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Article

In Silico Study Potential Secondary Metabolite Candidate of Citronella Grass (*Cymbopogon nardus*) on Immunity Cases

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Abstract. Foreign invaders in the form of bacteria, fungi, or viruses can cause various diseases if they enter the body, both molecularly and cellularly. Hence, the body needs a compound that can maintain and enhance the immune and immune systems to recognize abnormal cells that can become a source of disease for the body. Improving immunity/resistance for people with diseases is very important so that people undergoing treatment are given immunostimulant drugs or pharmaceutical preparations. This study aimed to determine the protein network associated with the body's immune system, which was activated by the administration of citronella (*Cymbopogon nardus*). The research method used is explorative descriptive with in silico analysis using a computational model with software including KNApSAck, Dr. Duke, Pubchem, Swiss ADME, Swiss Target Prediction, Gene Cards, Venny, STRING, and KEGG. Based on the results of pharmacological network analysis, *C. nardus* contains 40 secondary metabolites, 25 of them have high bioavailability. Based on pharmacological network analysis, (-)-menthol is an important compound that plays a role in the immune system because it is expected to interact with three crucial pathways related to immunomodulators.

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1. Introduction

Covid-19 (Coronavirus Disease 2019) is an immunity case that initially appeared in Wuhan at the end of 2019. In Indonesia, the first case of Covid-19 was announced on March 2020. As of 11 February 2023, Covid-19 had caused more than 672 million cases and 6.85 million confirmed deaths worldwide and 6.73 million cases and 0.16 million confirmed deaths in Indonesia [1]. Transmission was primarily person-to-person through droplet transmission during coughs and sneezes, personal contact (handshakes), or touching contaminated surfaces [2]. One of the ways that can be done to prevent transmission of the virus is to implement health protocols in daily life and increase immunity [3].

Foreign invaders in the form of bacteria, fungi, or viruses can cause various types of diseases if they enter the organism, both molecular and cellular. So we need a compound that can maintain and enhance the immune and immune systems to recognize abnormal cells that can potentially become a source of disease. The immune system is a system that can guard and protect an organism from harmful pathogenic substances, even destroying foreign invaders. The immune system will then record and mark these cell types to form antibodies [4]. Lymphocytes play an essential role in the immune system against microorganisms and tumors found in lymphoid organs such as the thymus, lymph nodes, spleen, and appendix in humans. The immune system is based on two types of lymphocytes divided into B cells and T cells, each of which is involved in the humoral immune system through the secretion of antibodies. T cells also have a role in cellular immunity, such as controlling viral infections [5].

Improving immunity/resistance in people with or without diseases is crucial so that people with diseases following treatment receive immunostimulants [4]. Certain medicinal plants have an immunostimulant effect and can be used as natural immunomodulators, including citronella grass (*Cymbopogon nardus*) [6]. During the Covid-19 pandemic, immunomodulators are compounds that play an essential role in the medical fields because of their function in helping to optimize the formation of the immune system to protect the body from attacks by viruses and other harmful pathogens that can harm the health of the body [7]. The use of immunomodulators can reduce the severity in Covid-19 [8-9].

C. nardus is a type of herbal plant often processed by the community to boost the immune system by brewing the plant [6]. *C. nardus* leaves contain essential oils, which contain various compounds with unique odors and can produce oil with 7-15% citronellal and 55-65% geraniol. Previous studies have shown that *C. nardus* essential oil has immunomodulatory activity on the proliferation of mouse lymphocyte cells. *C. nardus* also contains various chemicals: aldehydes, alkaloids, esters, phenols, flavonoids, polyphenols, saponins, steroids, and tannins [7]. Flavonoids are chemicals that benefit from maintaining and enhancing the immune system and can fights against Covid-19 [7,10]. Previously it was reported that citronella has anti-fungal, antibacterial, and antioxidant activities. However, there is little or no information explaining the immunomodulatory activity in C. nardus. This research was conducted to reveal or demonstrate the molecular and cellular processes/mechanisms that occur in humans when treated with C. nardus extract using pharmacological networks.

The activity of a plant compound can be investigated through three approaches, in silico, in vitro, and in vivo. The in silico test is scientifically valid, relatively new, and highly accurate [11]. The in silico method provides initial information on C. nardus plants which are thought to have pharmacological properties and can improve the optimization of compound activation. The several stages of this method are predictions, and hypotheses, and provide discoveries in the medical and therapeutic fields [12]. With rapid advances in bioinformatics, systems biology, and polypharmacology, a network-based drug discovery called network pharmacology approaches are considered promising for cost-effective drug development [13]. Network pharmacology has been used

specifically to explore protein/gene-disease connectivity pathways. It can describe the complexity between biological systems, drugs, and diseases in terms of networks, and provide a holistic approach [14].

2. Experimental Section

2.1. Materials and Tools

This research utilized the online database to gather and process the data. KNApSAck Family (http://www.knapsackfamily.com/) and Dr. duke's Phytochemicals and Ethnobotanical Database (https://phytochem.nal.usda.gov/) were used to collect the secondary metabolite of C. nardus. SwissADME (http://www.swissadme.ch/) was used to predict the bioavailability of the secondary metabolite of C. nardus. SwissTargetPrediction (http://www.swisstargetprediction.ch/) was used to predict target proteins related to plant secondary metabolite compounds. GeneCards (https://www.genecards.org/) collected target proteins associated with immunomodulation. StringDB (https://stringdb.org/) was used to gather, assess and integrate all existing information on protein-protein interaction.

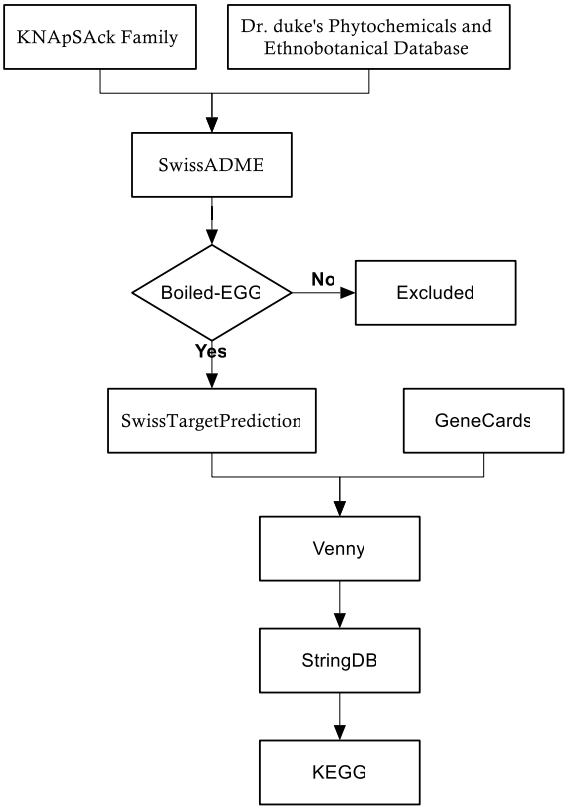

Figure 1. Schematic/flowchart of research

2.2. Methods

Identification of secondary metabolites of C. nardus was obtained using the KNApSAck Family [15] and Dr. Duke's Phytochemicals and Ethnobotanical databases [16]. Then prediction of bioavailability was carried out using SwissADME [17] and the BOILED-Egg method [18]. Only compounds that enter the BOILED-Egg area will be selected for the next step.

SwissTargetPrediction was used to collect target proteins predicted to interact with secondary metabolites [19]. Then, proteins that related to immunomodulators were collected using GeneCards

[20]. Then look for the intersection of proteins that are predicted to bind to compounds from plants using Venny [21]. The list of proteins that appeared on Venny was then entered into the StringDB database for further processing [22]. After that, predictions of protein interaction related to the immune system were searched using the KEGG Pathway method [23].

2.3. Data Analysis

Data analysis focused on what path plays the most significant role in the network. Then, analyze which protein interacts the most with the pathway. Finally, the secondary metabolites of *C. nardus* which interact with these proteins, were researched.

3. Results and Discussion

3.1. Identification of Secondary Metabolites of *C. nardus*

Secondary metabolites of *C. nardus* were obtained using the KNApSAck Family and Dr. Duke's Phytochemicals and Ethnobotanical Databases. KNApSAcK is the database with the highest number of compounds. KNApSAcK ranks first with more than 10,500 entries for therapeutic efficacy and biological activity records [24]. Dr. Duke's Phytochemicals and Ethnobotanical Databases was widely used to characterize bioactive compounds in plants [25-26]

There are several compounds in both databases. Compounds from the group of inorganic and long-chain fatty acid compounds were removed for further processing. There are 40 compounds identified in Dr. Duke and two in the KNaPSaCK Family are also present in Dr. Duke's Phytochemicals and Ethnobotanical Databases (Table 1).

Table 1. List of secondary metabolites of C. nardus from the KNApSAck Family and Dr. Duke's Phytochemicals and Ethnobotanical Databases

No.	Compound Name	Compound Code	Databases
1	1-Carvotanacetone	Mol 1	[9,10]
2	Alpha-Phellandrene	Mol 2	[10]
3	Alpha-Pinene	Mol 3	[10]
4	Alpha-Terpineol	Mol 4	[10]
5	Beta-Pinene	Mol 5	[10]
6	Bourbonene	Mol 6	[10]
7	Camphene	Mol 7	[10]
8	Camphor	Mol 8	[10]
9	Caryophyllene	Mol 9	[10]
10	Cis-Ocimene	Mol 10	[10]
11	Citronellol	Mol 11	[9,10]
12	Citronellyl-Butyrate	Mol 12	[10]
13	Delta-3-Carene	Mol 13	[10]
14	Elemol	Mol 14	[10]
15	Farnesol	Mol 15	[10]
16	Furfurol	Mol 16	[10]
17	Geraniol	Mol 17	[10]
18	Geranyl-Acetate	Mol 18	[10]
19	Geranyl-Butyrate	Mol 19	[10]
20	Geranyl-Formate	Mol 20	[10]
21	L-Borneol	Mol 21	[10]
22	L-Limonene	Mol 22	[10]
23	Linalol	Mol 23	[10]
24	Linalyl-Acetate	Mol 24	[10]

No.	Compound Name	Compound Code	Databases
25	Menthol	Mol 25	[10]
26	Methyl-Eugenol	Mol 26	[10]
27	Methyl-Heptenone	Mol 27	[10]
28	Methyl-Isoeugenol	Mol 28	[10]
29	Myrcene	Mol 29	[10]
30	Nerol	Mol 30	[10]
31	Nerolidol	Mol 31	[10]
32	P-Cymene	Mol 32	[10]
33	Perillaldehyde	Mol 33	[10]
34	Phellandral	Mol 34	[10]
35	Sabinene	Mol 35	[10]
36	Terpinen-4-OL	Mol 36	[10]
37	Terpinolene	Mol 37	[10]
38	Thujyl-Alcohol	Mol 38	[10]
39	Trans-Ocimene	Mol 39	[10]
40	Tricyclene	Mol 40	[10]

3.2. Bioavailability Prediction of the Secondary Metabolite of *C. nardus*

Bioavailability acts as a drug besides an essential parameter for determining the quantity and rate of drug absorption in the body [27]. Therefore, determining bioavailability is more critical than stating whether a compound has the potential as a drug. Bioavailability prediction was carried out using the SwissADME web server by BOILED-Egg (Brain Or Intestinal EstimateD permeation method). This method was proposed as an accurate predictive model that computes the lipophilicity and polarity of small molecules [28-29].

The BOILED-Egg model delivers a rapid, intuitive, easily reproducible yet statistically unprecedented robust method to predict the passive gastrointestinal absorption and brain access of small molecules useful for drug discovery and development [30-31]. This method uses an image model (Figure 2) to classify the absorption of compounds. The egg white area in the figure shows the ability of compounds to be absorbed in the digestive tract as long as the yolk area shows the capability of a compound to penetrate the blood-brain barrier based on WLogP and TPSA that describe the lipophilicity and polarity of a compound [18].

Table 2. Bioavailability prediction of the secondary metabolite of *C. nardus* using BOILED-Egg method

No	Bioavailability prediction	Total	Compound Code
1	High	25	Mol 1, Mol 4, Mol 8, Mol 11, Mol 12, Mol 14, Mol 15, Mol 16, Mol 17, Mol 18, Mol 19, Mol 20, Mol 21, Mol 23, Mol 24, Mol 25, Mol 26, Mol 27, Mol 28, Mol 30, Mol 31, Mol 33, Mol 34, Mol 36, Mol 38
2	Low	15	Mol 2, Mol 3, Mol 5, Mol 6, Mol 7, Mol 9, Mol 10, Mol 13, Mol 22, Mol 29, Mol 32, Mol 35, Mol 37, Mol 39, Mol 40

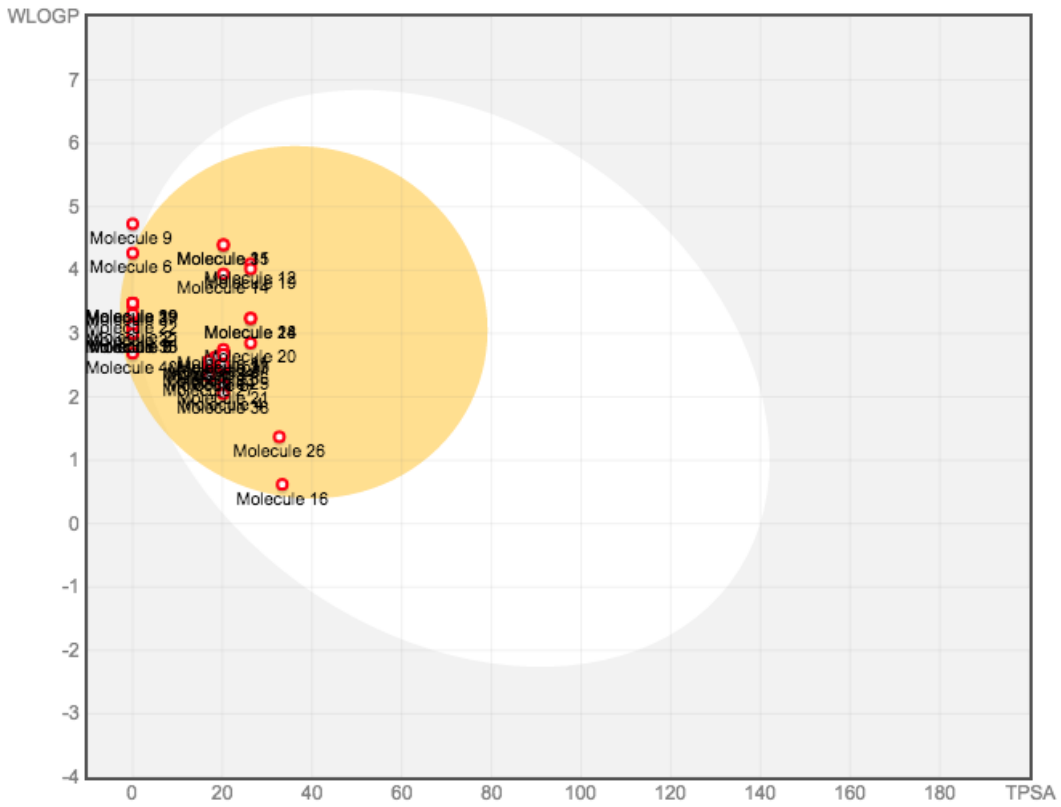

Figure 2. Bioavailability prediction of of the secondary metabolite of *C. nardus* using BOILED-Egg method. The egg white area in the figure shows the ability of compounds to be absorbed in the digestive tract as long as the yolk area shows the capability of a compound to penetrate the blood-brain barrier

3.2. Immunomodulator Protein Associated with the Secondary Metabolite of *C. nardus*

After obtaining the prediction of secondary metabolites of C. nardus which have high bioavailability, the next step is to predict the target proteins that can interact with the compounds using SwissTargetPrediction which is a computational drug target prediction tool that identifies the most probable macromolecular targets of a small molecule based on their similarity to known drugs in a library [32]. According to the research results, 502 proteins that predicted interacted with the secondary metabolites of *C. nardus*.

In order to obtain immunomodulator-linked protein data, GeneCards were used, and 1340 proteins were obtained. The GeneCards database of human genes was created in 1997 and has since been expanded to include gene-centric, disease-centric, and pathway-centric entities. GeneCards is gene-centric and a one-stop shop for detailed information about your genes of interest. GeneCards effectively navigate the world of human biological data, including genes, proteins, cells, regulatory elements, biological pathways, and diseases, and the connections between them [33].

Venny used it to find the intersection between proteins predicted to interact with secondary metabolites and immunomodulator-linked proteins. Based on the interaction results, 161 immunomodulator-linked proteins were predicted for interaction with secondary metabolites of *C. nardus* (Figure 3).

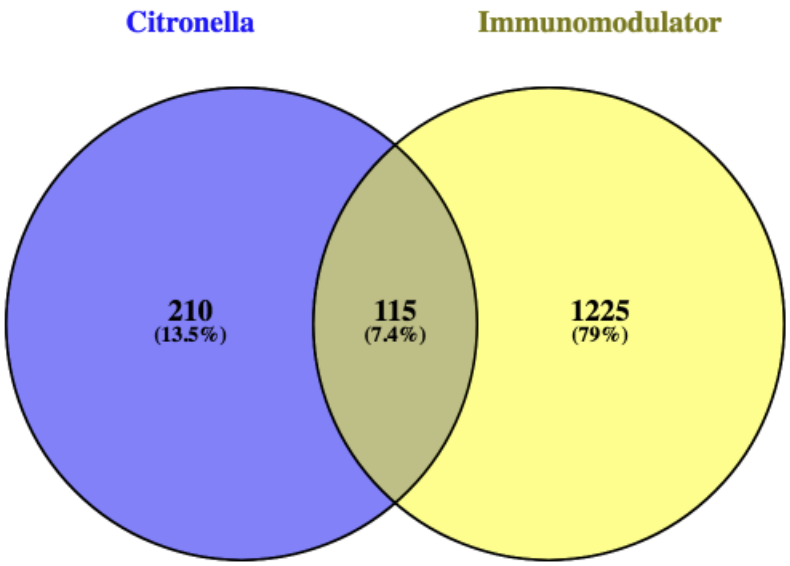

Figure 3. Venn diagram of protein that predicted *C. nardus* and immunomodulator-linked protein

The protein obtained from the intersection of the Venn diagrams is then further analyzed using StringDB, which aims to create a network of interactions between secondary metabolites and selected target proteins. This aims to determine the relationship between the selected proteins and to analyze the immunostimulating biological pathways affected by these proteins (Figure 4). StringDB is a database of known and predicted protein-protein interactions that integrate functional relationship data from various sources including > 9 million proteins [34-35]. StringDB collects various biological sources such as biochemical experiments, text mining, and co-expression studies to create an integrated score. This provides a very easy and fast way to see if groups of genes/proteins are functionally related [34].

After that, KEGG enrichment analysis was carried out. From the analysis results, the pathways associated with the immunomodulator were searched, and five pathways with the highest strength values were selected (Table 3). KEGG (Kyoto Encyclopedia of Genes and Genomes) is a collection of manually drawn pathway maps representing our knowledge of molecular interactions and reaction networks [23]. That determines the molecular mechanism of compounds in plants in interacting with target proteins to determine their role in the body's immune system. KEGG is utilized for bioinformatics research and education, including data analysis in genomics, metagenomics, metabolomics, and other omics studies, modeling and simulation in systems biology, and translational research in drug development [36].

Table 3. Pathway that related with immunomodulator by KEGG enrichment

No	Pathway	Strength
1.	PD-L1 *expression and PD-1 checkpoint pathway in cancer*	1.44
2.	*Th17 cell differentiation*	1.4
3.	*Fc epsilon RI signaling pathway*	1.31
4.	*T cell receptor signaling pathway*	1.28
5.	*IL-17 signaling pathway*	1.27

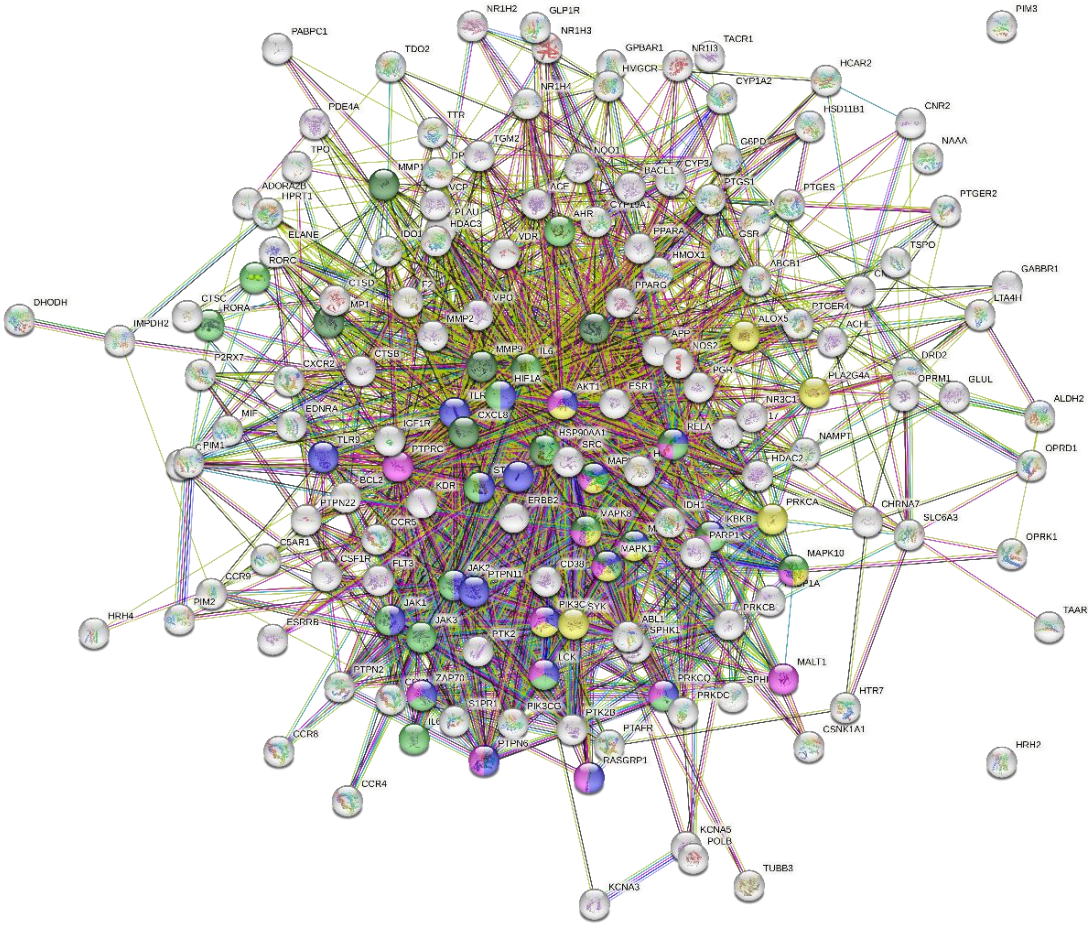

Figure 4. Network Pharmacology prediction results using StringDB. The color indicates which pathway is associated with the protein. Fc epsilon RI signaling pathway (red); PD-L1 expression and PD-1 checkpoint pathway in cancer (blue); Th17 cell differentiation (green); TNF signaling pathway (purple); IL-17 signaling pathway (yellow)

The following analysis looks for proteins connected to the five pathways, and based on the analysis, five proteins are interconnected with the five immunomodulator pathways (PARP-1, MAPK1, MAPK3, MAPK8, and MAPK14). The final step is to look for secondary metabolites from *C. nardus* that are predicted to interact with these compounds. Based on the analysis, the compound with the code Mol 25 (menthol) are the molecules with the most potential as immunomodulators that bind to PARP 1, MAPK 1, and MAPK 8 (Table 4).

Table 4. List of predicted proteins that can interact with the five immunomodulatory pathways along with molecules that are predicted to interact with them about your genes of interest.

No	Protein	Compound
1.	PARP-1	Mol25
2.	MAPK1	Mol25, Mol38
3.	MAPK3	Mol1, Mol33
4.	MAPK8	Mol4, Mol25, Mol31
5.	MAPK14	Mol14, Mol18, Mol19, Mol20, Mol30

Menthol is an organic compound found naturally in peppermint and spearmint drinks. Many studies have established that menthol has an immunomodulating mechanism that can affect the human immune tubular system. Immunomodulators are substances that can change the immune system's response to certain infections or diseases. Menthol can increase cytokine interleukin-10 (IL-10) production by immune cells of tubular cells. Menthol can help reduce inflammation in skin wounds by inhibiting the activity of inflammatory mediators and stimulating the production of anti-inflammatory cytokines such as interleukin-10 (IL-10) [37]. In addition, menthol can also stimulate the antioxidant defense system in skin cells, thereby helping to protect cells from damage caused by oxidative stress. This research was conducted by examining the response of immune cells to menthol under in vitro experimental conditions. The results showed that menthol could increase the production of IL-10 and reduce the production of pro-inflammatory cytokines in human immune cells. IL-10 is an anti-inflammatory agent that protects the tubular lining from damage result of high blood pressure [38].

4. Conclusion

Based on the results of pharmacological network analysis, *C. nardus* contains 40 secondary metabolites, 25 of them have high bioavailability. Based on pharmacological network analysis, (-)-menthol is an important compound that plays a role in the immune system because it is expected to interact with three crucial pathways related to immunomodulators

5. Acknowledgement

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