Antifungal Activity of Methanolic Extract of the Brown Seaweed Turbinaria ornata (Turner) J. Agardh, from Tomini Bay against Candida albicans

M S Zubair, Ardiana and A W Nugrahani
Pharmacy Study Program, Faculty of Sciences, Tadulako University, Palu, Indonesia
Email: sulaiman_zubair80@yahoo.co.id

Abstract. The brown seaweed Turbinaria ornata has been reported to possess diverse biological activities. This study aimed to evaluate the antifungal activity of Turbinaria ornata seaweed collected from Tomini Bay against Candida albicans and determine the compounds that might contribute to the antifungal activity. Turbinaria ornata seaweed extract was obtained by maceration using methanol 96% for 3 x 24 h. The filtrate obtained was then evaporated by rotary evaporator to obtain a viscous extract. Antifungal activity was tested using the agar diffusion method. Antifungal compounds were determined by bioautographic-TLC and identified using TLC reagent spray. The results showed that the methanolic extract of Turbinaria ornata possessed an inhibition zone with a diameter of 22.28 mm ± 0.63. Bioautographic-TLC test with eluent of n-hexane:ethyl acetate (7:3) found that a terpenoid (Rf value 0.71) appeared to contribute to the antifungal activity

1. Introduction
Indonesia is a maritime country, where two-thirds of the national territory is ocean. Indonesia is also known as the largest archipelagic country in the tropics, and is a country with exceptionally high marine biodiversity (megabiodiversity). One of the most abundant biological resources in Indonesian seas is seaweed. The total area of seaweed habitat in Indonesia is around 1.2 million hectares, and has thought to be the largest in the world [(Suparmi and Sahri, 2009)]. Seaweed resources need to be explored, especially considering the high diversity of seaweeds growing in Indonesian seas. Bisected by the equator, Tomini Bay is the largest bay in Indonesia, with an area of approximately 6 million hectares and abundant natural resources [(Tirtawinata, 2013)].

Seaweed, either harvested from the wild or cultivated, has been used traditionally for both food and medicine. Seaweeds tend to be rich in proteins, lipids, vitamins and minerals which are important for humans [(Wibowo, 2001)]. Turbinaria sp., one species of brown seaweed, has been reported to possess biological activity, including antibacterial [(Vijayabakar and Shiyamala, 2011)]; anti-tumour (Fajarningsih et al., 2008), anti-viral (Kumar et al., 2009), anti-coagulant (Manoj et al., 2013), antifouling (Kantida et al., 2012) and anti-fungal (Kumar et al, 2010, Kumari et al., 2011) activity. Meanwhile, Turbinaria ornata, one species of Turbinaria, has shown antioxidant and antibacterial activity against Bacillus subtilis and Escherichia coli. It contains terpenoids, saponins, and flavonoids (Vijayabakar and Shiyamala, 2012, Deepak et al, 2017). Extracts of Turbinaria conoides can be used as a natural antifungal agent for Beauveria bassiana infection in the protection for silkworm larvae [Kumari (2011)].
Diseases caused by fungi are still very common worldwide. *Candida albicans* is one of the fungi that most frequently infect humans, causing diseases such as candidiasis, respiratory infections, and thrush (Nobile and Johnson, 2015). Nowadays, there are many antifungal drugs available on the market. However, the intensive usage has resulted in reduced effectiveness of several antifungal drugs as pathogens develop resistance to existing antifungal agents. Some available antifungal products can also have toxic side-effects. This situation has prompted research to seek new antifungal agents from natural sources, such as seaweed. This study aimed to evaluate the antifungal activity of *Turbinaria ornata* from Tomini Bay against *Candida albicans*.

2. Methods

*Turbinaria ornata* seaweed was collected from Ambesia Village, Tomini Subdistrict, Tomini Bay on March 2018, and identified at the Tadulako University Biodiversity Unit. *Candida albicans* fungi were obtained from the Palu Health Laboratory. Major equipment used in this research included About 0.3 kg of powder extract was obtained through a maceration extraction method using methanol as a solvent for 3 x 24 hrs. The filtrate obtained was evaporated on a rotary evaporator (EYELA) at 54°C until a viscous extract was obtained.

Antifungal activity testing was carried out using an agar diffusion method. Potato dextrose agar (PDA) medium was poured into sterile petri dishes and allowed to solidify as a base layer. After the medium condensed, several wells were planted on the surface of the base layer. A total of 0.2 ml suspension of *C. albicans* (according to standard 0.5 McFarland 1 =1.5 x 10^8 CFU/ml) was inoculated into 25 ml of PDA medium and then poured evenly as a second layer. After solidifying, the wells were raised and filled with 50μl of sample solution at concentrations of 100%, 75%, 50%, and 25%. Each petri dish was then incubated in a reversed position at 37°C for 3 x 24 hours (Eyela® Incubator). The clear zone formed was then measured and noted. The tests were carried out with three replicates of each treatment [(Balouiri, 2016)]. Four types of controls were used: positive control using 0.1% Nystatin, negative control using DMSO, control media of PDA and control media of PDA + *Candida albicans*.

TLC Bioautography was used to identify the bioactive compounds in the extract by localizing the antifungal activity in a chromatogram (Djide, 2008). The procedure used a mixture of several eluents with different polarity with an elution distance of 7 cm to ensure sufficient separation between the compounds on the TLC plate. Before being spotted onto the TLC plate, methanol extract of seaweed *Turbinaria ornata* was first dissolved with chloroform and then sprayed on the TLC plate, then it was eluted using n-hexane:ethyl acetate (7:3). The choice of this eluent mixture was based on the results of observation to determine the eluent that produced the best separation with the most spots. The TLC plate was then dried for a while to remove the eluent liquid. The Rf value from each spot was calculated [(Djide, 2008)]. About 0.2 ml suspension of *Candida albicans* was aseptically inoculated on 10 mL of sterile PDA medium in a petri dish. After solidifying, the TLC chromatogram plate was placed in contact with the surface of the solid medium containing *Candida albicans* inoculum for 30 minutes. After 30 minutes diffusion, the plate was then removed and incubated at 30°C for 24 hours. The resulting chromatogram was observed under UV light at a wavelength of 254 nm and 366 nm. The inhibition zone was observed [(Djide, 2008)].

Positive bioautography TLC plates (showing inhibitory activity on *C. albicans*) were treated to determine the compounds responsible for the antifungal effect on *Candida albicans* fungi using spray reagents. These included FeCl₃ 1% for the detection of phenolic and tannin compounds; 10% H₂SO₄ in methanol reagent for the detection of saponin compounds; Liebermann-Burchard reagent for steroids; 1% AlCl₃ reagent for flavonoids and p-anisaldehyde-sulphate acid reagents for the detection of terpenoids [(Harborne 1987, Putiyanan et al, 2008).

3. Results and Discussion

The maceration of *Turbinaria ornata* seaweed using methanol solvent obtained 33.51 g of extract with the yield of 11.43%. The results antifungal activity tests of the methanol extract of *Turbinaria ornata*
seaweed with concentrations of 100%, 75%, 50%, and 25% against the fungal pathogen *Candida albicans* can be seen in Table 1. The TLC bioautography

| Concentration (%) | Loading dose (μg/μL) | Inhibition Zone (mm) | Average Inhibition Zone (mm) (mean±SD) |
|-------------------|----------------------|---------------------|----------------------------------------|
| 100               | 50                   | 22.22               | 22.77 21.87                            | 22.28±0.63                       |
| 75                | 37.5                 | 19.80               | 20.21 18.98                            | 19.66±0.57                       |
| 50                | 25                   | 14.54               | 15.65 14.26                            | 14.81±0.98                       |
| 25                | 12.5                 | 12.28               | 13.55 12.04                            | 12.62±1.06                       |
| Control (+)       | -                    | 31.57               | 31.88 31.47                            | 31.64±0.28                       |
| Control -         | -                    | -                   | -                                       | -                                  |

The results of the TLC bioautography showed that the methanol extract of *Turbinaria ornata* seaweed resulted in an inhibition zone against *Candida albicans* from one spot with an Rf value of 0.71 (Figure 1).

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Fig 1 Results of TLC chromatogram after eluting with n-hexane:ethyl acetate (7:3). (a) Visualization under 254 nm UV lamp. (b) Visualization under 366 nm UV lamp (c) Visible ray visualization (d) Visualization with p-anisaldehyde (e) TLC Bioautography on *Candida albicans* fungi

Antifungal activity tests are used to determine the ability of compounds present in samples to inhibit or kill certain fungi that are considered to be pathogenic in excessive amounts. In this study, the antifungal activity of the methanol extract of *Turbinaria ornata* seaweed against *Candida albicans* from Tomini bay was conducted. As well as determining the compounds that might responsible for antifungal effects using bioautographic TLC method. The results showed that the antifungal activity of methanol extract of seaweed *Turbinaria ornata* can inhibit the growth of *Candida albicans* with the average diameter of inhibitory zones depending on the concentration used: 25% (12.62 ± 1.06 mm), 50% (14.81 ± 0.98 mm); 75% (19.66 ± 0.57 mm); and 100% (22.28 ± 0.63 mm). An inhibitory zone formed in the agar diffusion test in the range 10-19 mm can be categorized as strong (Davis and Stout (1971)). It has been noted that, in general, the higher the concentration of an extract, the higher the active substance content and antifungal activity [Novi Yanti (2016)]. This study found the largest inhibition zone at 100% concentration. However, even at the lowest concentration, the diameter of the inhibitory zone formed (around 12-13mm) can be categorized as indicating strong antifungal activity.

This study found that terpenoids may responsible for antifungal activity against *Candida albicans*. This is because the results of visualization of TLC plate sprayed with anisaldehyde-sulphuric acid
caused the spot with Rf 0.71 to become purple. This reaction indicates that the compound was positive for terpenoids. This result is in accordance with previous studies [Deepak et el, (2017)] which have reported that Turbinaria ornata contains terpenoids.

Terpenoid compounds exposed to sulphuric acid spray reagents or anisaldehyde sulphuric acid will tend to become blue to blue violet although they can also sometimes form red, yellow, dark blue, purple, green or yellow brown spots under visible light [Putiyanan et al, (2008)]. Triterpenoid reaction with p-anisaldehyde-sulphuric reagent produces a purple colour based on the ability of triterpenoid compounds to form colour when exposed to H₂SO₄ in anhydrous acetic acid solvents. Terpenoids including triterpenoids and steroids are bioactive compounds that have antifungal activity. These compounds can inhibit fungal growth, either through the cytoplasmic membrane or interfere with the growth of fungal spores. However, the mechanism of inhibition by terpenoids is still not clearly known. It is possible that his presence of hydrophobic or lipophilic properties in terpenoids may cause damage to the cytoplasmic membrane, cell coagulation, and the occurrence of proton disorders in fungal cells.

4. Conclusion
Methanolic extract of Turbinaria ornata was found to inhibit Candida albicans with an inhibition zone diameter of over 20 mm at 100% and still showed strong activity at 25% concentration. Bioautographic-TLC testing with eluent of n-hexane:ethyl acetate (7:3) found that a terpenoid (Rf value 0.71) might contribute to the antifungal activity.

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