beta (PDB: 1AU1) and (size_x = 85.67Å; size_y = 12.23Å; size_z = −25.77Å) for Interleukin-6 (PDB: 4CNI) was designed using AutoDock tool 1.5.6 with an exhaustiveness value of 8. Grid box dimensions and exhaustiveness values are chosen based

on receptor-ligand properties and computational constraints. These parameters must be explicitly justified to demonstrate that the study thoroughly explores the binding interactions while maintaining computational efficiency. This study used an integrated experimental procedure integrating network pharmacology and molecular docking, based on the approaches published in previous research [29].

Results

PPI network analysis

The resulting PPI network, shown in (Figure 2), has 120 nodes and 163 edges, with each edge indicating a unique protein-protein interaction. The average node degree, which indicates how many connections each target has inside the network, was found to be 2.72. A local clustering coefficient of 0.386 suggests that the network's goals are well interconnected. Analysis of the PPI network revealed important targets, including TNF, C-C motif chemokine ligand 5 (CCL5), interferon gamma, DEAD (Asp-Glu-Ala-Asp) Box Polypeptide 58 (DDX58), interleukin 1 beta (IL1B), prostaglandin-endoperoxide synthase 2 (PTGS2), interferon induced with helicase c domain 1 (IFIH1) in relation to H9N2 influenza virus. Furthermore, the pivotal position of TNF, IL6, IFNB1, IFNG, and IL1B in the network suggests that they play an important role in the pathogenesis of the H9N2 influenza virus.

Compound-target network

Data were imported into Cytoscape software to investigate the signaling cascades and functional repercussions associated with the selected target genes, allowing for the creation of a complex compound-target network. (Figure 3) depicts the network, highlighting interactions between compounds, targets, and diseases, revealing the complex mechanisms behind the compounds' pharmacological effects in H9N2 influenza virus treatment. This network included four different components and their interactions with protein targets. Notably, the research emphasized the convergence of numerous components on different targets, implying the possibility of synergistic management by active biological entities. This complex interplay could improve the therapeutic efficacy of these medicines in treating H9N2 influenza virus and alleviating other related diseases and disorders.

GO annotation

A GO enrichment analysis was used to investigate the target proteins. ShinyGO settings were altered based on three criteria for evaluating target genes: GO biological processes (Table 2 and Figure 4A), GO molecular functions (Table 3, Figure 4B), and GO cellular components (Table 4, Figure 4C). The investigation focused primarily on major

Table 2 GO biological process

Description	*P*-value
Response to virus	1.07E−22
Defense response to virus	1.07E−22
Defense response to symbiont	1.07E−22
Response to cytokine	2.76E−17
Biological process involved in interspecies interaction between organisms	4.84E−16
Viral life cycle	8.70E−15
Cellular response to cytokine stimulus	8.70E−15
Regulation of viral process	1.38E−14
Viral process	2.45E−14
Response to external biotic stimulus	2.45E−14

Table 3 GO molecular process

Description	*P*-value
Double-stranded rna binding	0.000287
Cytokine receptor binding	0.000287
Cytokine activity	0.000919
Signaling receptor binding	0.001513
Protein dimerization activity	0.00177
Heparan sulfate binding	0.002052
Single-stranded rna binding	0.003444
Exogenous protein binding	0.003897
Interleukin-6 receptor binding	0.004765
Sirna binding	0.009144

Table 4 GO cellular components

Description	*P*-value
Cell surface	0.01178
Host cellular component	0.01178
Host cell	0.01178
Membrane raft	0.01178
Membrane microdomain	0.01178
T cell receptor complex	0.029333
External side of plasma membrane	0.031707
Endolysosome membrane	0.033749
Endoplasmic reticulum lumen	0.034164
Vesicle lumen	0.039368

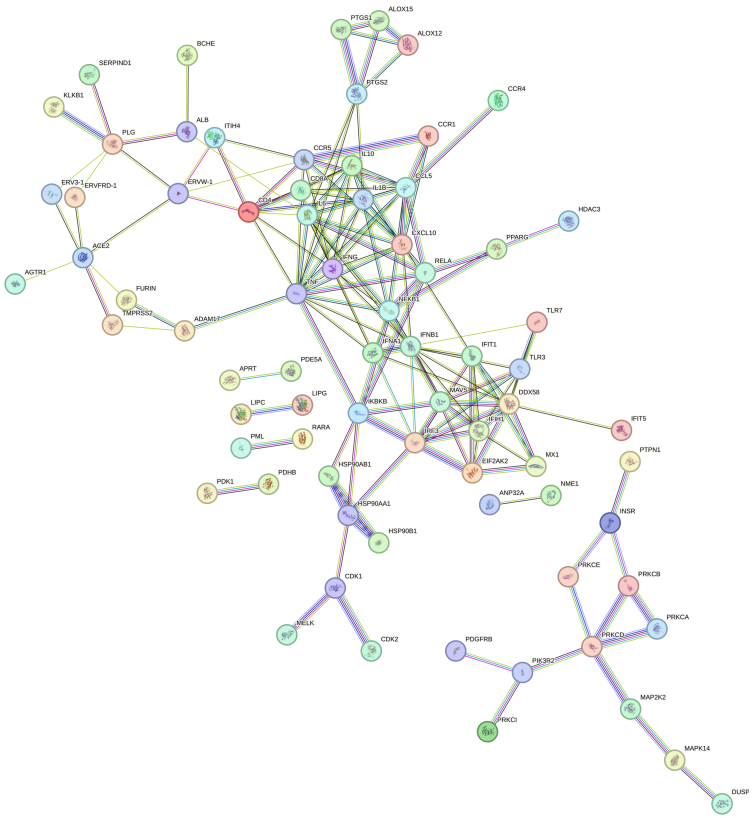


Figure 2 PPI network of kuwanon's in H9N2. Illustrating the functional and physical interactions between various proteins. Nodes represent individual proteins, while the connecting edges indicate interactions, providing insights into cellular processes, signaling pathways, and potential targets for therapeutic interventions.

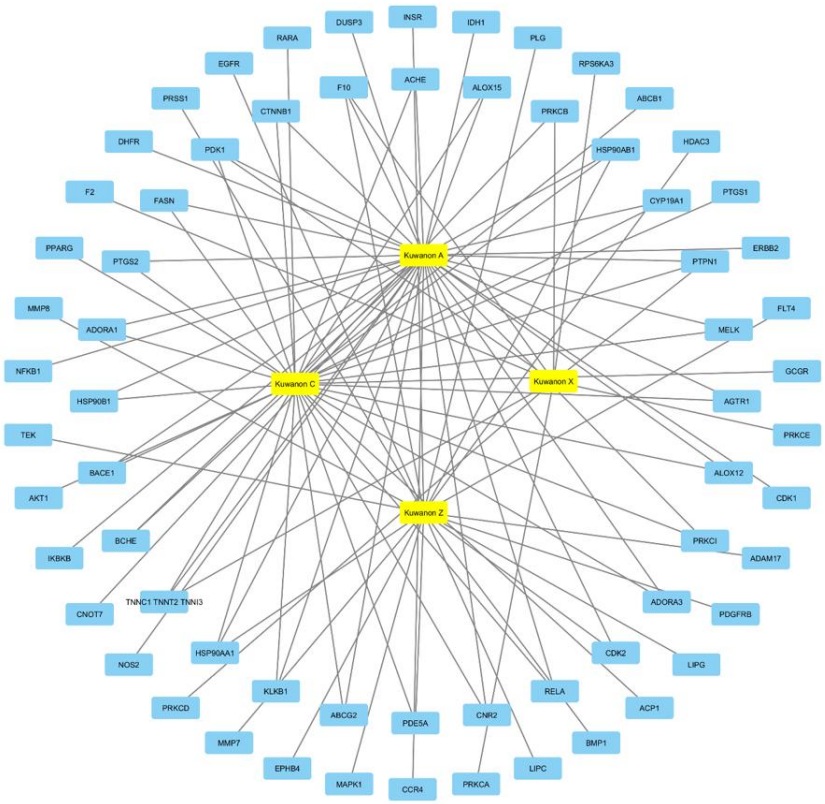


Figure 3 Compound target network. This network illustrates interactions among compounds, protein targets, and diseases, revealing synergistic mechanisms that enhance the therapeutic efficacy of agents against H9N2 influenza and related disorders.

KEGG pathways (Table 5, Figure 4D). The GO term fusion was confined to $P < 0.05$. Comprehensive GO and KEGG analyses revealed substantial relationships with pathways including H9N2 influenza virus. Influenza A, cytokine-cytokine receptor interaction, chemokine signaling pathway, and TNF signaling pathway (Figure 5–7). Kuwanon has shown promise in treating a variety of illnesses, including COVID-19, Measles, Hepatitis C, Yersinia infection, Human Cytomegalovirus infection, Tuberculosis, HIV-1 infection, Inflammatory Bowel Disease, and Viral Carcinogenesis. Although initially chosen to investigate the effects of the H9N2 influenza virus, the thorough KEGG analysis indicated that these genes are involved in a variety of illness pathways and disorders. These varied connections, as well as the visible network, show that Kuwanon has the potential to be a novel pharmacotherapeutic drug for treating a wide range of diseases and conditions.

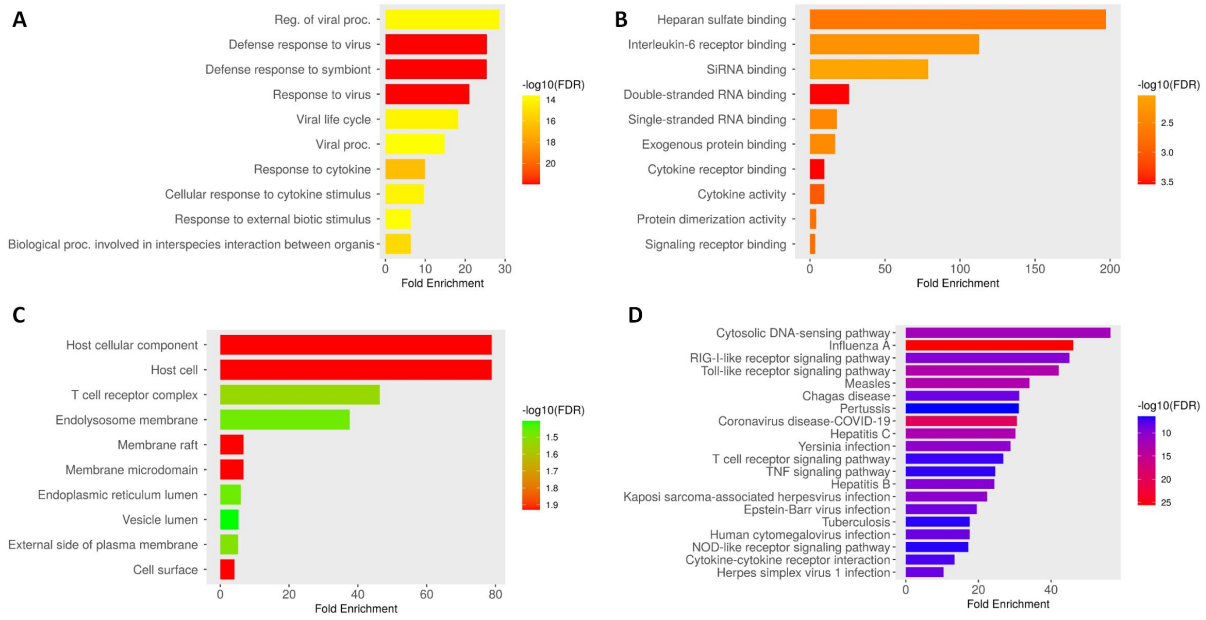


Figure 4 GO, (A) Biological process. (B) Molecular function. (C) Cellular component. (D) KEGG pathway

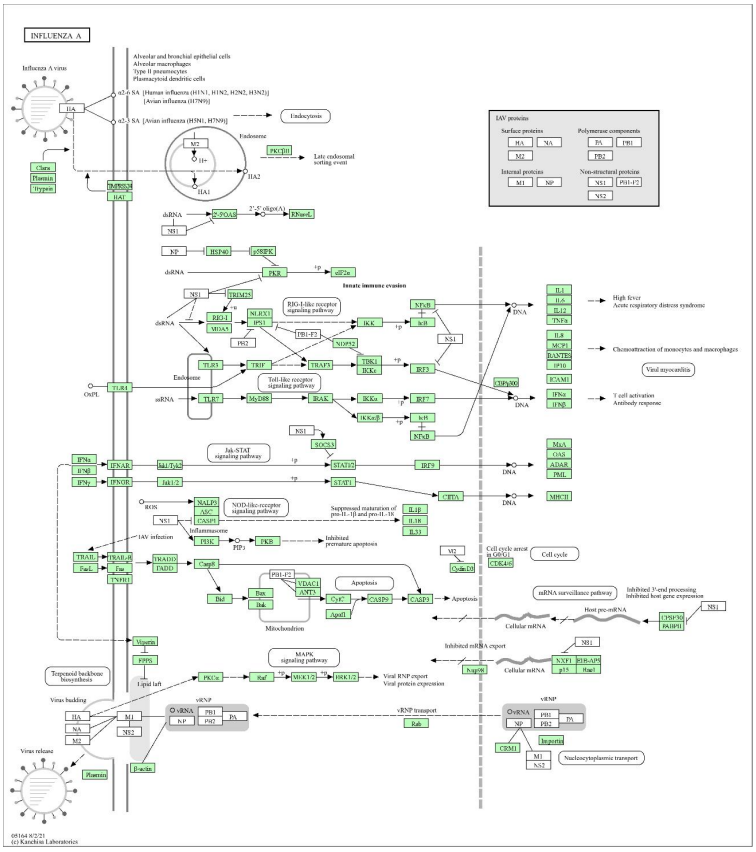


Figure 5 Influenza A pathway

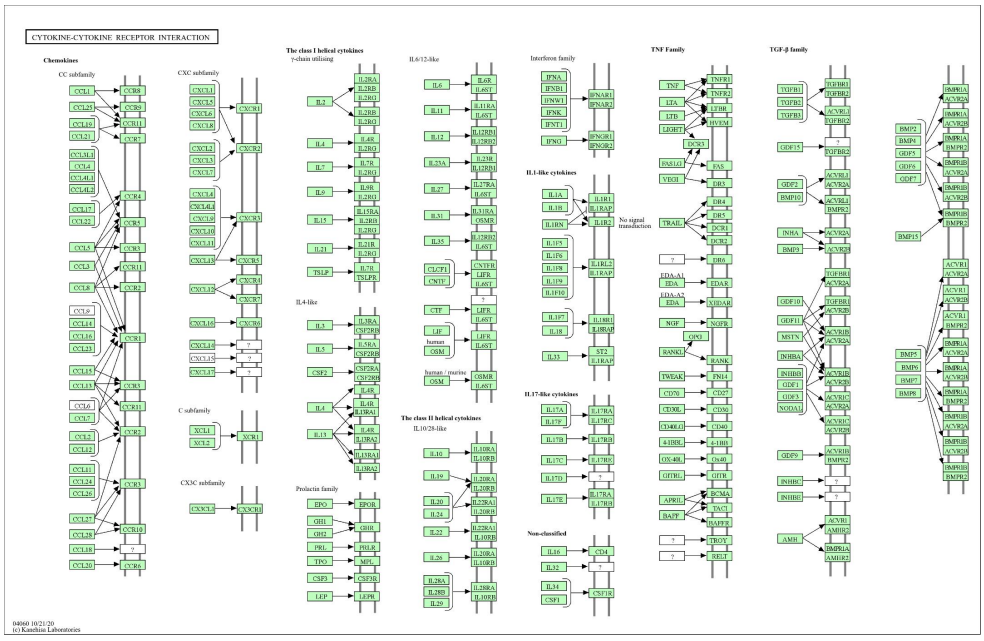


Figure 6 Cytokine-cytokine receptor interaction pathway

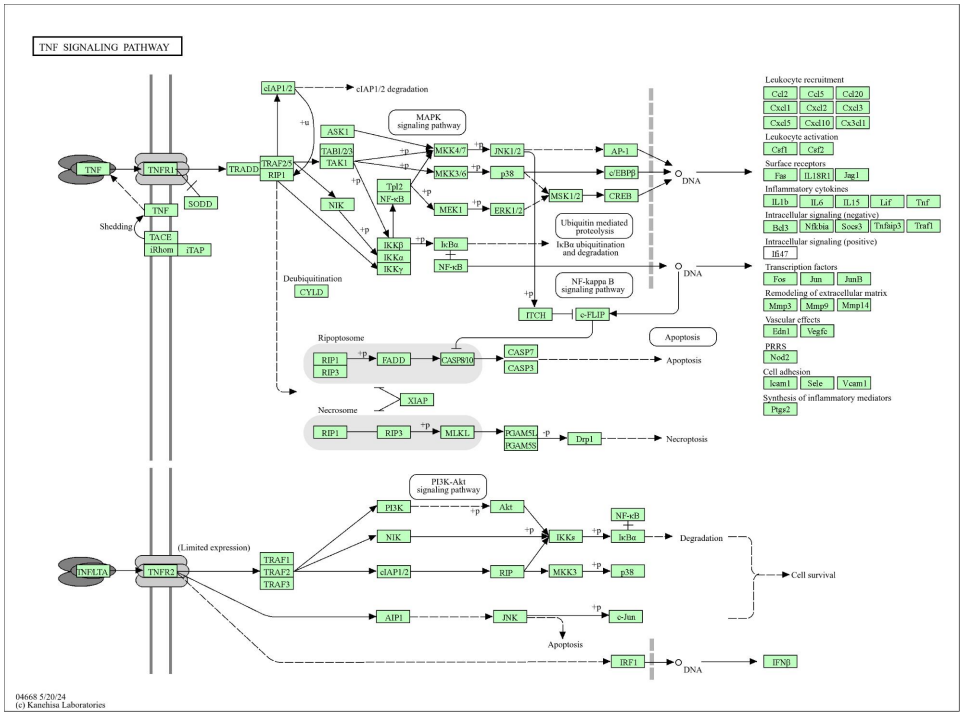


Figure 7 TNF signaling pathway

Table 5 KEGG Pathways

Description	*P*-value
Influenza A	2.56E-26
Coronavirus disease-COVID-19	1.94E-20
JAK-STAT signaling pathway	1.79E-05
Toll-like receptor signaling pathway	7.98E-14
TNF signaling pathway	1.51E-07
Th17 cell differentiation	2.14E-06
IL-17 signaling pathway	1.02E-06
Cytosolic DNA-sensing pathway	1.50E-12
RIG-I-like receptor signaling pathway	1.75E-10
Cytokine-cytokine receptor interaction	4.00E-08

Molecular docking

The chosen ligands were successfully docked to the targets to determine their binding affinity (kcal/mol). Table 6 shows the names of the compounds, their binding affinity (kcal/mol), and the interacting residues. The binding stance of ligands with receptors was depicted in Figure 8.

Molecular docking experiments revealed that the predicted free energy of binding docked ligand was between −5.2 and −9.4 kcal/mol for the human interferon-beta crystal structure (IFNB1, PDB: 1AU1) and between −5.4 and −9.6 kcal/mol for Interleukin-6 (IL-6, PDB: 4CNI).

The binding affinities of Kuwanon derivatives were compared with standard antiviral drugs (Baloxavir Marboxil, Umifenovir, and Favipiravir) for IFNB1 and IL-6 targets, revealing that Kuwanon compounds, particularly Kuwanon_Z, exhibit superior binding capabilities. For the IFNB1 target, Kuwanon_Z showed the strongest binding affinity (−9.4 kcal/mol), outperforming all standard drugs, including Baloxavir Marboxil (−8.4 kcal/mol), which was the best among the standard drugs. Other Kuwanon derivatives, such as Kuwanon_A (−8.3 kcal/mol), Kuwanon_X (−8.3 kcal/mol), and Kuwanon_C (−7.7 kcal/mol), also demonstrated stronger affinities than Umifenovir (−6.3 kcal/mol) and Favipiravir (−5.2 kcal/mol), which exhibited the weakest interactions.

Similarly, for the IL-6 target, Kuwanon_Z again displayed the strongest binding affinity (−9.6 kcal/mol), followed by Kuwanon_X (−8.7 kcal/mol), Kuwanon_A (−8.4 kcal/mol), and Kuwanon_C (−8.1 kcal/mol), all of which surpassed Baloxavir Marboxil (−7.9 kcal/mol), the best-performing standard drug. Umifenovir (−6.2 kcal/mol) and Favipiravir (−5.4 kcal/mol) showed weaker binding affinities than all Kuwanon compounds. These results highlight the potential of Kuwanon_Z as a superior antiviral candidate, with significantly stronger interactions with both IFNB1 and IL-6 targets compared to standard drugs. Other Kuwanon derivatives also show promising binding profiles, suggesting their potential as effective alternatives or complementary agents to existing antiviral therapies.

Table 6 Estimated free energy of binding, H-bond interactions and hydrophobic interactions between compounds and receptors

Targets	Compounds	Binding Affinity (kcal/mol)	Hydrophobic interactions	Hydrogen bonding	Salt bridges
IFNB1	Kuwanon_Z	−9.4	LEU9, LEU20, GLU42	GLN16, GLN23, I LE40, LYS45, ASN90, HIS93, GLN94, HIS97	–
	Baloxavir_Marboxil	−8.4	GLU42, ASN86	GLU42, ASN90, HIS93	HIS93, HIS121, ARG124, ARG128
	Kuwanon_A	−8.3	–	GLU42, GLN94, HIS97	–
	Kuwanon_X	−8.3	GLN16, GLN94	ASP39, HIS121, ARG128	–
	Kuwanon_C	−7.7	LEU9, PRO41, ASN90, HIS93, GLN94, HIS121	ASP39, GLU42, GLU43, ASN86, ASN90, HIS93, ARG128	HIS93
	Umifenovir	−6.3	GLN16, LEU20, ASN90, HIS121	GLN23, ASP39, ASN86, HIS121, ARG124, ARG128	HIS121
	Favipiravir	−5.2	LEU57, TYR60, LYS99, GLU103, LEU106, PHE111	LEU106	–
IL-6	Kuwanon_Z	−9.6	LYS45, TYR49, ALA55, PHE106, TYR109	TRP110	–
	Kuwanon_X	−8.7	GLN39, LEU115, GLU155, PRO156	GLY41, GLY42, LYS43, LEU115	–
	Kuwanon_A	−8.4	GLN38, GLN39	GLY41, GLY42, LYS43, THR85, TYR87	–
	Kuwanon_C	−8.1	ASP31, TYR32, PHE33, TYR103	PHE33, ASN53, ASN55, GLU101	TYR103
	Baloxavir_Marboxil	−7.9	VAL95, GLU165, PRO174	GLY42, TYR97, GLU155	GLU165
	Umifenovir	−6.2	GLN38, PRO41	GLN38, GLY42, TYR87, LYS103	–
	Favipiravir	−5.4	ILE117, PRO133	ILE117, CYS134, SER135, SER208	–

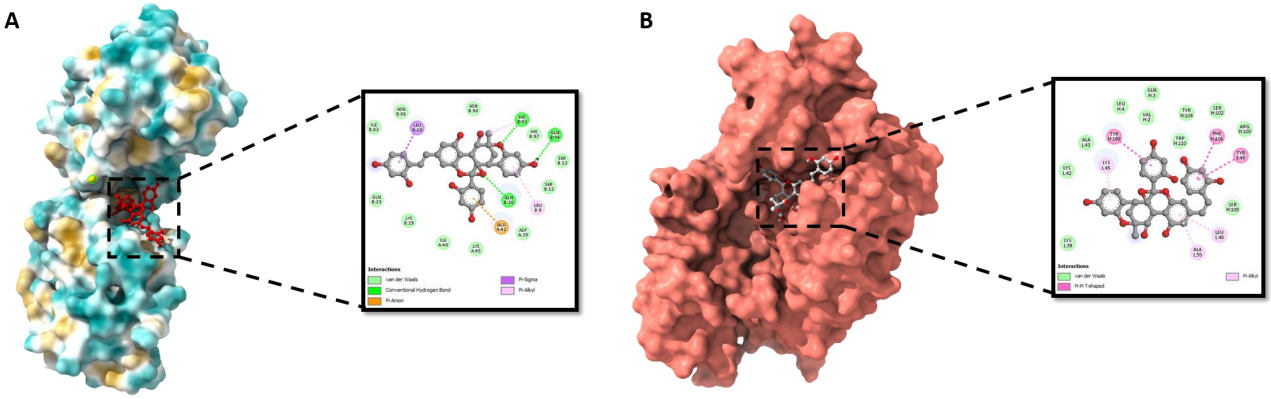


Figure 8 Amino acid interactions formed between docked complexes of A) Kuwanon Z with IFNB1 (PDB: 1AU1), B) Kuwanon Z with IL- 6 (PDB: 4CNI). PDB, Protein Data Bank.

Discussion

In silico screening is an effective technique for identifying new antiviral drugs. Computational approaches, such as molecular docking studies, allow for the effective study of natural substances and phytochemicals, decreasing resource demands in terms of time and money while also avoiding potential errors in clinical trials. Unlike the typical one medication, one target model, network pharmacology employs a multi-targeted treatment strategy. This method combines systems biology, network analysis, connectedness, and redundancy to clarify the complicated interactions between medications and disorders. Network pharmacology has been successful in identifying new targets and uncovering previously undiscovered signaling pathways connected with specific drugs. Network pharmacology not only provides fresh insights into the systemic interactions of treatment targets, but it is also a useful tool for understanding illness mechanisms and discovering possibilities of bioactive compounds [30, 31]. Recent research on the H9N2 avian influenza virus has revealed important details about its biology and possible antiviral targets. Jiang et al. (2022) used bioinformatics and system pharmacology to investigate the effects of kaempferol, a naturally occurring flavonoid, on COVID-19 and pulmonary fibrosis co-occurrence. Their findings showed that kaempferol impacts important targets like Epidermal Growth Factor Receptor, Mitogen-Activated Protein Kinase 3, AKT Serine/Threonine Kinase 1, as well as pathways like IL-17, TNF, and PI3K/AKT signaling. Although this work focused on SARS-CoV-2, the pathways discovered are equally applicable to H9N2, implying that comparable processes may be involved in its development. Another study looked at the inhibitory effects of chikusetsusaponin IVa on H9N2 replication. The researchers discovered that this chemical affects the Nrf2 pathway, resulting in decreased viral replication and modification of the host's oxidative stress response. This emphasizes the importance of the Nrf2-mediated oxidative stress response in H9N2 infection and implies that targeting this pathway could be an effective antiviral strategy [32, 33].

In this study, a new network was built to provide a thorough picture of the molecular pathways involved in natural phytochemicals. This network demonstrated the ability of bioactive compounds to alter the H9N2 influenza virus via interactions with 100 proteins across several pathways. PPI analysis revealed 120 nodes and 163 edges, with TNF, IL6, CCL5, IFNB1, IFNG, DDX58, IL1B, PTGS2, and IFIH1 emerging as significant genes in H9N2 Influenza Virus regulation. The PPI network reveals critical insights into the functional implications of key nodes and pathways in the context of H9N2 influenza virus pathogenesis. Key nodes such as TNF, IL6, IFNB1, IFNG, IL1B, and PTGS2 emerge as highly connected hubs, indicating their central role in organizing immune and inflammatory responses. TNF and IL6 are pivotal in mediating pro-inflammatory cytokine signaling, often linked to the overactivation of immune responses, which can result in cytokine storms and tissue damage during severe infections. IFNB1 and IFNG, as essential components of the interferon signaling pathway, drive antiviral responses by inducing interferon-stimulated genes that inhibit viral replication and modulate immune activity. IL1B, another key pro-inflammatory cytokine, amplifies the immune response by promoting chemokine release, such as CCL5, which recruits immune cells to the site of infection. Sensors like DDX58 and IFIH1 are crucial for detecting viral RNA, linking innate immune recognition to downstream interferon signaling pathways.

Moreover, the involvement of PTGS2 (COX-2) suggests its role in inflammation and fever regulation, connecting viral pathogenesis to broader physiological responses. The network's average node degree (2.72) and clustering coefficient (0.386) highlight its robust interconnectivity, suggesting a coordinated response where these pathways interact to mount a defense against the virus. Collectively, these nodes and pathways provide a comprehensive view of the host's response to H9N2 infection and highlight potential therapeutic targets to modulate inflammation, enhance antiviral defense, and prevent immunopathology. The compound-target network highlights the interactions of Kuwanon derivatives (Kuwanon_A, Kuwanon_C, Kuwanon_X, and Kuwanon_Z) with numerous molecular targets, emphasizing their therapeutic potential through synergistic effects and the possibility of off-target effects. Several targets, such as NF-κB1, IL-6, and IFNB1, are shared across multiple Kuwanon compounds, suggesting their ability to modulate interconnected inflammatory and immune signaling pathways. For instance, the simultaneous regulation of IFNB1 and IL-6 by Kuwanon_Z, Kuwanon_A, and Kuwanon_C may enhance antiviral and anti-inflammatory responses, while overlapping interactions with PPARG and PTGS2 could provide synergistic effects in inflammatory and metabolic conditions. This network-level synergy suggests that Kuwanon compounds may work collectively to achieve enhanced therapeutic outcomes. Additional study with GO and KEGG pathways found multiple important pathways and connections with various diseases and disorders connected to these genes. GO enrichment analysis indicated that these genes play a role in the regulatory processes of H9N2 influenza virus infection. Additionally, KEGG pathway analysis revealed the critical functions of pathways such as Influenza A, Cytokine-cytokine receptor interaction, Chemokine signaling, and TNF signaling in the network. These findings suggest Kuwanons' potential as viable choices for treating the H9N2 influenza virus. Aside from the known pathways associated with Influenza A, Cytokine-cytokine receptor interactions, and Jak-STAT signaling, our research revealed a variety of complex molecular networks involved in Human papillomavirus infection, Malaria, Shigellosis, Alzheimer's disease, Diabetic cardiomyopathy, Pancreatic cancer, and Asthma. Overall, these findings show that Kuwanons could be effective multitarget therapeutic agents.

Molecular docking studies revealed that the binding free energy for the docked ligands ranged between −5.2 and −9.4 kcal/mol for the human interferon-beta crystal structure (IFNB1, PDB: 1AU1) and −5.4 and −9.6 kcal/mol for Interleukin-6 (IL-6, PDB: 4CNI). The ligand-protein interaction models included hydrophobic interactions, hydrogen bonding, π-stacking, and salt bridge forms. Among the drugs studied, Kuwanon Z had the highest affinity for both the IFNB1 and IL-6 receptors. Kuwanon Z has a binding energy of −9.4 kcal/mol to IFNB1, creating eight conventional hydrogen bonds with residues GLN16, GLN23, ILE40, LYS45, ASN90, HIS93, GLN94, and HIS97. It also formed π-σ, π-anion, and π-alkyl bonds with LEU9, LEU20, and GLU42. Kuwanon Z had a binding energy of −9.6 kcal/mol with IL-6, formed two conventional hydrogen bonds with TRP110, and had multiple hydrophobic interactions with LYS45, TYR49, ALA55, PHE106, and TYR109. IFNB1 plays an important role in antiviral defense via interferon signaling, whereas IL-6 is a key modulator of inflammation during viral infections. The roles of IFNB1 and IL-6 are critical to the pathogenesis of H9N2 influenza virus and the host's antiviral response. IFNB1 is an important component of the innate immune response that is triggered quickly upon virus identification by pattern recognition receptors (PRRs) such as RIG-I and TLR7. It induces an antiviral state in infected and surrounding cells by activating the JAK-STAT pathway, which results in the production of interferon-stimulated genes like MxA, OAS1, and PKR, which impede viral replication and protein synthesis. However, the H9N2 virus uses viral proteins such as NS1 to inhibit IFNB1 synthesis, emphasizing its importance in viral defense [34]. In contrast, IL-6 is a pro-inflammatory cytokine that serves two functions during H9N2 infection. Moderate IL-6 levels promote immune cell recruitment, inflammation, and effective virus clearance by activating adaptive immunity processes such as T-helper cell differentiation and B-cell antibody generation. However, high IL-6 levels can trigger a cytokine storm, resulting in severe lung pathology and worsening disease severity. This combination of protective and pathogenic activities emphasizes the need of IL-6 control. IFNB1 acts as a crucial initiator of antiviral defenses, whereas IL-6 links innate and adaptive immunity, with dysregulation risking immunopathology. These processes are critical for understanding H9N2 pathophysiology and creating targeted antiviral treatments [35]. The high binding affinities suggest that Kuwanon Z may have a dual effect, increasing antiviral immunity

while also inhibiting excessive inflammatory responses, addressing two crucial components of influenza pathogenesis.

Kuwanon Z was chosen as a lead analogue because to its excellent binding characteristics, which highlight its potential for targeting various biological processes. This interaction profile not only demonstrates Kuwanons' therapeutic potential, but also provides a framework for refining analogues to improve specificity and efficacy. These discoveries pave the path for the development of personalized antiviral medicines that take advantage of these molecular interactions to more effectively combat influenza and other viral diseases.

Recent research on structurally related chemicals to kuwanon, such as other flavonoids from the mulberry family, has emphasized their potential for antiviral and anticancer applications. Kuwanon C, for example, has shown considerable antiviral activity in several experiments, indicating potential against cervical cancer cells. This flavonoid alters the mitochondrial membrane potential, causes reactive oxygen species generation, and initiates apoptosis, which has implications for both cancer therapy and maybe antiviral medicines by targeting comparable pathways in viral-infected cells . Interestingly, the presence and number of prenyl groups in these compounds has a major impact on their biological efficacy. For example, Kuwanon H, which has two prenyl groups, showed more ACE inhibition and may have potential benefits for regulating illnesses such as hypertension, which indirectly links to viral disease management by reducing comorbidities. Other similar chemicals, such as isopentenyl flavonoids, are being studied for their wide range of biological activities, including antiviral, anti-inflammatory, and antioxidant properties. These drugs' antiviral activities frequently entail altering immune response pathways and limiting viral replication through cellular signaling mechanisms [36, 37].

Conclusion

In this study, we used a comprehensive strategy combining network pharmacology and substantial database mining tools to discover critical target proteins linked with Kuwanon's effects on the H9N2 influenza virus. We created a thorough target protein network and discovered that Kuwanon largely interacts with domains of Influenza A, cytokine-cytokine receptor connections, chemokine signaling pathways, and other key biological pathways. These interactions have an impact on key target proteins including as TNF, IL6, CCL5, and IFNB1, resulting in considerable therapeutic effects against the H9N2 influenza virus. The network analysis provides significant evidence for Kuwanon's unique features in relation to the H9N2 influenza virus, indicating their potential as novel pharmacological treatments for virus management. Our findings show that Kuwanon has a strong affinity for H9N2 influenza virus targets, potentially causing structural alterations in proteins that disrupt viral function and reduce overall virus activity. Kuwanon, in particular, has showed promise as a viral protease inhibitor, making it an intriguing drug discovery option. The study's findings provide crucial insights for future antiviral medication design beyond H9N2, indicating the broad applicability of natural molecules such as Kuwanons. By targeting conserved pathways and processes shared among diverse influenza strains and other viral diseases, this strategy can inform the development of multi-target antiviral medicines. Furthermore, the strong binding affinities demonstrated in molecular docking suggest Kuwanons as a model for developing derivatives or analogues with broader antiviral effectiveness.

Limitations and future work

In this paper, we use a computational technique to discover candidate drugs for treating the H9N2 influenza virus and propose a prediction mechanism. However, we recognize a few restrictions. Even when using publically available resources to build comprehensive drug-target networks, partial data and unvalidated drug-target interactions may still exist. To improve the accuracy of our network-based approaches, we need to extensively map the H9N2 virus-host interactome and investigate biological impacts using functional genomics experiments. Furthermore, preclinical investigations examining in vivo efficacy and potential side effects, supported by cell-based tests, are required before proceeding to clinical trials. As a result, while computational predictions provide significant insights and identify prospective treatment targets, they are based on theoretical models that may not necessarily correspond to the complex biological milieu found *in vitro* and in vivo research. Future research will require Molecular Docking simulations for compound target stability and experimental validation to corroborate the predictions and justify our proposed hypothesis. This technique will assure the strength and integrity of our methodology.

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