

Antifungal Potential Of The Sponge *Styllisa Flabelliformis* Against The Pathogenic And Resistant *Aspergillus* Fungi

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Abstract

Together with bacterial and viral infection, fungal infection represents the world's top ten killer diseases, desperately requiring new antifungal drugs. This research aimed to evaluate the antifungal activity of the sponge *Styllisa flabelliformis* against the pathogenic fungi *Aspergillus flavus* and *Aspergillus parasiticus* by the standard agar diffusion technique. Three concentrations (1, 10, and 10 mg/mL) for the extract and 0.5 mg/mL for ketoconazole and fluconazole were prepared and evaluated in triplicate against the tested fungi. Whereas ketoconazole poorly inhibited *A. parasiticus* and fluconazole weakly inhibited *A. flavus*, the extract of *S. flabelliformis* exerted antifungal activity against *A. parasiticus* (6.8 ± 1.8 ; 8.3 ± 3.2 ; and 9.5 ± 2.1) mm and *A. flavus* (6.8 ± 1.1 ; 11.5 ± 1.4 ; and 14.3 ± 1.1) mm 1, 10 and 100 mg/mL respectively. PASS analysis showed jasplakinolide as a promising antifungal agent with potential activity (Pa) of 0.736. STITCH analysis further confirmed that jasplakinolide worked by inhibiting the expression of cytoskeleton genes that prevented fungi from synthesizing chitin and inhibiting the formation of the fungi's cell walls and hyphae, different from the ergosterol synthesis inhibition in ketoconazole and fluconazole, implying the potential of jasplakinolide as an antifungal agent.

Keywords: Fluconazole, Jasplakinolide, Ketoconazole, Sangihe, STITCH

INTRODUCTION

Fungal infection represents an emerging and serious medical problem, associated with life-threatening diseases such as fungemia, meningitis, and chronic pulmonary aspergillosis. Not surprisingly, the World Health Organisation listed it along with bacterial and viral infections as the top ten killer diseases. This problem mainly roots in the increase of drug-resistant fungi, many of which have acquired resistance to most antifungal drugs (Perlin et al., 2017). Despite the current threatening antifungal drug resistance, antifungal drugs remain scarce (Wiederhold, 2017) and ineffective mainly due to the lack of interest in antifungal drug research compared to other therapeutic areas. Consequently, there have been only

three classes of antifungal drugs (i.e. azole, polyene, and echinocandin) used in clinical practice in the last 50 years and one new antifungal has been successfully developed in the last 30 years (Roemer & Krysan, 2014) with the most common from the azole family. Moreover, most antifungal drugs are inflicted with adverse side effects (Wiederhold, 2017), leading to high mortality, particularly in patients with a compromised immune system (Perlin et al., 2017). In particular, *Aspergillus flavus* and *Aspergillus parasiticus* are the major source of aflatoxins that cause acute and chronic liver damage, liver cirrhosis, tumor, and teratogenic effects, aggravating the already frightening antifungal resistance problem. Taken together, these suggest that there is great urgency in discovering new, safer, and more effective antifungal

drug leads with marine sponges as a promising source. This marine invertebrate was reported to contribute to 35% of the total antifungal compounds discovered from all marine invertebrates between 2012 and 2015 (El-Hossary et al., 2017).

In our ongoing search for new anti-infective drug leads from Sangihe marine sponges (Balansa et al., 2020; Riyanti et al., 2020), we screened the extracts from 15 sponges collected from Enepahembang coral reef against *A. flavus* and *A. parasiticus*. Of these, only the extract from *Styllisa flabelliformis* showed a noticeable antifungal activity. A literature search indicated that *S. flabelliformis* was a good source of antimicrobial bromopyrole-type compounds (Hassan et al., 2007) and the antifungal cyclic depsipeptide jasplakinolide (Setyowati et al., 2005). However, despite the alarming threat of the resistant pathogenic *Aspergillus* fungi against human health (Wiederhold, 2017), and the current unmet medical condition for antifungal drugs (Roemer & Krysan, 2014) neither bromopyroles nor jasplakinolide nor crude extracts from *S. flabelliformis* has been evaluated as the antifungal agents against the pathogenic *Aspergillus* fungi. Importantly, very little is known in the literature today about the interaction mechanism of jasplakinolide as an antifungal agent. Fortunately, the advent of bioinformatics has provided rich 'omics' information, making a significant breakthrough towards analysis and interpretation of the data. In this context, a database like STITCH is beneficial because this database works by integrating information on interaction, metabolic cascade, molecular structure, binding, and relation to targeted protein (Kuhn et al., 2008). Similarly, PASS is a computer program that works by predicting bioactive profiles of a given compound that resemble drugs based on the compound's structure and activity (Filimonov et al., 2014).

This study reports the antifungal activity of extract from Sangihe *S. flabelliformis* and two commercial antifungals (fluconazole and ketoconazole) against *A. flavus* and *A. parasiticus* by comparing zone of inhibition of the extract

and commercial antifungal drugs using a slightly modified agar dilution method. We also used STITCH and PASS analysis to predict antifungal interaction between jasplakinolide and between the commercial antifungals and their targeted enzymes, giving insight into their interactions with targeted proteins or enzymes, very beneficial and promising complementary tools in facilitating the slow-paced discovery process in antifungal compound discovery.

METHOD

Sponge collection

Sponges were collected by scuba diving in Enepahembang East Tahuna, Sangihe Islands, North Sulawesi, Indonesia at a depth of 1 m in July 2017 at the geographical position around 3°36'00.7"N, 125°29'44.5"E (Fig. 1). After morphological description and underwater documentation by the photograph (GoPro Hero 4.0), each specimen was cut, kept in a zipped plastic bag, and transferred to the laboratory in Politeknik Nusa Utara (POLNUSTAR) where the specimens were stored at a -16 °C freezer until used.

Extract preparation

The sponge (132 g wet weight) was defrosted, cut into small pieces, and soaked in 300 mL methanol overnight. A portion of the solution (50 mL) was filtered and spread onto 50 Petri dishes (1 ml each) and air-dried at r.t overnight to yield 0.7 grams of crude extract from which 3 methanolic solutions (1, 10, and 100 mg/mL) was prepared.

Sample identification

The spicules were purified using a modified acid digestion method (Hooper & van Soest, 2002) and measured using our previous method employing a combination of light microscopy and CoreIDRAW that we previously reported (Riyanti et al., 2020). Shortly, following the drying of a portion of the sponge (1 cm³) at 105 °C for 1 – 2 hr to completely remove water, it was left to cool down for 15 min before maceration with the commercial bleach Bayclin, washing with distilled water and rinsing with alcohol (70 %). It was followed

by the observation of free spicules under a light binocular microscope (Olympus, XSZ107BN) with 4x and 60x magnification and taking the picture of spicules through

the microscope eyepiece before the conversion of the value to a micrometer scale.

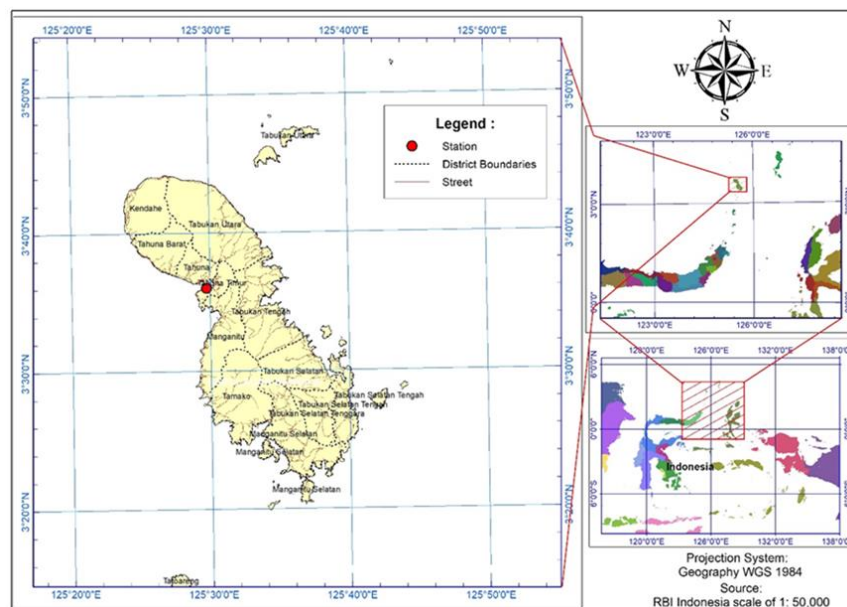


Figure 1 Sampling Location at Enengpahembang, Sangihe Islands, North Sulawesi

Antifungal assay

The standard agar diffusion method (Balouiri et al., 2016) was used to evaluate the antifungal activity of the extract of *Styllisa flabelliformis* against the two pathogenic fungi, *Aspergillus flavus* and *Aspergillus parasiticus*. The fungi were taken from the stock then streak on the media containing Sabouraud Dextrose Agar (SDA). Sterilized paper discs (6 mm in diameter) previously impregnated with the extract and antibiotic individually at different concentrations were placed on top of the media. After diffusion, the plates were incubated at 28 °C. The inhibition zone was measured after 24 hr of incubation with the assay carried out in triplicate.

STITCH and PASS analysis

A previous study showed that *S. flabelliformis* produced antifungal jasplakinolide as an antifungal agent against *Candida albicans* (Setyowati et al., 2005). Although it was not obtained experimentally in this study, it can be assumed that the same depsipeptide may be the responsible antifungal compound(s) in the extract of Sangihe *S. flabelliformis*.

To predict that antifungal activity, we used the database PASS dan STITCH (Search Tool for Interactions of Chemicals). PASS analysis was conducted by inserting canonical SMILE (Simplified Molecular-Input line-Entry System) of jasplakinolide (<https://pubchem.ncbi.nlm.nih.gov/compound/9831636>), ketoconazole (<https://pubchem.ncbi.nlm.nih.gov/compound/12854716>), and fluconazole (<https://pubchem.ncbi.nlm.nih.gov/compound/3365>) to PASS database (<http://way2drug.com/PassOnline/index.php>). STITCH analysis was performed by inserting the name of the jasplakinolide, ketoconazole, and fluconazole in the STITCH database (<http://stitch.embl.de/cgi/input.pl>) (Riyadi et al., 2020; Rumengan et al., 2021).

RESULTS AND DISCUSSION

Sponge identification

This bright orange, soft, compressible, and easily torn sponge had a foliaceous or leaf-like shaped growth form and corrugated surface structure. The sponge was collected at a depth of 1 m in Enengpahembang coral reef, attached to a

rocky bottom. It contained megascleres style typed spicule about 277.65 – 286.33 μm long. The morphological and spicule characteristics of the sponge perfectly matched those for *S. flabelliformis* (Hooper & van Soest, 2002), suggesting the assignment to *Styllisa flabelliformis* (Fig. 2).

Antifungal Test

The antifungal test showed that the extract of *S. flabelliformis* inhibited the growth of *A. flavus* and *A. parasiticus* in a dose-dependent manner with stronger antifungal activity against *A. flavus* than *A.*

parasiticus. For example, the extract exhibited an inhibition zone of (6.8 \pm 1.8; 8.3 \pm 3.2; and 9.5 \pm 2.1) mm at the concentration (1, 10, and 100) mg/mL against *A. parasiticus* respectively. Similarly, it showed the same but stronger inhibitory zones of (6.8 \pm 1.1; 11.5 \pm 1.4; and 14.3 \pm 1.1) mm at concentrations (1, 10 and 100) mg/mL respectively against *A. flavus* (Fig. 3). In contrast, whereas fluconazole showed a weak inhibitory zone against *A. flavus* (7.3 \pm 1.8 mm), ketoconazole (0 mm) failed to inhibit the growth of *A. parasiticus*.

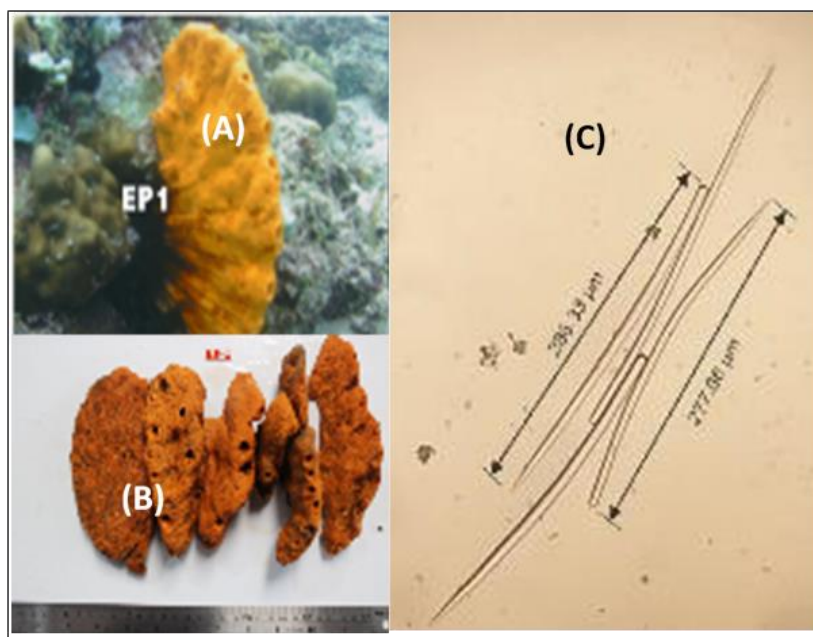


Figure 2 The sponge *Styllisa flabelliformis* underwater picture (A), laboratory picture (B), and its spicule, slightly bent megascleres style 277.6 – 286.33 μm (C).

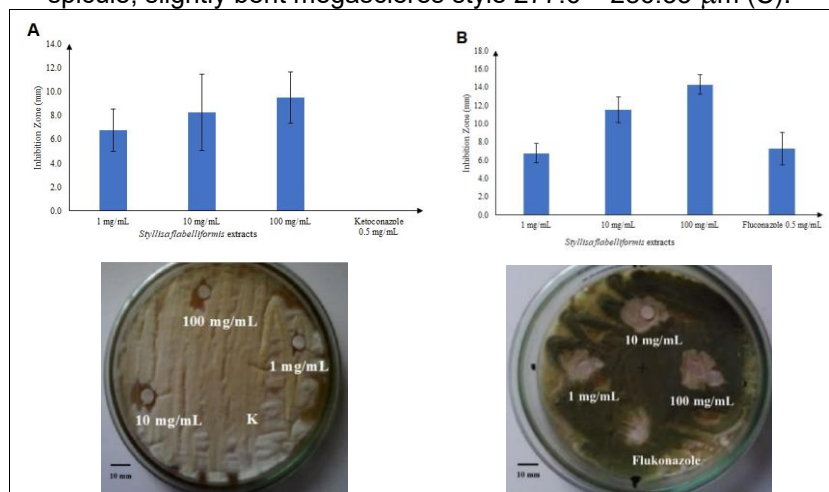


Figure 3 Inhibition of *S. flabelliformis* extract (1, 10, 100) mg/mL, ketoconazole, and fluconazole (0.5 mg/mL) against *A. parasiticus* (A) and *A. flavus* (B)

STITCH and PASS Prediction

In PASS analysis, the probability to be active ($P_a \geq 0.7$) and inactive ($P_i \leq 0.3$) mean a strong correlation between a compound structure and its bioactivity. Hence, the observed $P_a = 0.736$ and $P_i = 0.008$ for jasplakinolide confirmed a strong correlation between jasplakinolide and its antifungal activity, slightly stronger than fluconazole ($P_a = 0.726$ and $P_i = 0.008$) and much stronger than ketoconazole ($P_a = 0.645$ and $P_i = 0.014$). The STITCH analysis showed that jasplakinolide exerted its antifungal activity by modulating CTGF (Connective tissue growth factor), CDH1 (Cadherin-1) and TAGLN (transgelin) as well as by inhibiting the expression of cytoskeleton genes such as ACTA1 (α -skeletal-actin1), ACTC1 (Cardiac -Actin) and further to ACTA2 (α -skeletal-actin2) and VCL (Vinculin) (Fig. 4). In contrast, fluconazole and ketoconazole imparted their antifungal activity on *Aspergillus flavus* by interacting via cytochrome 450 (CYP) enzyme metabolism including CYP51A (14-alpha-sterol demethylase CYP51A) and CYP51B (14-alpha sterol demethylase CYP51B) and cytochrome 450 putative including CADAFLAP004358, CADAFLAP000000143, CADAFLAP004358, CADAFLAP004358 and CADAFLAP004358 (Fig. 5).

Although marine sponges are known as a prolific source of antifungal compounds, most research on sponge antifungals has been devoted to fungi of the genus *Candida* and much less on the genus *Aspergillus*. Earlier studies reported a cyclic depsipeptide from *S. flabelliformis* such as jasplakinolide as an antifungal agent against the genus *Candida* (Scott et al., 1988). As for the members of *Aspergillus* genera, the only known antifungal compound against the fungi is the alkaloid batzelladine L from the sponge *Amphimedon* sp. (Arevabini et al., 2014). Hence, to the best of our knowledge, the present study represents the first study on antifungal activity of the sponge *S. flabelliformis* against the pathogenic and aflatoxin-producing fungi, *A. flavus*, and *A. parasiticus*.

Our results also showed that extract of *S. flabelliformis* had a stronger antifungal activity against *A. parasiticus* and *A. flavus* than the commercial antifungals. Apart from confirming the resistance of *A. flavus* and *A. parasiticus*, the result suggests the potential of the extract as an antifungal agent, which shows relevance to the current unmet and acute need for antifungal agents (Krysan, 2017). However, this result should be interpreted cautiously because the antifungal activity was observed on a crude extract, not pure compounds. Although improved activity has been reported for many compounds after purification from the crude extracts, some of them suffered from the loss of activity (Nwodo et al., 2010). The authors discovered that while pure compounds showed lower antifungal activity compared to fractions and crude extracts from *C. americana*, the combination of isolated compounds and subfractions had improved antifungal activity. The result indicated the importance of purification and combination of isolated compounds from an active antifungal extract for improving antifungal activity and for the discovery of new antifungal compounds (Mendes De Toledo et al., 2015). Moreover, out of 15 extracts of Sangihe sponges, only the extract of *S. flabelliformis* showed a noticeable antifungal activity against the tested fungi, standing in stark contrast to our antibacterial screening with nearly half of 15 extracts exhibiting antimicrobial activity against Gram-negative bacteria. Nonetheless, this result agreed with earlier reports on antimicrobial screening of sponges and ascidians. Tadesse et al. in 2018 discovered that while 29% and 15% of Norwegian sponge and ascidian extracts were active against Gram-positive and Gram-negative bacteria respectively, only 5% of the extract were active against fungi (Tadesse et al., 2008). In addition, it was reported that the discovery of antimicrobial hits was much higher than that of antifungal agents. Similarly, from their intensive screening of marine invertebrates from the Caribbean, Amade et al. had arrived at the same conclusion, claiming the probability of

discovering antimicrobial being 75% for Gram-positive bacteria, 35% for Gram-negative bacteria, and only 23% for antifungal. Together, they strongly

indicated the low probability, difficulty, and challenge in discovering new antifungal agents (Amade et al., 1987).

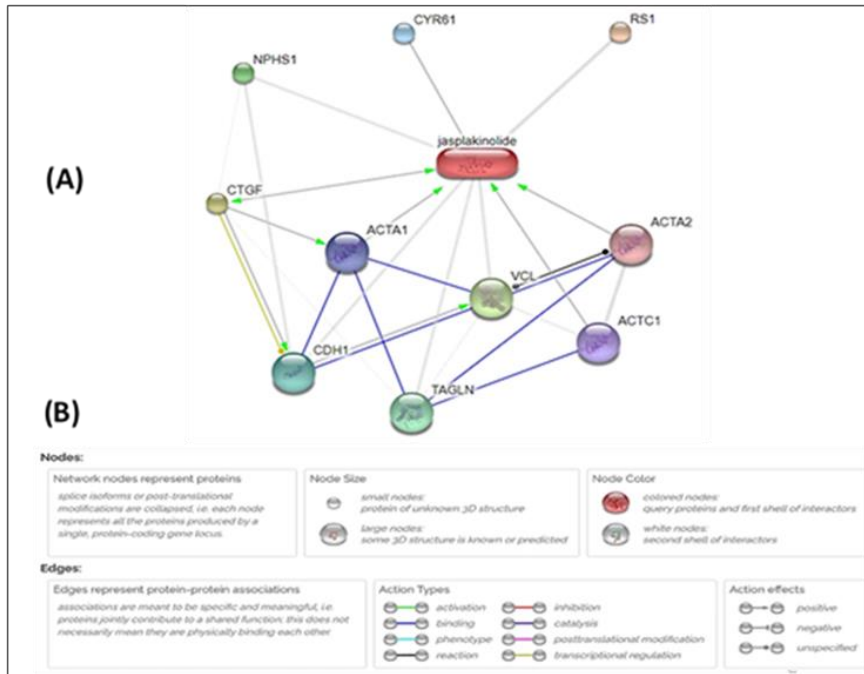


Figure 4 Pathway of jasplakinolide using STITCH database, showing an interaction between jasplakinolide and various protein (A), Network nodes representing protein, edge representing protein association action types (B).

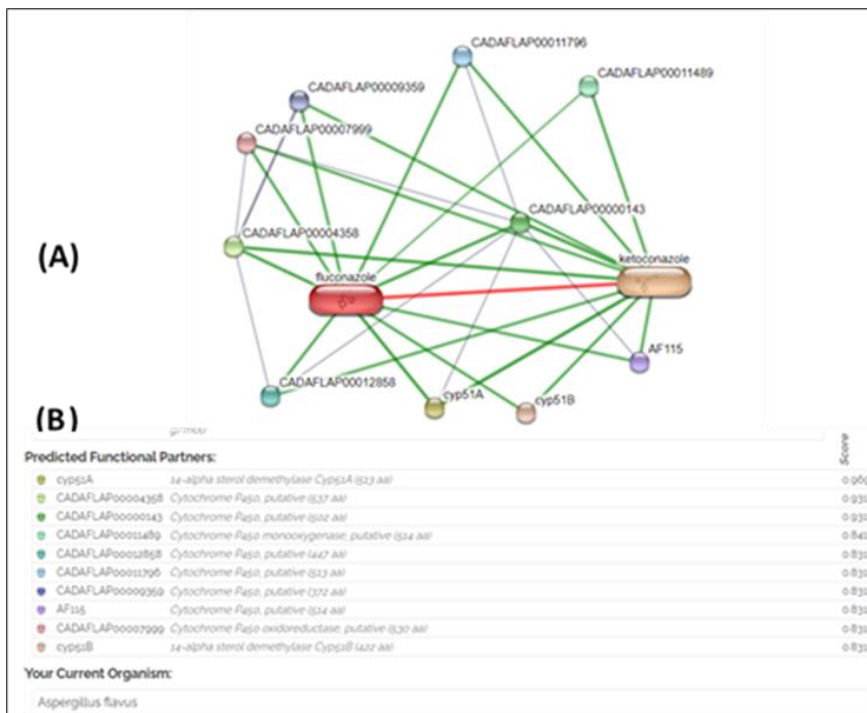


Figure 5 Pathway of antifungal ketoconazole and fluconazole using STITCH, showing superimposed connections between the antifungal and their targeted enzymes (i.e. CYP51A, CYP51B (A) and other members of the cytochrome P450 (B).

We also found that both fungi responded differently towards the commercial antifungal and the extract of *S. flabelliformis*. For instance, weak antifungal activity was observed for fluconazole against *A. flavus* but the loss of activity for ketoconazole against *A. parasiticus*. This result was in line with the report from Marichal & Vanden Bossche, (1995), who found a much higher concentration of ketoconazole was required to inhibit test fungi rather than fluconazole due to a greater affinity of fluconazole against fungal cell membranes. The result indicated the resistance of the test fungi against the commercial antifungals and the antifungal potential of the extract as an antifungal agent.

Unfortunately, our result was constrained by the lack of spectroscopic data, preventing us from elucidating the molecule(s) responsible for the antifungal activity of the extract. To this end, however, we used PASS and STITCH analysis to predict the antifungal activity of the putative jasplakinolide in the extract of *S. flabelliformis* and compare it to those of the ketoconazole and fluconazole. Because in PASS analysis, the relationship between a compound and its activity is determined to be strong when the value of probability to be active or $P_a \geq 0.7$ and the value of probability to be inactive or $P_i \leq 0.3$, then the PASS analysis showed that the relationship between jasplakinolide and antifungal activity was very strong, slightly stronger than fluconazole and much stronger than ketoconazole, indicating the potential of jasplakinolide as an antifungal agent. Indeed, this result was in agreement with a previous report on the antifungal effect of jasplakinolide from *S. flabelliformis* against *Candida glabrata*, *C. parapsilosis*, *C. albicans*, *C. pseudotropicalis*, and *C. tropicalis* on 0.96 to 2.17 ng concentration depending on incubation period (Scott et al., 1988; Setyowati et al., 2004).

STITCH analysis showed that ketoconazole and fluconazole owed their antifungal activity to the inhibition of the heme P-450 dependent 14- α -demethylation of lanosterol (CPY51A) that

led to the depletion of ergosterol, which played a crucial role in maintaining the membrane integrity of fungal cells by acting as a bioregulator of membrane fluidity and asymmetry, and hence disintegration of membrane cell integrity.

The earlier generation of azole antifungals such as ketoconazole targeted the heme protein involved in catalyzing the cytochrome P-450 dependent 14- α -demethylation of lanosterol (CPY51A), leading to depletion of ergosterol, accumulation of sterol precursors, and formation of membrane plasma with different structure and function (Ghannoum & Rice, 1999). The more recent generation including fluconazole also exerted its antifungal activity at least partly by inhibiting cytochrome P-450-dependent 14- α -sterol demethylase (Sanati et al., 1997).

In contrast, we found evidence to suggest that jasplakinolide imparted its antifungal activity mainly in two ways. First, the cyclodepsipeptide suppressed the expression of CTGF, CDH1, and TAGLN, leading to the suppression of inflammation and stimulating the macrophage's phagocytosis process. As shown in Fig. 4, following the modulation from jasplakinolide, the cognitive tissue growth factor (CTGF), a matricellular protein involved in various signaling pathways, modulated multiple signaling pathways leading to cell adhesion and migration, angiogenesis, myofibroblast activation, and extracellular matrix deposition and remodeling. CTGF also interacted with cytokines with growth factors such as transforming growth factor- β (TGF- β) (Lipson et al., 2012) and during fungal infection, CTGF and TGF- β , which are pro-fibrotic mediators, were released together with macrophages and stimulate signal transduction pathways, resulting in the proliferation and activation of fibroblasts, thereby ensuring the maintenance of normal tissue structure (Neary et al., 2015). At chronic infection, TGF- β contributed to limiting inflammation and promoting the release of lysozyme, which had an antifungal effect (Shao et al., 2005).

In addition, CTGF upregulated the transcriptional expression of CDH1 (Cadherin-1). Located at adherens junctions of the epithelial tissue, CDH1 functions in cell-cell adhesion, tension sensing, and signal transduction (Lecuit & Yap, 2015). Because this transmembrane protein has a key role in reducing inflammation due to microorganism infection, maintaining CDH1 prevented the fungus from releasing destructive protease enzymes in host tissues during the infection process (Seleem et al., 2016). Moreover, CDH1 bound to the ACTA1 gene (α -skeletal-actin1), which was a cytoskeleton that plays an important role in supplying energy during endocytosis (transport of very small macromolecules and materials into host cells) (Pooja et al., 2015) Thus, the binding prevented supply energy that inhibit endocytic processes. Furthermore, ACTA1 bound to TAGLN (transgelin), which had an important role in host defense against infection by microorganisms by modulating macrophage performance (Yu et al., 2013). Also, as TAGLN induced cytoskeletal genes and activated a phagocytic function, this upregulation of cadherin-1 by TAGLN protected host cells from sepsis (Kim et al., 2017).

Second, jasplakinolide inhibited the expression of cytoskeleton genes (ACTC1, ACTA1, ACTA2, and VCL), preventing fungi from synthesizing chitin and inhibit cell wall and hyphae formation. This process was initiated by TAGLN binding to ACTC1 (Cardiac-Actin) and ACTA2 (α -skeletal-actin2), the cytoskeleton genes involved in the contraction of myosin protein filaments (Lu et al., 2015) Because myosin was the main driver in chitin synthesis, in cell walls compiling (Treitschke et al., 2010) in endocytosis, and hyphae formation in fungi (Lamichhane et al., 2019) then the positive binding of jasplakinolide to the ACTC1 interfered with cell wall assembly, material transport, and fungal hyphae formation. Moreover, ACTA2 reacted with VCL (Vinculin), which was a functional regulator of cadherin-based cell-cell adhesion (Fig. 4) (Peng et al., 2015). Actin-based cytoskeletal genes such as

ACTA1 and ACTA 2 promoted VCL activation (Bays & DeMali, 2017). Vinculin was a gene that was often the target of pathogenic microorganisms in invading host cells (Thwaites et al., 2015). During the microbial invasion, VCL recruited actin on the plasma membrane, allowing invading microorganisms to infect the host's cells, cause inflammation (Pimentel et al., 2015) and release toxic materials (Liu et al., 2020). Therefore, inhibition of VCL activation prevented fungi from invading the host's cells. Jasplakinolide mechanism was similar to chitin synthesis inhibitors, such as nikkomycin (Ghannoum & Rice, 1999) but was different from that of the azole family.

Therefore, this study had important implications for the current effort in antifungal drug discovery which has been in desperate need of antifungal molecules for years. with a new mode of action and a new way of interpreting the omic data. In terms of molecular structure, either the extract contains bromopyrole or cyclic depsipeptide (jasplakinolide) would serve as new additional antifungals to the commercial azole antifungals whose mechanism of action targeted the ergosterol synthesis. Likewise, the different mechanisms of action shown through the STITCH and PASS data analysis for jasplakinolide and the commercial azole antifungals could contribute to the current knowledge in antifungals whose structure is limited to a few classes of antifungal compounds. Moreover, STITCH and PASS analysis, which allow the understanding of the mechanism of action of jasplakinolide and the commercial antifungal, will certainly contribute to speed up the current slow-paced and time-consuming in the traditional bioassay-guided fractionation in antifungal drug discovery.

CONCLUSION

The present study indicates that whereas the crude extract from *Sangihe S. flabelliformis* was active against *A. flavus* and *A. parasiticus*, the commercial antifungal drugs were ineffective against the test fungi. In addition to confirming the resistance of *Aspergillus* against the azole

type antifungal agents, this finding suggested the extract may contain a compound(s) either bromopyrroles or jasplakinolide, which was different from the antifungal mechanism known from the azole antifungal family such as imidazole (ketoconazole), triazole (fluconazole). In addition, both STICH and PASS analysis showed the cyclic depsipeptide jasplakinolide exerted its antifungal activity against *A. flavus* and *A. parasiticus* by inhibiting the expression of cytoskeleton genes. This inhibition prevented the fungi from synthesizing chitin and inhibiting the formation of the fungi's cell walls and hyphae and was different from that of the ergosterol synthesis inhibition known for ketoconazole and fluconazole, thus potentially contributing to the control of the resistant toxicogenic and pathogenic *Aspergillus* fungi. Similarly, the database and computer programs such as STITCH and PASS was shown to be able to contribute to speed up the slow-paced and time-consuming antifungal compound discovery. Nevertheless, further work is needed to elucidate the structure of the active compound(s) responsible for the antifungal activity of *Sangihe S. flabelliformis*. An equally important question for future studies is to determine the antifungal effect of the extracts individually or in combination with the commercial antifungals against the resistant and pathogenic *Aspergillus* fungi.

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