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The pharmacological network of *Tinospora cordifolia*: Its role in regulating ınflammation and cathelicidin production

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ABSTRACT: This study aims to promote a pharmacological network strategy to investigate the potential anti-inflammatory activity and molecular mechanisms of the bioactive compounds in *Tinospora cordifolia* (TC) for controlling inflammation and regulating the production of the antimicrobial peptide cathelicidin. Using the Knapsack database and several recent research findings, SwissADME, PubChem, and PASS Online, we screened the drug-likeness of various TC compounds. Utilizing the SwissTargetPrediction, String-DB, GeneCards, and Venny Diagram, we identified 468 potential targets related to inflammation target protein and cathelicidin production. Further refinement using Cytoscape with CytoHubba highlighted 15 core targets, including BCL2, JUN, STAT3, HSP90AA1, MTOR, AKT1, ESR1, SRC, BCL2L1, TNF, MDM2, PTGS2, HSP90AB1, MMP9, and MMP2. GO and KEGG pathway analysis revealed that the core targets for inflammation control and cathelicidin production are predominantly enriched in TLR, NOD, MAPK, and NFKB inflammatory pathways. Molecular docking conducted with Autodock confirmed strong binding between TC ligands and several proteins in these pathways, such as JAK1, AKT1, IKBKB, and IRAK4. Overall, these findings suggest that TC is predicted to inhibit inflammation by inhibiting the activity of these four target proteins in the inflammatory pathways. This research provides a theoretical basis for understanding the molecular mechanisms of TC in inhibiting inflammation and controlling the production of the antimicrobial peptide cathelicidin.

KEYWORDS: Cathelicidin, inflammation, network pharmacology, *Tinospora cordifolia*

1. INTRODUCTION

Infectious diseases remain a significant problem today, particularly in low- and lower-middle-income nations. Severe infectious diseases like SARS, MERS, *Tuberculosis*, and *Ebola* cases may be found worldwide, and the COVID-19 pandemic, which occurred most recently. These diseases are produced by introducing new variants of microorganisms and re-emerging old variants that turn into epidemics [1]. Infectious diseases accounted for 28% of all ailments worldwide in 2017, and they pose a threat [2]. Moreover, it is reported that infectious diseases cost the lives of 30 million youths [3].

The immune system, the body's defense mechanism, naturally has the potential to ward against infection. The body's immune system is classified into innate immunity and adaptive immunity. Innate immunity refers to the human body's innate immune system, which is ready to prevent bacteria from entering the body and rapidly eradicate them so they cannot spread to deeper organs. Innate immunity can also be called natural, native, or native immunity [4]. While adaptive immunity, which expresses receptors on its cell membrane, can more precisely recognize the presence of pathogenic microbes and distinguish them from standard body parts, innate immunity can recognize the presence of various groups of microbes in general [4]. The two immune systems protecting the body possess specialized functions and work together to prevent infection. Through innate immune cells and the quick yet non-specific synthesis of antimicrobial peptides, innate immunity has a wide range of capabilities for avoiding infection [5]. In contrast, adaptive immunity is more focused on antigen exposure. It is recognized by B and T lymphocyte cells with a long-term memory system that is beneficial in the event of reinfection [6].

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Plants' immunomodulatory potential must be continuously investigated, as plants have historically been one of the most essential sources of therapeutic substances. *Curcuma domestica* and *Curcuma xanthorriza*, with their main content curcumin, are known to have anti-inflammatory and immunomodulatory activity in pre-clinical trials. Likewise, *Echinache purpurea*, *Quillaja saponaria*, *Chenopodium quinoa, Astragalus membranaceus, Glycyrrhiza glabra*, and some of the plants that have phytochemical substances that have been proven by pre-clinical tests and even in some clinical tests to have immunomodulatory activity [7].

As one of the 17 most diverse nations, Indonesia is home to a wide variety of plants that have the potential to be important sources of raw materials for the development of immunomodulatory treatments, among other forms of medicine [8]. *Tinospora cordifolia* (TC) is a plant species distributed in Indonesia and almost throughout Southeast and South Asia. TC is one of the plants used as a traditional medicine in Indonesia. Traditionally, TC is used as an antidiabetic drug by Indonesian people [19] for malaria fever and heart disease [20]. The *Tinospora* genus is one of Southeast Asia's indigenous plants with immunomodulatory properties [17, 18]. Regarding its immunomodulatory activity, TC is known to have anti-inflammatory activity in in vitro and in vivo tests. Administration of TC ethanol extract had a therapeutic effect on mice infected with Salmonella typhimurium, which was demonstrated by higher survival rates in experimental animals, reduction of inflammation in the liver, and normalization of antioxidant enzymes (superoxide dismutase and catalase) in experimental animals [15].

Antimicrobial peptides like cathelicidin are key immune system components in addition to pro- and anti-inflammatory components. Cathelicidin is an antimicrobial peptide with a wide range of activity [9]. Multiple active plant components also influence the synthesis of cathelicidin [16]. Thus, this study uses insilico-based analysis with computer-aided drug discovery (CADD) technologies to investigate how TC compounds interact with immune system target proteins in regulating inflammation as well as the synthesis of the antimicrobial peptide cathelicidin.

The use of computers to search for drug candidates and explain the pharmacological mechanisms of drug candidates has been overgrown in recent years. Currently, various plant phytochemical databases are available [10]–[12], in silico test tools for plant efficacy such as absorption, distribution, metabolism and excretion (ADME) [13], bioactivity [14], and protein networks that respond to the presence of these phytochemicals in the body. This artificial intelligence device, or what is usually called computer-aided drug discovery (CADD), has helped a lot in research on drug discovery and its predictive molecular mechanisms [11]. This new approach in pharmacology and molecular biology research helps reduce trial and error methods, thereby reducing research costs and increasing the speed of finding therapeutic methods for various diseases [17], including finding immunomodulatory candidates.

The molecular potential of TC in regulating or modulating the immune system has not been fully elucidated, particularly in controlling inflammation and how it plays on the production of antimicrobial peptides like cathelicidin as a first line of defense against infection. Through an in silico approach, the study intends to unveil a pharmacological network consisting of the interaction between ligands (bioactive chemicals TC) and target proteins as well as protein-protein interactions in the immune system in controlling inflammation and cathelicidin production.

2. RESULTS

2.1 Compound data collection, preparation, and ADME analysis

A total of 44 bioactive chemicals TC were found after data was extracted from multiple databases and searches through reliable international publications. To further analyze these compounds, we used swissADME http://www.swissadme.ch/, a web-based platform that offers free access to reliable prediction models for physicochemical properties, pharmacokinetics, drug-likeness, and medicinal chemistry friendliness. The platform uses various in-house methods, such as the BOILED-Egg, iLOGP, and Bioavailability Radar. The website http://www.swissadme.ch has a user-friendly interface, making it easy to input and interpret data [13]. According to the results of the swissADME analysis, nineteen of the forty-four compounds met the drug-likeness criteria. These fifteen compounds have features that do not get past the deep pink zone of the Swiss ADME bioavailability radar and meet all five of the drug chemical similarity standards defined by Ghose, Veber, Lipinski, Muege, and Egan (Table 1 & Supplement 2.)

The findings showed that the fifteen compounds were classified into five groups, as shown in Table 1. Alkaloid: berberine, isocolumbin, jatrorrhizine, magnoflorine, palmatine, Tembetarine, tetrahydropalmatine, tinosporin, (+)-Corytuberine, (+)-Reticuline, (+)-N-Methylcoclaurine, (S)-Norcoclaurine; Glycosides: Tinocordifolioside, tinocordiside; Isoquinolines: Coclaurine; Carboxylic acid: N-transferuloyltyramine; Terpenoid: columbin, tinosporide, tinocordifolin.

2.2 Biological activity prediction

Based on the results of analysis carried out using PASS Online software (Way2Drug; https://www.way2drug.com/passonline/) (Figure 1), it is known that substances that correlate with the innate immune system and have a Pa of more than 0.7, include coclaurine (histamine release stimulant Pa: 0.746, MAPK stimulant Pa: 0.73), columbin (antiinflammatory Pa: 0.77), N-trans-feruoyltyramine (JAK2 expression inhibitor Pa: 0.794; MAPK stimulant Pa: 0.743, MMP9 expression inhibitor Pa: 0.734), corytuberine (MAPK stimulant Pa: 0.773), isocolumbin (antiinflammatory Pa: 0.77; nitric oxide antagonist Pa: 0.812), tetrahydropalmatine (MAPK stimulant Pa: 0.944), n methyl coclaurine (histamine release stimulant Pa: 0.739; MAPK stimulant Pa: 0.733), norcoclaurine (MAPK stimulant Pa: 0.703), tinocordifolioside (immunosuppressant Pa: 0.735), reticuline (histamine release stimulant Pa: 0.721; MAP kinase stimulant Pa: 0.73), tinocordifolin (immunosuppressant Pa: 0.692), tinocordiside (transcription factor NFKB stimulant Pa: 0.772; immunosuppressant Pa: 0.722; caspase 8 stimulant Pa: 0.706), tinosporide (nitric oxide antagonist Pa: 0.933; antiinflammatory Pa: 0.891), tinosporin (nitric oxide antagonist Pa: 0.812; antiinflammatory Pa: 0.77), and vitamin D (immunosuppressant Pa: 0.805; CYP21A1 substrate Pa: 0.735; anti-inflammatory Pa: 0.702).

2.3 Target Fishing Protein, Construction of protein-protein, protein-ligand and immune-related signaling pathways

Nineteen TC compounds and vitamin D were subjected to protein target fishing analysis using the Swisstargetprediction platform. The results showed that 468 target proteins were interacting with the compounds (Figure 2. B). Using the keywords "Innate Immune Protein" (IIP), "Adaptive Immune Protein" (AIP), and "Cathelicidin Protein" (CAPP), 468 target proteins were found through a search intersection on GeneCard (https://www.genecards.org). The red circles represent the target proteins for vitamin D and TC (VDETC), which were found on SwissTargetPrediction (https://www.swisstargetprediction.ch). The 468 proteins that make up the IIP+AIP+CAPP+VDETC intersection interact with TC compounds and are involved in synthesizing cathelicidin (CAP) and the innate and adaptive immune systems (Figure 2.A).

A total of 468 IIP, AIP, VD, and ETC target proteins were then constructed for a pharmacological network related to the immune system using String-DB (https://string-db.org/) and an image of the network can be seen in Figure 2.B. Next, protein centrality analysis was carried out using the Cytoscape platform with the Cytohubba add-on tool, the Maximal Clique Centrality (MCC) method [18], to obtain data on the proteins with the most central role in the immune system from the 468 proteins that had been constructed. The results of the Cytohubba analysis using the MCC method show that the fifteen proteins with the highest scores are in Figure 2. C. These proteins are BCL2, JUN, STAT3, HSP90AA1, MTOR, AKT1, ESR1, SRC, BCL2L1, TNF, MDM2, PTGS2, HSP90AB1, MMP9, MMP2. The construction of protein-protein networks, bioactive compounds-proteins, and protein pathways is arranged as in Figure 2.D.

The investigation of 468 proteins involved in immune system pathways revealed interactions between the proteins and nineteen TC active chemicals, which were further enriched by the Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg/). Figure 3 depicts the 21 pathways found as a result of this analysis that are closely associated with the immune system.

2.4 Patterns of Interaction between TC Bioactive Compounds and Target Proteins

Based on an immune system pathway study across many platforms, data on 21 signaling pathways was collected. According to data gathered from analysis conducted using the String-DB platform, these 21 pathways are related to the control of the immune system (Figure 3). The KEGG Pathway platform was then used to analyze the data and identify which of the several pathways controls the production of pro- and anti-inflammatory cytokines, such as cathelicidin (CAP). Six major pathways exhibited a close association with the production of pro- and anti-inflammatory cytokines and CAP, according to the results of the investigation of the 21 signaling pathways utilizing the KEGG Pathway platform. Figure 4 provides an overview of this path. The six mechanisms in immunocompetent cells that produce CAP, proinflammatory cytokines including TNFa, IFNg, IL-1, IL-6, IL-8, and IL17, and other antimicrobial peptides such defensins are briefly summarized in Figure 4.

Six pathways are known to initiate the production of proinflammatory cytokines and CAP in immunocompetent cells, as Figure 4 briefly demonstrates. The first pathway, pro-inflammatory cytokines, chemokines, and antimicrobial peptides, such as CAP and defensin, are produced by transcription factors that are activated by the IFN-JAK-STAT pathway. The activation of TLR-IRAK4 is the second pathway. TLR-AKT activation is the third pathway. TLR-VDR is the fourth pathway. Activation of the NOD1-IKBKB-NFKB receptor is the fifth pathway. The NOD2-JUN is the sixth pathway.

Table 1. The displays of the ADME prediction results for 19 active molecules in TC, indicating that these compounds are suitable candidates for medication based on the ADME criteria. According to Lipinski, Ghose, Veber, Egan, and Muegge, every chemical meet the drug likeness criteria in terms of their similarity to drug candidates (http://www.swissadme.ch).

No	Compound Class	Compound Name	PubChem CID	Formula	MW	Caco2+ Permeability	HIA	LD50, mol/kg, rat	BBB+
1	Alkaloid	Berberine	2353	C20H18NO4+	336.36	0.8726	0.5	2.7834	0.9279
2		İsocolumbin	24721165	C20H22O6	358.39	0.5837	0.9945	3.9368	0.9139
3		Jatrorrhizine	72323	C20H20NO4+	338.38	0.7902	0.6233	2.6103	0.8663
4		Magnoflorine	73337	C20H24NO4+	342.41	0.7275	0.9462	2.6986	0.8895
5		Palmatine	19009	C21H22NO4+	352.4	0.8444	0.8017	2.6332	0.9287
6		Tembetarine	167718	C20H26NO4+	344.42	0.7043	0.9696	2.6917	0.9004
7		Tetrahydropalmatine	5417	C21H25NO4	355.43	0.8393	0.9739	3.3933	0.9777
8		Tinosporin	188289	C20H22O6	358.39	0.5837	0.9945	3.9368	0.9139
9		(+)-Corytuberine	160500	C19H21NO4	327.37	0.8212	0.9526	2.695	0.9476
10		(+)-Reticuline	439653	C19H23NO4	329.39	0.7957	0.9183	2.691	0.9537
11		(+)-N-Methylcoclaurine	440595	C18H21NO3	299.36	0.8464	0.987	2.6888	0.9668
12		(S)-Norcoclaurine	440927	C16H17NO3	271.31	0.6222	0.9749	2.5795	0.6774
13	Glycoside	Tinocordifolioside	100926541	C21H32O8	412.47	0.849	0.6453	3.3205	0.5932
14		Tinocordiside	177384	C21H32O7	396.47	0.8143	0.6702	2.6103	0.7449
15	Isoquinolines	Coclaurine	160487	C17H19NO3	285.34	0.5493	0.9843	2.5245	0.7392
16	Carboxylic acid	N-Transferuloyl-tyramine	5280537	C18H19NO4	313.35	0.5778	0.9884	2.0305	0.6816
17	terpenoid	Columbin	442015	C20H22O6	358.39	0.5837	0.9945	3.9368	0.9139
18		Tinosporide	442068	C20H22O7	374.38	0.6033	0.9837	3.4251	0.8514
19		Tinocordifolin,	100926540	C15H22O3	250.33	0.6558	0.9951	3.0924	0.8998

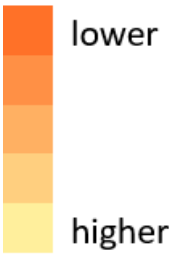
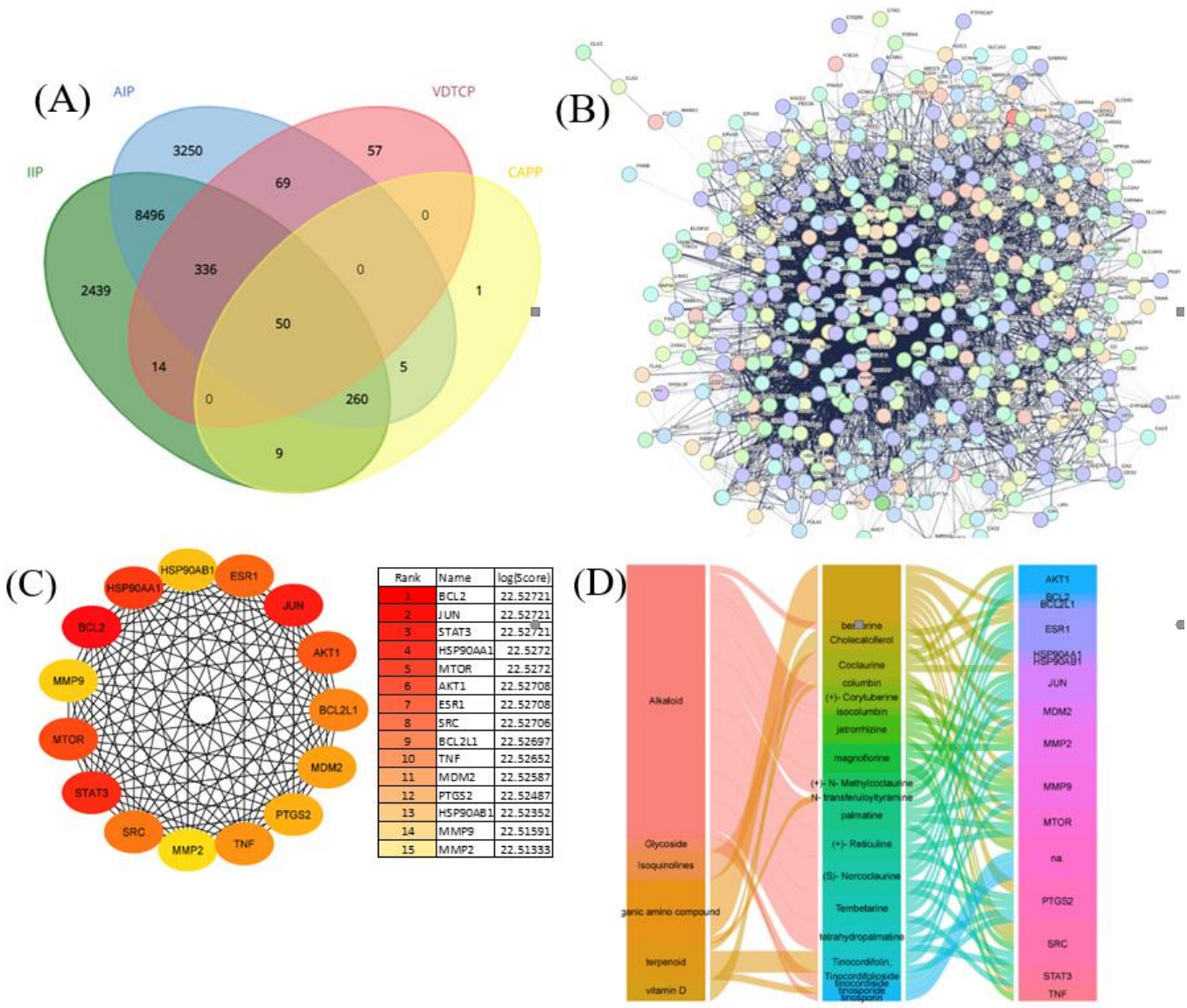


Figure 1. Prediction of percent value of biological activity (Pa), primarily related to the immune system of compounds contained in TC extracts using PASS Online (Way2Drug) Software analysis.



Rank	Name	log(Score)
1	BCL2	22.52721
2	JUN	22.52721
3	STAT3	22.52721
4	HSP90AA1	22.5272
5	MTOR	22.5272
6	AKT1	22.52708
7	ESR1	22.52708
8	SRC	22.52706
9	BCL2L1	22.52697
10	TNF	22.52652
11	MDM2	22.52587
12	PTGS2	22.52487
13	HSP90AB1	22.52352
14	MMP9	22.51591
15	MMP2	22.51333

Figure 2. (A) venny diagram (https://bioinfogp.cnb.csic.es/tools/venny) number of proteins involved in the innate immune system with keywords entered in the Genecards database (https://www.genecards.org) "Innate Immune Protein" (IIP) green circle; "Adaptive Immune Protein" (AIP) blue circle; "Cathelicidin related Protein" (CAPP) yellow circle; meanwhile the vitamin D and TC target protein (VDETC) was obtained from Swisstargetprediction (https://www.swisstargetprediction.ch) in the red circle. The IIP+AIP+CAPP+VDETC intersection contains 468 proteins involved in the innate, adaptive immune system and cathelicidin biosynthesis and interacts with TC compounds. **(B)** Network of 468 innate immunity-related TC target proteins using string-DB software (https://string-db.org). **(C)** 15 central proteins from analysis using Cytoscape with cytohubba add-on software with Maximal Clique Centrality (MCC) criteria. **(D)** Illustration of the relationship between 19 active TC compounds and 15 central proteins of the immune system related to cathelicidin biosynthesis and inflammation.

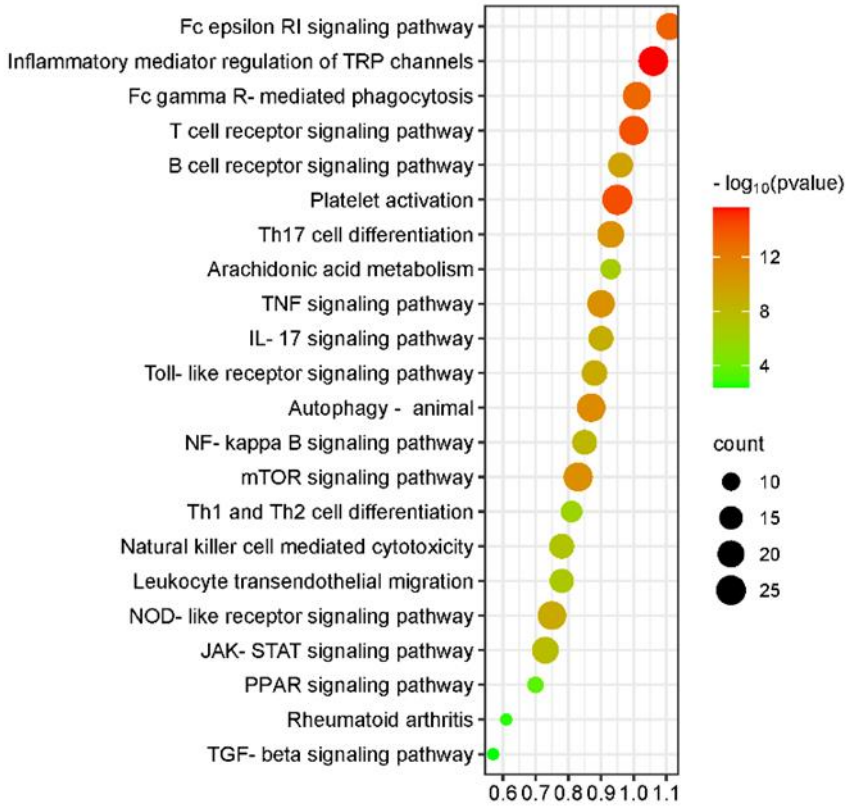


Figure 3. Results of analysis of 468 proteins involved in immune system pathways that interact with 19 TC active compounds using the String-db platform (https://string-db.org/) and enriched with the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database (https://www.genome.jp/kegg/). Based on the results of analysis using the STRING platform, there are 21 pathways that are closely related to the immune system as listed in this figure. Data visualization using the SRPLOT platform (https://www.bioinformatics.com.cn). Data Notes: The x-axis represents the strength of protein-protein interactions (PPI), the y-axis represents pathway enrichment, the dot size represents the number of genes and the dot color represents the P value.

The six pathways enrichment from the KEGG Pathway platform, shown in Figure 4, were then examined for interactions with 19 TC compounds. The findings are displayed in Figure 4, which shows how 19 TC compounds were interacting with the JAK protein in lane 1, IRAK4 and NFKB in lane 2, IRAK4 and JUN in lane 3, AKT1 and NFKB in lane 4, IKBKB and NFKB in lane 5, and JUN in lane 6. The enzyme proteins IRAK, JAK, IKBKB, and AKT all have active sites. The results of molecular docking of TC compounds with these proteins (Figure 5) demonstrate that some TC compounds bind to amino acid residues in the active site of these enzymes, implying the possibility of competitive inhibitors to their function. This is predicted to lead to a reduction in activity on this route. Details about the TC compounds that bind to the active sites and those that attach to non-active sites are provided in the Supplement.

3. DISCUSSION

3.1 Compound data collection, preparation, and ADME analysis

TC is a plant used as a traditional medicine in Indonesia. Traditionally, TC is used as an antidiabetic drug by Indonesian people [19] for malarial fever and heart disease [20]. Related to its anti-inflammatory activity, TC is known to have anti-inflammatory activity in in vitro and in vivo tests. The ethanol extract of TC has an anti-inflammatory effect on mice infected with Salmonella typhimurium. Anti-inflammatory activity is demonstrated by higher survival rates in mice, reduction of inflammation in the liver, and normalization of antioxidant enzymes (superoxide dismutase and catalase) [15]. TC extract inhibits the production of proinflammatory cytokines in dendritic cells, such as TNF-α IL-1β [21].

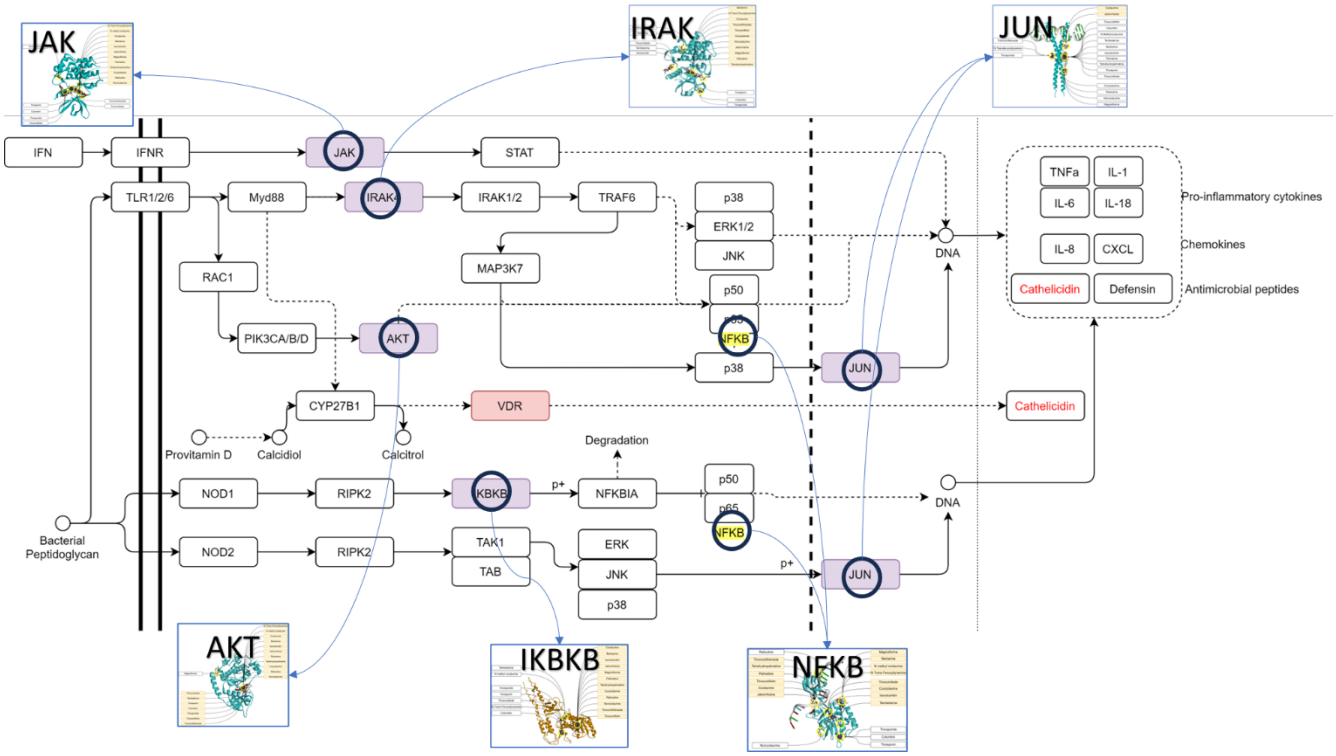


Figure 4. Diagram illustrating the interactions between 19 TC compounds and various proteins in the production route of antimicrobial peptides and pro-inflammatory cytokines. Yellow boxes represent TC bioactive compounds that bind to the active site of proteins in signaling pathways; purple boxes represent proteins that interact with TC bioactive compounds. Two transcription factor proteins, NFKB P50 and JUN (AP-1), and four enzyme proteins, JAK1, IRAK4, IKBKB, and AKT1, are involved in this pathway.

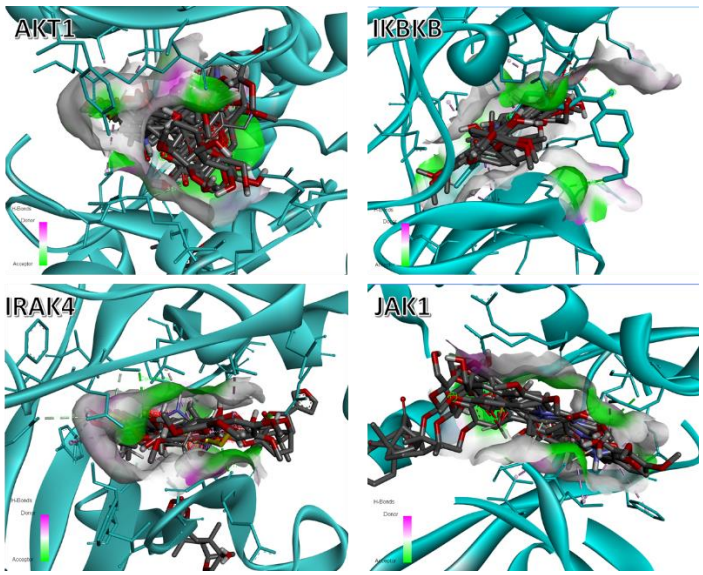


Figure 5. Illustration of molecular docking on four proteins (JAK1, IRAK4, IKBKB, and AKT1) involved in the biosynthesis pathway of proinflammatory cytokines TNFα, IL-1, IL-6, IL-18, and the production of cathelicidin. This illustration shows that some bioactive compounds of *Tinospora cordifolia* bind to the active sites of these four proteins and potentially act as competitive inhibitors, thereby reducing the function of the pathways responsible for the biosynthesis of these proinflammatory cytokines and cathelicidin production.

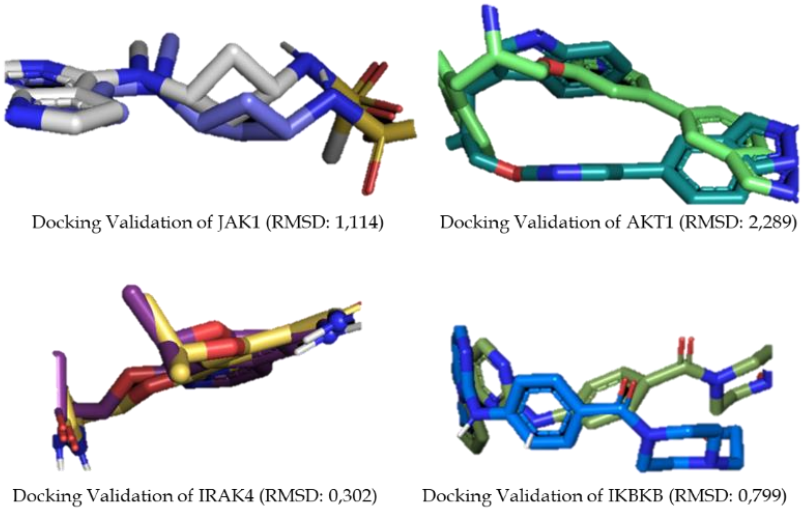


Figure 6. Validation of docking for JAK1, IRAK4, IKBKB, and AKT1 using Pymol 8.0 confirmed the docking validity, with an average RMSD value below 3Å.

The bioavailability radar shown on the compound analysis results page using swissADME contains six axes for six properties important for oral bioavailability (Supplement 2). A SwissADME descriptor defines each property, and the optimal value range is depicted as a pink area. The first axis is the saturation of the compound. For saturation, the ratio of sp hybridization carbons to the total carbon number of the molecule must be at least 0.25. The second axis is molecular weight; the molecular weight (MW calculated with OpenBabel) should be between 150 and 500 g/mol. The third axis is polarity; TPSA must be between 20 and 130Å. The fourth axis is solubility; logS should not exceed 6 for solubility. The fifth axis is lipophilicity; for lipophilicity, XLOGP should range from − 0.7 to + 6.0. The sixth axis is flexibility; a molecule cannot have more than nine rotatable bonds for the flexibility criterion [13]. According to the results of the swissADME analysis, 15 of the 21 compounds met the drug-likeness criteria.

The fifteen compounds that passed the ADME criteria were then identified and categorized. The Classyfire software (http://classyfire.wishartlab.com/) was used to classify these compounds. ClassyFire automatically classifies all known chemical compounds into one of more than 4800 categories by utilizing chemical structures and structural characteristics. It is simple to determine all of a compound's parents using ClassyFire, which offers a list for every compound. Classyfire created the labels Kingdom, SuperClass, Class, and SubClass for each signifying the first, second, third, and fourth-level chemistry taxonomies, the taxonomy structure used by this software inspired by original Linnaean biological taxonomy[22].

Based on the results of analysis using Classyfire, it is known that the 19 compounds confirmed to be in TC are grouped into five groups of metabolite compounds. Alkaloid: berberine, isocolumbin, jatrorrhizine, magnoflorine, palmatine, Tembetarine, tetrahydropalmatine, tinosporin, (+)-Corytuberine, (+)-Reticuline, (+)-N-Methylcoclaurine, (S)-Norcoclaurine; Glycosides: Tinocordifolioside, tinocordiside; Isoquinolines: Coclaurine; Carboxylic acid: N-transferuloyltyramine; Terpenoid: columbin, tinosporide, tinocordifolin.

Vf-1 alkaloid isolated from *Voacanga foetida* stems showed immunosuppressant activity on RAW 264.7 cells [23]. A fraction derived from *Unicaria tomentosa*, it was discovered that oxindol pentacyclic-alkaloid enhanced human granulocyte and macrophage phagocytosis, inhibited myeloid cell line growth, and suppressed TNF-alpha production [24]. The next class of compounds identified in TC are isoquinolines. Isoquinolines are a type of alkaloid. One that is well-known to have immunomodulatory activity is berberine. Berberine triggered an immunological suppressive population in the liver of mice, characterized as a granulocytic-myeloid-derived suppressor cell (G-MDSC))---like population, reducing alcohol-induced hepatic damage. Berberine significantly reduced cytotoxic T cells and increased G-MDSC-like cells in the liver and blood [25]. Recent studies have shown that isoquinoline alkaloids may modulate an animal's innate immune system, metabolism, and digestive system [26]. According to several investigations, certain terpenes and terpenoids can lessen inflammation symptoms by reducing the release of pro-inflammatory cytokines such as tumor necrosis factor-alpha, interleukin 1, and nuclear transcription factor-kappa B [27]. Therefore, this kind of predictive research helps determine the immunomodulatory activity of these compounds through a similar approach to drug compounds as stated in the drug-likeness criteria of Lipinski, Weber,

Goose, and others in determining the efficacy of bioactive drug compounds. With this approach, compounds tested and compiled in the database of various tools used in this research can be used as comparison and prediction tools.

3.2 Biological activity prediction

The PASS Online (Way2Drug) software examined the biological activity of TC compounds, which uses computation to predict a compound's biological activity. Using these tools, it is possible to predict various types of biological activity, including interactions with molecular targets, pharmacotherapeutic effects, side effects, metabolism, acute toxicity for rats, cytotoxicity, influence on gene expression, and other properties that assess the potential of a drug-like compound[14].

Several substances show biological activity correlated with the immune system Figure 1. Certain substances, like coclaurine, and tinocordiside, exhibit immunomodulatory properties. N-trans-feruylpyramine are a few other substances that exhibit immunosuppressive properties. Furthermore, tinocordiside functions as both an immunosuppressant and an immunomodulator simultaneously. These properties of plant bioactive chemicals are beneficial in therapeutic approaches based on many targets and several substances. Because target cells or their modulation and inhibition pathways differ, one substance may have two distinct actions simultaneously. For instance, it was discovered that the plant fraction of *Arisaema jacquemontii* significantly decreased mitogen-induced T- and B-cell proliferation while also significantly stimulating humoral immune responses [28].

In this study, the tinocordiside molecule with Caspase 3 stimulant activity had the highest immune system activity, with a Pa value of 0.892. Caspase-3 has been linked to hematopoietic self-renewal and differentiation, which is essential for creating distinct blood cell lineages and balancing the number of immune cells[29]. Caspases are a group of genes that play a crucial role in homeostasis by controlling cell death and inflammation. Caspases have been generally classified based on their known functions in apoptosis (caspase-3, -6, -7, -8, and -9 in mammals) and inflammation (caspase-1, -4, -5, and -12 in humans and caspase-1, -11, and -12 in mice)[30].

Another interesting finding from this study was that one TC-containing substance was predicted to interact significantly with the transcription factor protein NFKB. With a Pa score of 0.772, the prediction of tinocordiside action as an NFKB stimulant seems fairly robust. NFKB was identified as a critical regulator of inducible gene expression in the immune system. The NFKB family of transcription factors regulates innate and adaptive immune responses and the growth and maintenance of the cells and tissues that belong to the immune system [31]. NFKB family members regulate several aspects of innate and adaptive immune responses by controlling the transcription of genes encoding cytokines, antimicrobial effectors, and cellular differentiation, survival, and proliferation. Furthermore, NFKB aids in forming and surviving cells and tissues that carry out mammalian immunological responses [32].

It is interesting to note that there are N-trans feruoyl tyramine with its biological activity as MMP9 expression inhibitors with a Pa value 0.734. MMP9 is a crucial enzyme generated by neutrophils and macrophages. MMP9 promotes the migration of numerous types of leukocytes to the site of infection. MMP9 activity is often regulated by the presence of tissue inhibitors of metalloproteinases (TIMPs). An imbalance between MMP9 and TIMPs 23 causes inflammation [33].

3.3 Target Fishing Protein, Construction of protein-protein, protein-ligand and immune-related signaling pathways

Network pharmacology is a relatively new field that combines pharmacology, bioinformatics, system biology, and computer science [34]. The paradigm of network pharmacology is identical to a particular compound's multicomponent, multitarget, and multichannel interactions. This systems approach is compatible with a holistic view of symptom differentiation and the application of multicomponent-based treatments [35]. The construction of protein-protein networks, bioactive-protein compounds, and protein pathways is arranged as shown in Figure 2. The target protein fishing analysis results using the SwissTargetPrediction platform revealed that 19 TC compounds interact with 468 target proteins (Figure 2.B). These 468 proteins are the outcome of analyses performed using SwissTargetPrediction and GeneCard. SwissTargetPrediction is an AI-equipped platform commonly used to accurately predict protein targets for specific ligands/bioactive compounds based on molecular property similarity with real laboratory test results (non-in silico) compiled in the database [36]. The 468 target proteins were obtained through an overlay search on GeneCard (https://www.genecards.org) [37-38] with the keywords "Innate Immune Protein" (IIP) green circle, "Adaptive Immune Protein" (AIP) blue circle, and "Cathelicidin related Protein"

(CAPP) yellow circle. Meanwhile, in the red circle, vitamin D and TC target proteins (VDETC) were obtained from SwissTargetPrediction (https://www.swisstargetprediction.ch). The intersection of IIP+AIP+CAPP+VDETC represents 468 proteins involved in the innate and adaptive immune systems, as well as cathelicidin biosynthesis (CAP), interacting with TC compounds (Figure 2.A). The pharmacological network paradigm is synonymous with a specific compound's multi-target, multi-component, and multi-pathway interactions. This system's approach is compatible with a holistic view of symptom differentiation and multi-component-based treatment applications. In the context of the immune system, homeostasis achieved by multi-component plant therapy can support overall health [39].

The 468 target proteins (IIP, AIP, VD, and ETC) were subsequently used to construct an immune system-related pharmacological network using String-DB (https://string-db.org/), as depicted in Figure 3B. Protein centrality analysis using Cytoscape's CytoHubba add-on with the Maximal Clique Centrality (MCC) method [18] identified the fifteen most central proteins out of the 468. The construction of protein-protein, bioactive compound-protein, and protein pathway networks, as shown in Figure 2.D, illustrates that one bioactive compound can be linked to several targets. This demonstrates that TC is an immunomodulator with synergistic multi-component and multi-target effects.

The GO and KEGG pathway analysis in Figure 3 show that the Fc epsilon RI signaling pathway is most strongly impacted by these fifteen TC compounds, according to GO and KEGG pathway analysis results. Fc epsilon RI is a high-finite IgE receptor that is crucial in IgE-mediated allergy reactions. It is a multimeric immunological receptor that binds IgE with high affinity and has a tetrameric structure on the mast and basophilic cell surfaces, consisting of one IgE-binding alpha chain, one beta chain, and two gamma chains [47]. Many signaling pathways that regulate a range of effector responses, such as mast cell degranulation, cytokine/chemokine production, and leukotrien creation, are activated when the Fc epsilon RI signaling pathway is activated [40]. Fc epsilon is also expressed by human monocytes and dendritic cells (DCs), including both plasmacytoid and ordinary DCs [41].

3.4 Patterns of Interaction between TC Bioactive Compounds and Target Proteins

Inflammation is the body's natural process in response to infection. TNFα, IFNγ, IL-1, IL-6, and IL-8 are some of the proinflammatory cytokines produced during infection. Based on pathway analysis (Figure 3) through Gene Ontology (GO) and KEGG Pathway enrichment, six pathways are associated with the production of proinflammatory cytokines as well as cathelicidin production, and one independent pathway for cathelicidin production via vitamin D receptor activation (Figure 4). Four proteins are known to be inhibited by TC compounds: JAK1, IRAK4, IKBKB, and AKT1.

Janus-associated kinase (JAK) and signal transducer and activator of transcription (STAT) mediate cellular signals from cytokine receptors and are involved in inflammation. Active compounds that can inhibit the functional activity of JAK proteins can suppress inflammation and are clinically useful for treating hyperinflammatory diseases [42]. The AKT1 protein is responsible for inflammation, and it has been found that docking results against AKT1 with good binding affinity correlate closely with reduced inflammation in animal experiments [43]. Similarly, for the IKBKB protein, molecular docking results with low binding energy correlate with in vivo research findings indicating that IKBKB inhibition as a key therapeutic target can suppress inflammation related to the NFKB pathway and provide analgesic effects [44]. For the IRAK4 protein, in vitro and in vivo tests support the findings that inhibition of the TLR-IRAK4-NFKB pathway can inhibit inflammatory activity [45].

4. CONCLUSION

The results of this study indicate that TC has potential as an anti-inflammatory agent by reducing the production of pro-inflammatory cytokines through the TLR-IRAK-NFKB, TLR-AKT-JUN, NOD-IKBKB, NOD-JUN, and JAK-STAT pathways while maintaining the production of the antimicrobial peptide Cathelicidin, as TC compounds do not interfere with the VDR-Cathelicidin independent pathway. This study also shows that the TC compounds have a good predictive potential as anti-inflammatory drugs with good bioavailability in terms of ADME and drug-likeness properties. However, this in silico study requires further validation through in vitro and in vivo studies to confirm the predictive validity.

5. MATERIALS AND METHODS

5.1 Phytochemical Data Retrieving and ADME Prediction

TC metabolite data was taken from the Knapsack plant metabolite database (http://www.knapsackfamily.com/KNApSAcK_Family). This site is one of the most up-to-date sources of

plant metabolite data [10]. The metabolite compound data was then unified with the molecular data from the PubChem database (https://pubchem.ncbi.nlm.nih.gov) [21] in tabular form. The table contains data on compound name, PubChem CID, Canonical Simplified Molecular-Input Line-Entry System (SMILES), synonyms, molecular weight, 2D structure, 3D structure, and other necessary data. The ADME content of TC compounds was analyzed using SwissADME (http://www.swissadme.ch) [13]. Data from the web server for each of these TC compounds included ADME radar, physicochemical properties, lipophilicity, water solubility, pharmacokinetics, and drug-likeness.

5.2 Biological Activity Analysis

The biological activity of TC bioactive compounds (ligands) is predicted using Prediction of Activity Spectra for Substances Online (PASS Online) (http://way2drug.com/passonline), where the Pa score is analyzed (Pa is the predicted score of the probability of being an active compound) [14]. Bioactive compounds were also analyzed for aspects of Human Intestine Absorption (HIA), Blood-Brain Barrier (BBB), Caco2, and LD50 using the Laboratory of Molecular Modeling and Design web server (http://lmmd.ecust.edu.cn)[46].

5.3 Protein target fishing

Target proteins of related TC bioactive compounds were predicted using the Swisstargetprediction software (http://www.swisstargetprediction.ch) [36]. Ligand from TC is entered individually in the Swisstargetprediction software page. The list of proteins from the analysis results was downloaded and recapitulated.

5.4 Network analysis of proteins, protein-ligands, and immune-associated signaling pathways

The results of the target protein recapitulation are then entered into the STRING-DB software page (https://string-db.org) [47]. The "KEGG Pathway" analysis menu is opened, and proteins related to immune system regulation are selected. The existing protein network file is then "Exported to Cytoscape." The exported file to Cytoscape software version 3.8.2 is in the form of target protein networks merged with stitch to analyze ligand networks and target proteins. The pharmacological networks are combined to visualize the pharmacological network between the protein target, ligands and the pathways.

5.5 Gene Ontology (GO) and KEGG Pathway Enrichment

The primary immune system pathways are filtered out by examining a network of "core immune system target pathways" using the Kyoto Encyclopedia of Gene and Genome Paths (KEGG) and Gene Ontology (GO) enrichment analysis from the String-DB software. Using bioinformatics analysis, the KEGG enriching bubble diagram and GO term enrichments are created online using SRPLOT platform (https://www.bioinformatics.com.cn/) to represent interactions between the drug target and the pathway associated with the target immune systems.

5.6 Patterns of Interaction between TC Bioactive Compounds and Target Proteins

The target protein's 3D crystal structure was retrieved from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) database (https://rcsb.org/). JAK1 (PDB ID: 5KHX), IRAK4 (PDB ID: 5UIT), IKBKB (PDB ID: 3RZF), and AKT1 (PDB ID: 2OH0). Then, we obtained a 3D structure of a molecule corresponding to the protein that we intended to study from the Knapsack and PubChem databases, followed by importing the protein and compounds into the Discovery Studio 2019 and PyRx 0.8 software for molecular docking. Ligand docking for JAK1 was set to center_x = 10.6344; center_y = 5.7115; center_z = -16.75; size_x = 50.1121601772; size_y = 56.3999415398; size_z = 55.3799414825. Ligand docking for AKT1 center_x = 9.8445; center_y = 1.9653; center_z = -2.0844; size_x = 50.5999909401; size_y = 66.7113408279 size_z = 47.0987982368. Ligand docking for IKBKB was set to center_x = 73.4626; center_y = -19.7365; center_z = 32.939000187; size_x = 66.0479410172; size_y = 66.7760091019; size_z = 116.013708582. And the ligand docking for IRAK4 was set to center_x = -27.8055; center_y = -32.0015; center_z = 14.8585; size_x = 46.1974002504; size_y = 59.5149385262; size_z = 51.4303600121. Docking validation employs Pymol as a reference tool, aiming for an RMSD value under 2 Angstroms to be considered valid.

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