Antimacrofouling Assays of *Sargassum duplicatum* Extract in the Settlement Inhibition of *Balanus* sp. on Wooden Plank Substrate

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ABSTRACT

Marine macro-biofouling by Balanus sp. is a source of problems in marine industry. Chemicals have been employed in dealing with biofouling, but are harmful to the environment. The bioactive compounds in seaweeds offer an environment-friendly anti-biofouling alternative. The aim of this study was to examine the ability of Sargassum duplicatum crude extract to inhibit macrofouling by Balanus sp. S. duplicatum was extracted by using the maceration method. Balanus sp. settlement inhibition assays were conducted by applying extract on wooden planks at varying extract and paint ratios. The wooden planks were later immersed in the seawater. Observation of the number of Balanus sp. attached on the planks was carried out after 10, 20, and 30 days of immersion in seawater. The results from the observation showed that treatment P4 (75% extract, 25% paint) was the most effective in inhibiting macrofouling by Balanus sp. This was evident from the 10-day and 30-day observations in which the number of Balanus sp. attached was only 12.3 or 3%. The number was lower compared to in other treatments in 30-day observation, i.e., treatment P3 (50% extract, 50% paint) at 24 or 6%, treatment P5 (100% extract) at 48.3 or 12%, P2 (25% extract, 75% paint) at 73.6 or 19%, treatment P1 (100% paint) at 102 or 26%, and control P6 (no paint and extract) at 135.6 or 34%. For this reason, S. duplicatum extract was considered to have the potential to be developed as an anti-fouling agent on marine materials.

Keywords: Biofouling, Antifouling, Wooden plank, *Sargassum duplicatum*, *Balanus* sp.

INTRODUCTION

The sea hosts a variety of marine organisms that naturally adhere to materials immersed in water and cause biological fouling and damage to such materials. This is referred to as biofouling or the adverse growth of marine organisms on immersed artificial structures, such as ship hulls, jetty pilings, navigational instruments, aquaculture net cages, and seawater pipes as well as wooden poles of coastal structures (Fusetani, 2004).

These organisms typically belong to bacterium, plant, and animal groups. In general, biofouling can be divided into two types: microfouling, which involves many microorganisms, especially bacteria, and macrofouling, which involves organisms like barnacles (*Balanus* sp.) (Almeida et al., 2007; Schultz, 2007).

Macrofouling involves algae and invertebrates like soft corals, sponges, anemones, tunicates, hydroids, barnacles, mussels, tubeworms, bryozoans, and seaweeds (Bannistera et al., 2019). These organisms live in attached colonies and cause damage to materials in the sea, leading to important problems in marine technology due to cost implications in the marine environment and artificial materials (Almeida et al., 2007).

Balanus sp. is a type of fouling organisms belonging to the Crustacea group in the sea. *Balanus* sp. adheres to a substrate by secreting a substance that is capable of inflicting damages to its host (Yule and Walker, 1984). The damage shortens the life of coastal constructions and ships, sends operating costs up, and corrodes substrates (Anil et al., 1995; Bannister et al., 2019). Marine biofouling has been causing significant losses as it shortens the service life of marine equipment (Silkina et al., 2009).

Over these years, biofouling of coastal structures or ships are handled with tributyltin-based anti-fouling paints, which have been proven effective but causing side effects to the environment for their toxicity to non-target organism and for their accumulative and mutagenic nature (Burgess et al., 2003; Bangkedphol et al., 2009; Kotrikla, 2009; Silkina et al., 2009; Pereira et al., 2019). Widespread uses of the commercial anti-fouling agent tributyltin (TBT) in maritime industries can directly and indirectly increase the heavy metal contents in sea environment (Boxall et al., 2000; Giacomazzi and Cochet, 2004). This agent is toxic to marine biota and has the potential to damage marine ecosystems (Clare et al., 1999; Evans et al., 2000; Fernandez et al., 2005).

TBT was ultimately banned in many countries (Bellas, 2006), with the International Maritime Organization issuing a resolution on the restriction of the use of TBT-based paints on ships (IMO, 2002). Other harmful chemicals that are frequently used as anti-fouling agents in paints are arsenic, organo-mercury, DDT, and lead (Evans et al., 2000; Fernandez et al., 2005; Kotrikla, 2009). Therefore, a breakthrough is needed to replace chemical materials for paint with natural and environmentally friendly materials.

Physical and chemical methods come with several disadvantages. Biological methods as natural natural in biofouling handling are needed to produce eco friendly anti-fouling compounds (Yebra et al., 2004; Perez et al., 2006). Several kinds of natural anti-fouling agents that possess the ability to inhibit the growth of fouling organisms have been isolated from marine organisms like bacteria and marine algae (Abarzua and Jakubowski, 1995; Silkina et al., 2009).

Marine bioactive compounds can provide a good and environmentally friendly anti-fouling alternative to existing chemicals (Silkina et al., 2009). *Sargassum*, a seaweed from the brown algae group (*Ochrophyta*), is known to contain plenty of bioactive compounds, which are good at deterring the growth of the organism (Sastry and Rao, 1994). Seaweeds, notably *Sargassum*, are able to release secondary metabolites during metabolism for self-defense against predators and pests. The bioactive compounds produced by *Sargassum* are effective in protecting them from predators and bacteria (Santi et al., 2014). For the bioactive properties it possesses, *Sargassum* is seen to have the potential to be a marine anti-fouling source (Hellio et al., 2001).

The *Sargassum* diversity in Indonesia is quite vast, with 12 species that been identified, namely, *Sargassum duplicatum*, *S. histrix*, *S. echinocarpum*, *S. gracilimun*, *S. obtusifolium*, *S. binderi*, *S. polycystum*, *S. crassifolium*, *S. microphylum*, *S. aquofilum*, *S. vulgare*, and *S. polyceratium* (Kadi, 2005). This rich diversity is very conducive for the development of naturally sourced antifouling agents.

One of the *Sargassum* species that has been widely studied for their bioactive compounds is *Sargassum duplicatum* (Johnson et al., 2019). It contains a wide variety of chemicals with a great number of potential applications in the biotechnology industry, including as antifouling agent (Hellio et al., 2001; Chambers et al., 2006). Thus, it was considered necessary to conduct assays to find out the potential of *S. duplicatum* crude extract to inhibit fouling by marine organisms like *Balanus* sp.

MATERIALS AND METHODS

Sampling of *S. duplicatum*

Samples of the seaweed *S. duplicatum* (Figure 1) were collected *in situ* from Barang Lompo Island, Makassar, South Sulawesi, Indonesia, where large beds of *S. duplicatum* and other seaweeds were present in abundance. The samples were collected from 2 to 6 m depth by scuba-diving. The samples were kept in plastic bags. In the laboratory, specimens were washed and cleaned of epiphytes, sand, and any attached material, then placed at room temperature in the shade to air-dry. The samples were stored at -20 °C prior to extraction. Dried teak wooden plank (25 cm x 35 cm in size) was used as a substrate. Commercial green-colored paint that was used was marine paint that was known as alkyd

paints that contain biocides, which are most commonly used to paint ships sides or to coat the boats.

Figure 1. The samples of seaweed *S. duplicatum* that were collected from the beach of Barrang Lompo Island, Makassar, South Sulawesi, Indonesia.

S. duplicatum extraction

Extraction of *S. duplicatum* was conducted using the maceration method (Fahruddin et al., 2019). Methanol (1:3 w/v) was used as an extraction solvent for dried *S. duplicatum*. Samples were placed in 60 °C water bath for 24 hours, then filtered using fast qualitative filter paper Whatman No. 1. The filtrate was stored at 10 °C, then concentrated with a rotary evaporator. *S. duplicatum* extract solution was prepared by dissolving 0.01 mg/L of extract with 2 mL of methanol and after that, homogenized using a vortex mixer. The formula used to calculate the extract yield is as follows:

$$EYV(\%) = \frac{X2}{X1} x100\% \quad (1)$$

where EYV is the extract yield value (%), $X1$ is the initial sample weight, and $X2$ is the final weight (extract value).

S. duplicatum extract treatments and anti-macrofouling assays

Treatments were established by mixing the *S. duplicatum* extract solution with paint in the following ratios: P1: 100% paint; P2: 25% extract, 75% paint; P3: 50% extract, 50% paint; P4: 75% extract, 25% paint; P5: 100% extract; and P6: control (no paint and extract). For additional information, 100% of extract concentration is equivalent 0.005 mg/mL of methanol. Every treatment was applied on a teak plank substrate with a brush and was left for 4–5 hours to dry. Afterwards, the plank was immersed into the seawater near a deck at 1–1.5 m depth with a suspension rope at the same site at Popsa dock, Makassar, South Sulawesi, Indonesia (Figure 2). All treatments were performed in triplicate, resulting in comparable results. Observation of the number of organisms attached

to the plank was conducted on day 10, day 20, and day 30. Immersing the treatment was only until the 30th day, because the maximum *Balanus* sp. attachment occurs on 20-25th days (Minchinton and McKenzie, 2008). The average number of *Balanus* sp. attached to every plank was counted manually with the help of a loop (Figure 3).

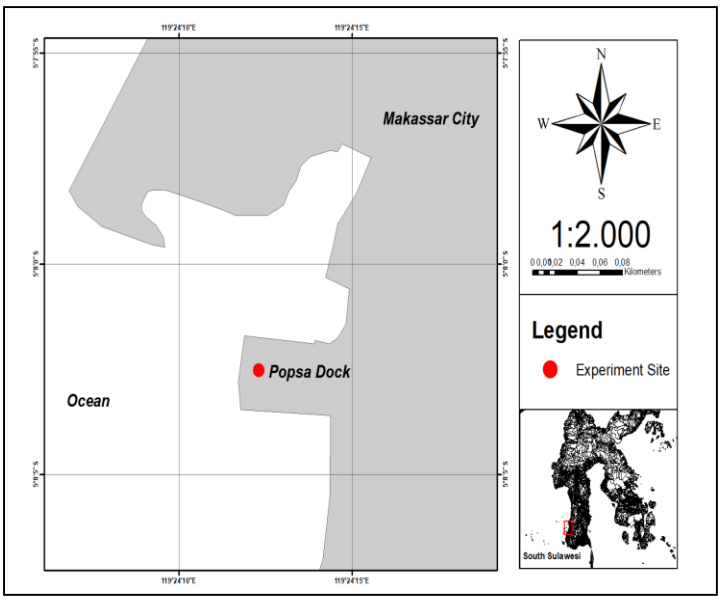

Figure 2. Map and the experiment site of anti-macrofouling activity at Popsa dock, Makassar, South Sulawesi, Indonesia.

Figure 3. Visualization of the number of *Balanus* sp. attached to plank using loop.

RESULTS

S. duplicatum extraction

A *S. duplicatum* extract yield value of 27.6% was obtained from the extract weight of 85 g that was divided by the dry weight of 306 g and then multiplied by 100%. The extract yield value was considered high based on the resulted weight, which did not differ much from the initial weight. This suggested that a high value of polar bioactive compounds was contained in the *S. duplicatum*.

Anti-macrofouling activity

From the observation on day 10, it was found that the wooden planks treated with *S. duplicatum* extract were subject to biofouling, marked with sticky and slick surfaces of the wooden planks. There were *Balanus* sp. adhering to the wooden planks, but the sizes were small. Whitish larval colonies in a large quantity were also found (Figure 4). However, only a small fraction was showing the structure of macroscopic individuals. Therefore, the fouling was not observed and counted as macrofouling, except for one that occurred on the wooden plank substrate in control P6 on the tenth day of observation, with 18 *Balanus* sp. spotted. In treatments P1, P2, and P5, only 8, 6.3, and 6.6 *Balanus* sp. were attached to the wooden plank, respectively, while in treatment P3 and P4, only 3 and 1 *Balanus* sp. were spotted (Figure 5).

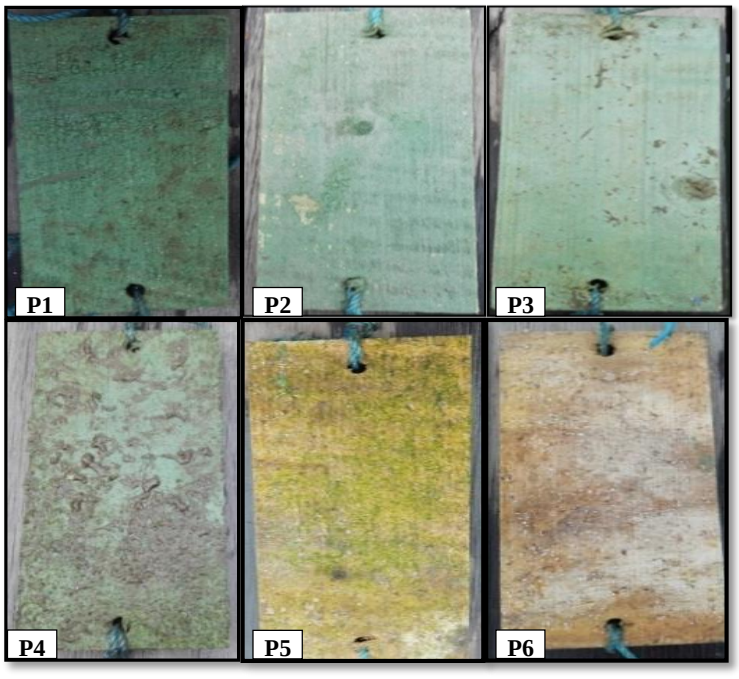

Figure 4. Appearance of wooden plank surfaces after being treated and immersed into the seawater for 10 days: P1: 100% paint; P2: 25% extract, 75% paint; P3: 50% extract, 50% paint; P4: 75% extract, 25% paint; P5: 100% extract; and P6: control (no paint and extract).

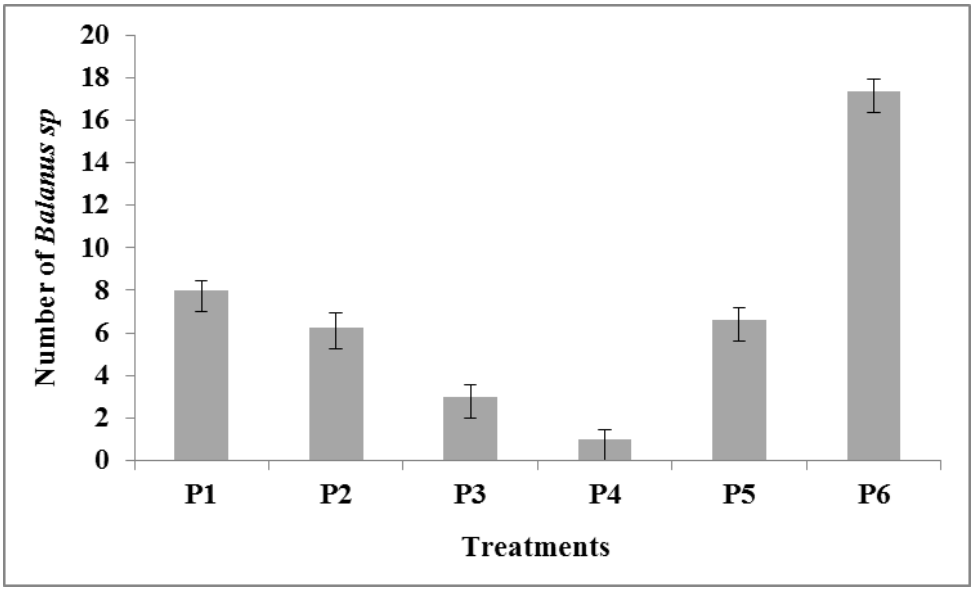

Figure 5. Number of *Balanus* sp. attached to the wooden plank on 10 days of submersion. P1: 100% paint; P2: 25% extract, 75% paint; P3: 50% extract, 50% paint; P4: 75% extract, 25% paint; P5: 100% extract; and P6: control (no paint and extract).

In the 20th day observation, the number of *Balanus* sp. attached was clearly visible on the plank surfaces. Coarse, dark shells attached to the substrates were especially apparent in treatment P6 (Figure 6). A number of *Balanus* sp. was attached in all treatments, but at varying rates. The least numbers of *Balanus* sp. were found in treatments P4, P3, and P2 as many as 4.3, 14.6, and 52, respectively, while the largest was found in treatment P1 as many as 66.6. The number of *Balanus* sp. in control P6 was even larger (94.3) (Figure 7).

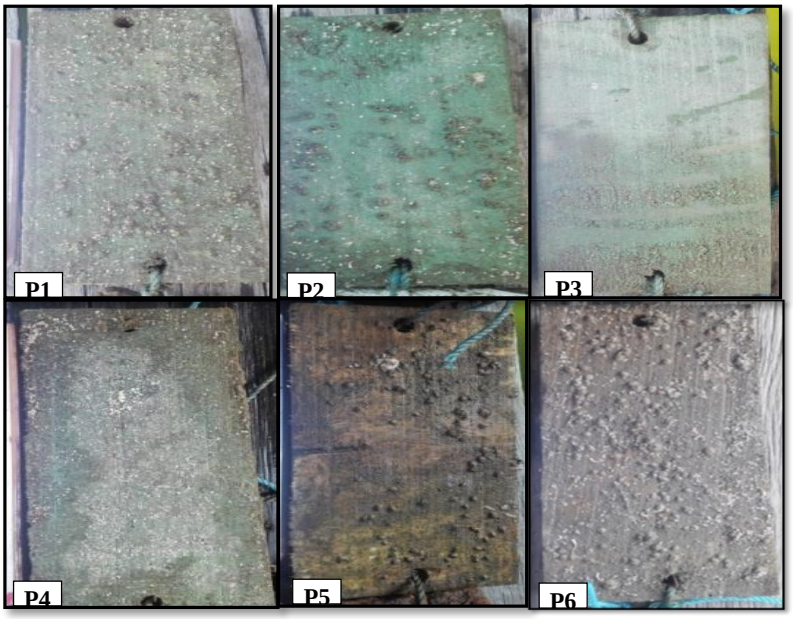

Figure 6. Appearance of wooden plank surfaces on 20th day of observation after submersion in the seawater P1: 100% paint; P2: 25% extract, 75% paint; P3: 50% extract, 50% paint; P4: 75% extract, 25% paint; P5: 100% extract; and P6: control (no paint and extract).

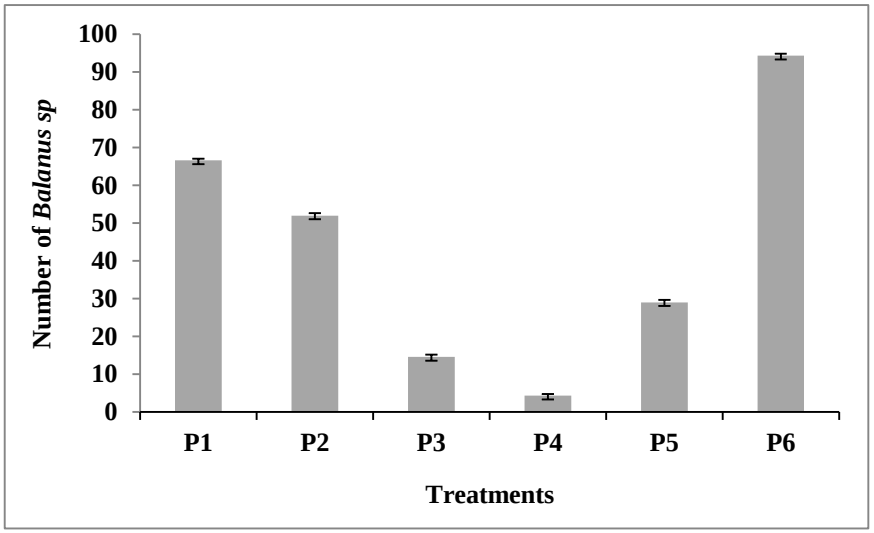

Figure 7. Number of *Balanus* sp. attached to the wooden plank on 20th day of observation P1: 100% paint; P2: 25% extract, 75% paint; P3: 50% extract, 50% paint; P4: 75% extract, 25% paint; P5: 100% extract; and P6: control (no paint and extract).

In the observation on day 30, the *Balanus* sp. density on the plank surfaces in treatments P1, P2, P5, and P6 increased, marked with acorn barnacles adhering to the substrates (Figure 8). The smallest numbers were found in treatments P4 and P3 as many as 12.3 and 24, respectively. The numbers of *Balanus* sp. in

treatments P1, P2, and P5 were 102.3, 73.6, and 48.3, respectively, while control (P6) had the most *Balanus* sp. with 135.6 (Figure 9).

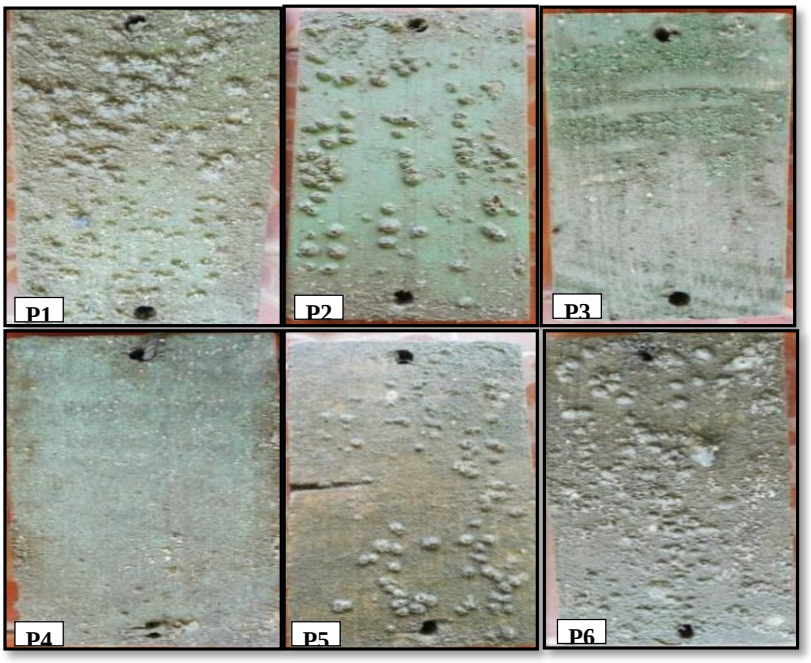

Figure 8. Appearance of wooden plank surfaces on day 30 after submersion P1: 100% paint; P2: 25% extract, 75% paint; P3: 50% extract, 50% paint; P4: 75% extract, 25% paint; P5: 100% extract; and P6: control (no paint and extract).

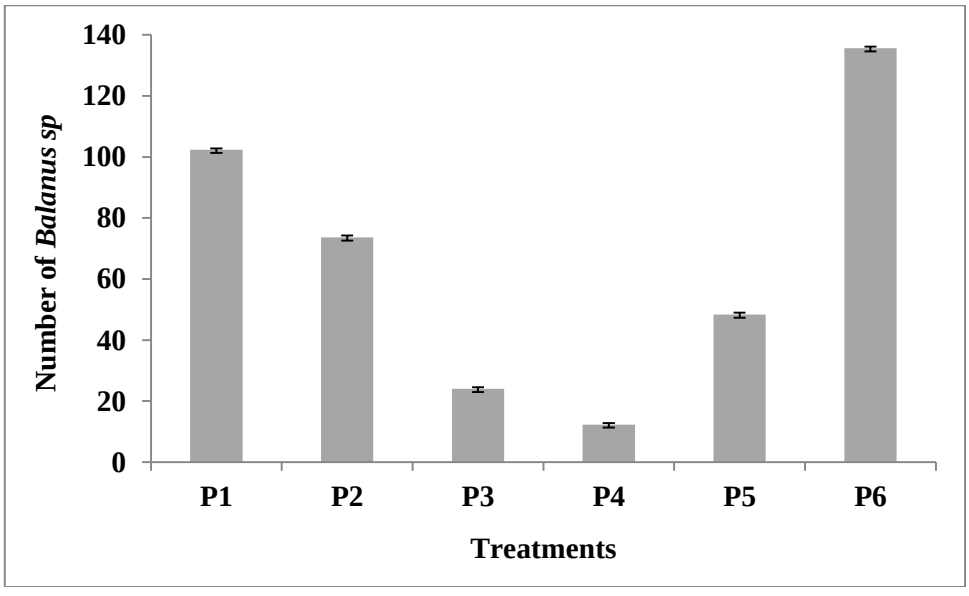

Figure 9. Number of *Balanus* sp. attached to the wooden plank on 30th day P1: 100% paint; P2: 25% extract, 75% paint; P3: 50% extract, 50% paint; P4: 75% extract, 25% paint; P5: 100% extract; and P6: control (no paint and extract).

Based on the percentage of the number of *Balanus* sp. attached to the wooden plank in the last observation on the 30th day, then the treatment P4 (75% extract, 25% paint) exhibited the highest effectiveness in inhibiting fouling *Balanus* sp. on wooden planks at 3%, followed by treatment P3 (50% extract, 50% paint) at 6%, treatment P5 (100% extract) at 12%, treatment P2 (25% extract, 75% paint) at 19%, treatment P1 (100% paint) at 26%, and control P6 (no paint and extract) at 34% (Figure 10).

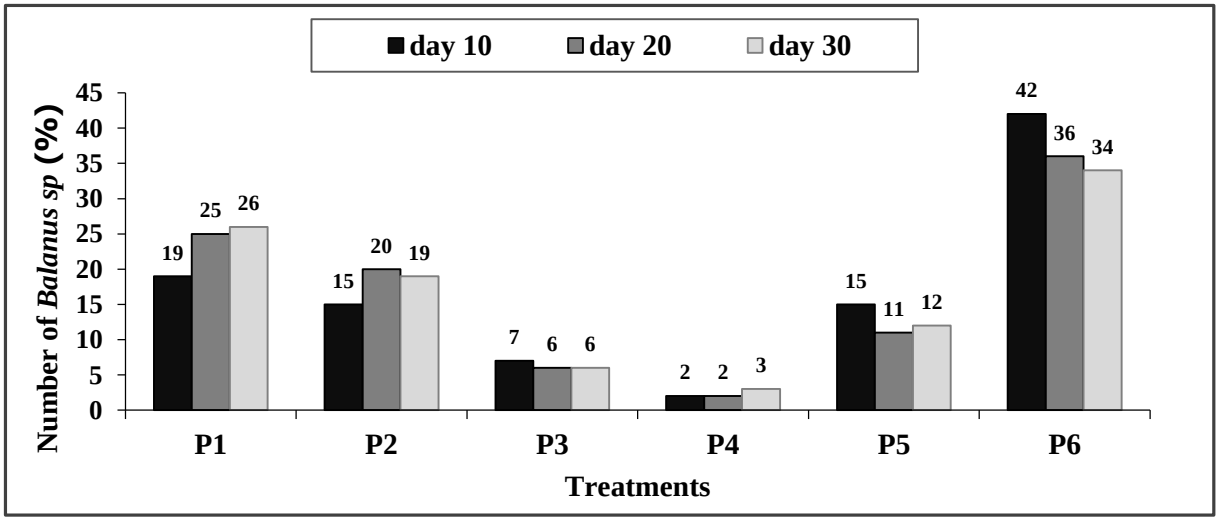

Figure 10. The effectiveness of each treatment in the inhibition of fouling *Balanus* sp. on wooden planks with treatments: P1: 100% paint; P2: 25% extract, 75% paint; P3: 50% extract, 50% paint; P4: 75% extract, 25% paint; P5: 100% extract; and P6: control (no paint and extract).

DISCUSSION

Extraction yield

An extract yield value of 27.6% indicated that methanol was a good solvent for obtaining bioactive compounds that are polar molecules (Sastry and Rao, 1994; Septiana and Ari, 2012). *S. duplicatum* generates bioactive compounds that tend to be polar rather than non-polar, while methanol, which is a polar solvent, will bind the hydrogen in bioactive compounds. As a result, methanol allows easier bioactive compounds extraction than n-hexane solvent (Santi et al., 2014; Fahruddin et al., 2019). The bioactive compounds contained in *S. duplicatum* like alkaloids, saponins, quinones, phenols, steroids, and flavonoids are deterrent to organism growth (Sastry and Rao, 1994). Based on the laboratory bioassays conducted, these metabolic compounds were found to not only be toxic to a number of organisms, but also be cytotoxic and antimicrobial (Targett et al., 1986).

Reason for using teak plank and green paint

Consideration was given to the selection of teak for this study as the type of timber that was used would influence the fouling organisms adhesion. According to Boesono (2008), teak-based materials are easy for fouling organisms to attach to on accounts of the materials elasticity and lack of chemicals that allow resistance to fouling organisms. In addition, the use of wooden planks is in accordance with boats or ships as well as pier support poles in Indonesia, especially in the islands in the Province of South Sulawesi, that are made mostly of wooden boards (Lanoeroe et al., 2005; Latifah et al., 2019).

The paint used was dark green paint for the reason that macrofouling adhesion can be affected by the selection of the paint color, and in this study, green paint was used in the paint-extract mixture (Rafael et al., 2014). Paints of dark colors are favorable to biofouling adhesion process. Fouling organisms are fonder of dark colors like brown, black, red, and green than bright colors like orange, yellow, and white. As stated by Almeida et al. (2007), dark colors have higher light absorbance rates and allow heat and light absorbance by the substrate. Organisms like barnacles demonstrate aversion to bright colors because in the larval phase, they are negatively phototrophic, which means they avoid light (Rafael et al., 2014).

Anti-macrofouling activity of *S. duplicatum* extract

On day 10, the wooden planks felt slippery to the touch. This indicated that microfouling organisms that secreted mucopolysaccharides attached themselves to the wooden planks. Biofouling occurs due to accumulation of nutrients on the substrate surfaces (Kerr et al., 1999). First, these nutrients attract microfouling organisms, then the accumulated organisms propagate and provide a source of nutrition for the development of higher-trophic-level multicellular organisms to the substrate (Myers et al., 2006; Bell et al., 2015). The bacterial biofilm on the substrate surfaces plays an important role in the adhesion and metamorphosis of some invertebrate larvae (Maki et al., 1988).

The treatment P4 (extract/paint ratio of 3:1) was the most effective in inhibiting macrofouling adhesion until day 30 with 12.3 *Balanus* sp. attached, followed by P3 (extract/paint ratio of 1:1) with 24 *Balanus* sp. attached. This suggested that increasing the *S. duplicaticum* extract portion in the mixture applied on the wooden substrate would influence the outcome.

That being said, the application of 100% extract was less effective in inhibiting *Balanus* sp. adhesion than the application of treatments P3 and P4, in which extracts were mixed with paint. *S. duplicatum* extract alone without paint would not stick to the planks for a long time, causing the anti-fouling activity to last only for a short time (Schultz, 2007; Boesono, 2008). Similarly, the application of 100% paint in treatment P1 was also proven ineffective in inhibiting the adhesion of macrofouling, showing the number of *Balanus* sp. attached more than 26%.

Treatment P4 showed the highest effectiveness in inhibiting *Balanus* sp. adhesion, proving that the bioactive compounds contained in the extract possessed the ability to inhibit the attachment of *Balanus* sp. in the sea. The differences in the quantity and the rate of bioactive compounds release led to the differences in the number of *Balanus* sp. between treatments, when extract was added to paint in varying quantities. According to Burgess et al. (2003), the paint will facilitate in the rate of release of antifouling active compounds from the extract and therefore, when the extract is incorporated into a paint, active components that are released from the painted surface wil be against a range of target organisms. In a previous study, extracts of *Renilla reniformis*, which is the sea pansy, that were added to paint and encapsulated in microtubules were found to be effective in controlling biofouling in the marine environment (Price et al., 1992), and paint formulations incorporating extracts of sponges were also shown to be active to inhibit macrofouling in barnacle settlement assays (Willemsen and Ferrari, 1993). In a separate study, extract of sponges was shown to be active against macrofouling when it was mixed with abietic acid, which is one of the main ingredients of paint and usually coated onto panels (Bakus et al., 1994). It was also stated by Matsumura et al. (2000) in the field trials that the paints had effect on either the onset or degree of macrofouling.

According to Holmström and Kjelleberg (1999), bioactive compounds produced by several marine organisms can inhibit the settlement or development of a range of surface colonizing species. This occurred because the bioactive compounds are able to cause disturbance to cell wall formation, which in turn, can lead to death of colonizing species (Johnson et al., 2019). Moreover, quinone in *S. duplicatum* is destructive to cell membranes, thus causing impediment to initiate growth and attachment on a substrate (Ganesan et al., 2008). This mechanism makes bioactive compounds able to inhibit the attachment of biofouling (Batubara et al., 2016; Johnson et al., 2019). On this account, the crude extract of *S. duplicatum* can be used as an antifouling agent containing bioactive compounds that are effective in controlling marine organisms settlement (Ganesan et al., 2008; Silkina et al., 2009).

CONCLUSION

The *S. duplicatum* extraction with methanol generated a high yield value of 27%, with a dry weight of 306 g and an extract weight of 85 g. Based on the last observation on the 30th day, mixture of extract and paint was more effective in settlement inhibition of *Balanus* sp. In the treatment of P4 (extract 75%, paint 25%), the number of the *Balanus* sp. attached was only 3%. It was the lowest compared to in other treatments. Treatment P1, which only used paint, showed 6% of *Balanus* sp attached, while treatment P5, which only used *S. duplicatum* extract, showed 12% of *Balanus* sp. attached. This shows that the combination of extracts containing bioactive compounds with paint will be more effective in

inhibiting the attachment of macrofouling rather than only extracts or only paints because in the mixture, the paint stimulates the release of the active component in the extract to control the barnacles settlement on the substract. This work demonstrated the potential of *S. duplicatum* extract as paint coating and provide a solution to prevent macrofouling settlement on materials in the sea.

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