PRELIMINARY SCREENING OF MARINE ALGAE FROM SOUTH SULAWESI COAST FOR CYTOTOXIC ACTIVITY USING BRINE SHRIMP *ARTEMIA SALINA* LETHALITY TEST

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Abstract: Fifteen algae extracts, isolated from seven species of marine algae belonging to the classes Phaeophyta (*Padina boergesenii, Sargassum prismaticum, Rosenvingea orientalis Dictyopteris acrostichoides*), Chlorophyta (*Codium dwarkense*), and Rhodophyta (*Sarconema filiforme, Wrangelia tanegana*) were investigated for their cytotoxic activity against brine shrimp *Artemia salina*. The algae extracts were prepared successively first by n-hexan followed by dichloromethane (DCM) and finally with ethyl acetate (EtOAc). Of all the extracts tested, only the dichloromethane (DCM) extract of *Sarconema filiforme* showed no significant activity with LC_{50} value >1000 µg/mL. Other 12 extracts such as *Rosenvingea orientalis* (n-hexan, DCM), *Wrangelia tanegana* (n-hexan, DCM), *Padina boergesenii* (n-hexan, DCM, EtOAc), *Codium dwarkense* (n-hexan), *Sargassum prismaticum* (n-hexan, DCM) and *Dictyopteris acrostichoides* (DCM, EtOAc) showed significant activity with LC_{50} value below 62.5 µg/mL. The n-hexan extract of *Dictyopteris acrostichoides* and the dichloromethane extract of *Codium dwarkense* exhibited intermediary cytotoxicity with LC_{50} value between 62.5 and 250 µg/mL.

***Keywords*:** Marine algae, cytotoxicity, brine shrimp *Artemia salina* lethality test.

INTRODUCTION

Over the past several decades, algae and their extracts have generated an enormous amount of interest in the pharmaceutical industry as a fresh source of bioactive compounds with different bioactivity such as cytotoxic (Tang *et al*., 2002), antibacterial (Vallinayagam *et al*., 2009), antifungal (Aliya and Shamaeel, 1999), antiviral (Serkedjieva, 2004; Garg *et al*., 1992), antitumour (Kaori, 2002), antioxidant (Yuan and Walsh, 2006) and larvasidal (Thangam *et al*., 1993). Until now more than 2400 marine natural products have been isolated from seaweeds of subtropical and tropical populations (Faulkner, 2001; Munro and Blunt, (1999).

In the world, also in Indonesia, diseases crisis caused by non-controlled cell proliferation as tumour- and cancer cells have a significant position. National Cancer Institute USA reported that almost one third of human death caused by cancer disease have correlation with human diet. As reported by Harvard School of Public Health in USA that pre-menopause women in Japan have a chance on severe breast cancer three times lower than that severe by American women. This caused by they always include algae on their food menu. Algae have a big potential as food and medicine since long ago since they rich on vitamin, mineral, crude fibrous, protein and polysaccharide (Ito and Hori, 1989; Lahaye, 1991; Darcy-Vrillon, 1993). Algae have a low fat content with unsaturated form which can protect human from cardiovascular pathogen. Their non-toxic phycocolloid role as low calorie nutrition and stabilization agent in food industry (Van den Hoek *et al*., 1993; Critchley and Ohno, 1998; Lee, 1999). In Japan traditional food, algae were used as sushi wrapper, seasonings, condiments and salad or vegetables which composed 10-25% of food consume from most Japan people (Skibola, 2004; Teas, 1981).

Global economy crisis, high price of medicinal drugs, difficult access of population on medical treatment and pharmacy, and side effect of synthetic chemical drugs, are the reasonable factor to use herbal medicine for disease treatment. Many

experts suggest consuming various foods help to prevent the developing of cancer cells or reduce the tumour cells size. Algae have ingredients like ß-carotene, protein, vit B12, fibrous, chlorophyll, and essential fatty acid which protect women from breast cancer. The omega-3 fatty acids (eicosapentaenoic- and stearidonic acid) and iodine in seaweed is thought to reduce breast cancer risk (Vinayak *et al,*.2010). Diets with three algae, *Porphyra tenera*, *Laminaria religiosa* and *L. japonica* var. *ochotensis*, showed an inhibitory effect on mammary tumorigenesis with tumor incidences of 35%, 35%, and 50%, respectively, while in control only 69% (Yamamoto *et al.,* 1987).

Most anticancer and antitumour drugs for chemotherapy treatment are synthesized from the synthetic chemical compounds and for the long term chemotherapy, many cancer patients could not be stand from suffering the drugs side-effect and can finally die. It is therefore urgent to find a new anticancer drug which safely for the long term therapy. For this, non-synthetic or natural product especially marine natural product could be a promised resource. Several study showed that marine environment which cover almost 70% of earth is a rich sources on bioactive compounds, many of them have unique structure different with those from terrestrial one. Marine secondary metabolites are organic compounds produced by marine organisms like microbes, sponges, and seaweeds. The organism biosynthesizes these compounds to protect themselves and to maintain homeostasis in their environment (Selvin and Lipton, 2004). The problem which always appears in marine drug discovery is raw material supply for industrial scale. Marine algae or seaweeds are the renewable resources that easy to cultivate in Indonesia. With the coast line of ±81.000 km, Indonesia has a big challenge or a high potential on marine algae cultivation. Until now there are 555 species of algae that have been found in Indonesia water (Bengen, 2001) and one of the areas which has a big potential for algae culture is South Sulawesi. The aim of this study was to assess the cytotoxic effects of organic crude extracts of marine algae collected from South Sulawesi coast, towards the nauplii of the brine shrimp *Artemia salina*, as a potential source of marine bioprospecting for anticancer drugs candidate.

Brine shrimp bioassay has been used in this study since it is known as an efficient, safe, fast and reproducible procedure to assess biological and pharmacological potential of new compounds (Meyer *et al*., 1982; Manilal *et al*., 2009a) and requires only a small amount of the assayed substance (Svensson *et al*., 2005). This assay has presented satisfactory correlation with citotoxicity property to some solid human tumours (Badisa *et al*., 2007). Moreover, it is an inexpensive test and without ethical constraints (Aristides *et al*., 2008).

MATERIALS AND METHODS

Algae Collecting

Algae were collected during the low tide along the coasts of Takalar and Pangkep in South Sulawesi, Indonesia. The materials were washed with cleaned sea water and put into plastic bags before kept in an ice box to prevent photolysis and thermal degradation during transportation.

Sample Preparation

In the laboratory of Marine Science and Fishery Faculty of Hasanuddin University, algae species were washed with filtered seawater to remove the epizoones, epiphytes, animal castings, sand, calcareous and other adhering detritus matters. Small samples of the species were separated for identification and the rest were washed with freshwater to remove the salt. After draining off the water, the cleaned plant materials were wiped with a blotting sheet and were sun-dried carefully under shade for 24-48 hour. Dried materials were weighed and cut into small pieces before finely grounded in a mechanical grinder. The powdered algae were kept airtight in plastic bags and put in the room temperature for further experiment.

Extraction of Algae

Extraction of algal materials was conducted as described previously (Zainuddin, 2006). 50 g of finely powdered algal material were extracted with 500 mL n-hexan in a 1-L capacity round bottom flask (1:10, w/v). The extraction was run on a stirrer plate for 24 h under room temperature. The extracts were filtered through a Whatman no. 1 filter paper then evaporated under reduced pressure in a rotary evaporator until 5-10 mL volume. The concentrated extracts were kept on small vials and let dry under room temperature to yield thick oily crude extract and stored airtight at -20ºC for further analysis. The algae residue from n-hexane extraction were dried at room temperature for 24 h and re-extracted successively with higher polarity solvents (dichloromethane and ethyl acetate) using the method as described above.

Brine Shrimp Lethality Test

Brine shrimp *Artemia salina* eggs were hatched in a flask containing filtered seawater (1 g cyst per litre) under continuous illumination at 27–30ºC for 48 h. The air stone was placed in the bottom of the jar to ensure complete hydration of the cysts. After incubation period, newly hatched nauplii were collected for the assay.

Brine shrimp cytotoxicity assay was performed using the freshly hatched free-swimming nauplii of *Artemia salina*. Stock solution was prepared by dissolving ten mg of crude algae extract in 1% methanol in an eppendorf and filled with filtered seawater until 1 mL volume. Test solutions were prepared by diluting the stock solution with filtered seawater to obtain two-fold serial dilution (1000, 500, 250, 125 and 62.5 µg). Ten brine shrimp nauplii were transferred into vials containing 4 mL filtered seawater. One mL of the test solution contained each extract concentration were added into vials to make 5 mL of total solution. Parallel positive (only methanol and seawater) and negative (only seawater) controls were included in experiment set up. The cytotoxicity was determined after 24 h exposure under constant illumination. The number of live brine shrimp nauplii in each vial was determined with a hand lens, and the mortality rate was calculated. The mortality end point of the bioassay was determined if no internal or external movement of larvae was observed during 30 seconds. Each test was run in duplicate and it was repeated until two or three times.

Based on the percent mortality, the results were expressed as LC_{50} value. It was defined as the concentration needed to cause half of the tested brine shrimp died within 24 h. If the LC_{50} value is <1000 µg/mL the assayed compound was regarded as cytotoxic and if the value is >1000 µg/mL, the assayed compound was regarded as non-toxic (Meyer *et al*., 1982; Badisa *et al*., 2007; Parra *et al.,* 2001).

RESULTS

The algae species are collected along the coasts of Takalar and Pangkep, South Sulawesi Province, Indonesia. They are distributed in the intertidal zone of sandy beaches and belonging to classes Phaeophyta, Chlorophyta and Rhodophyta. Five species consist of four Phaeophyta (*Padina boergesenii, Sargassum prismaticum, Rosenvingea orientalis, Dictyopteris acrostichoides*) and one Chlorophyta *(Codium dwarkense)* were collected from Takalar coast, whereas two Rhodophyta (*Sarconema filiforme* and *Wrangelia tanegana*) were collected from Pangkep coast (Table 1).

Percent of dry biomass ranging from 6.57% to 19.62%. The highest percent were shown by brown alga *Rosenvingea orientalis* and red alga *Sarconema filiforme* (both have dry weight of 19.62%) whereas the lowest was shown by green algae *Codium dwarkense* with 6.57% (Table 2).

Table 1. Figure and classification of marine algae collected from Takalar and Pangkep coast of Sulawesi Selatan

No.	Algae Species	Class, Order and Family (Location)	Algae Figure
1.	*Padina boergesenii*	Phaeophyta, Dictyotales, Dictyotaceae (Takalar)	
2.	*Sargassum prismaticum*	Phaeophyta, Fucales, Sargassaceae (Takalar)	
3.	*Rosenvingea orientalis*	Phaeophyta, Scytosiphonales, Chnoosporaceae (Takalar)	
4	*Dictyopteris acrostichoides*	Phaeophyta, Dictyotales, Dictyotaceae (Takalar)	
5	*Codium dwarkense*	Chlorophyta, Bryopsidales, Codiaceae (Takalar)	
6	*Sarconema filiforme*	Rhodophyta, Gigartinales, Solieriaceae (Pangkep)	
7	*Wrangelia tanegana*	Rhodophyta, Ceramiales, Wrangeliaceae (Pangkep)	

Table 2. Wet- and dry-weight of marine algal biomass

No.	Algae Species	Class	Wet Weight (ww) (g)	Dry Weight (dw) (g)	Percent of dw/ww
1	*Padina boergesenii*	Phaeophyta	540	58.22	10.78
2	*Sargassum prisnaticum*	Phaeophyta	597.3	74	12.39
3	*Rosenvingea orientalis*	Phaeophyta	265.04	52	19.62
4	*Dictyopteris acrostichoides*	Phaeophyta	1008	110,9	11
5	*Codium dwarkense*	Chlorophyta	1129	74.14	6.57
6	*Sarconema filiforme*	Rhodophyta	158	31	19.62
7	*Wrangelia tanegana*	Rhodophyta	514	78.67	15.31

A total of 15 organic extracts of seven marine algae species were obtained by extraction with three different polarities of solvent. The dry weight of crude extracts ranging from 110.5 mg to 662 mg (0.22-1.32%). The highest percent of dry weight was shown by dichloromethane extract of brown alga *Dictyopteris acrostichoides* (1.32%), whereas the lowest one was shown by n-hexane extract of red alga *Wrangelia tanegana* (0.22%.) (Table 3).

The fifteen organic extracts were evaluated for their cytotoxicity using brine shrimp *Artemia salina* lethality test. Twelve of 15 organic extracts showed high cytotoxicity with LC_{50} <62.5 µg/mL, whereas two extracts only presented intermediary cytotoxicity with LC_{50} ranging from 62.5 to 250 µg/mL. Of all the organic extracts tested, dichloromethane extract of *Sarconema filiforme* exhibited no cytotoxic activity against *Artemia salina* larvae with LC_{50} >1000 µg/mL (Fig. 1).

Table 3. Percentage of crude extracts obtained from 50 mg of biomass dry weight in 500 mL organic solvent.

No.	Algae species	Class	Extracts	Crude Extracts (mg)	Crude Extracts (%)
1	*Rosenvingea orientalis*	Phaeophyta	Hex	218.1	4.36
2	*Wrangelia tanegana*	Rhodophyta	Hex	110.5	2.21
3	*Padina boergesenii*	Phaeophyta	Hex	226.9	4.54
4	*Codium dwarkense*	Chlorophyta	Hex	147.5	2.95
5	*Sargassum prismaticum*	Phaeophyta	Hex	215.7	4.31
6	*Dictyopteris acrostichoides*	Phaeophyta	Hex	519.6	10.39
7	*Codium dwarkense*	Chlorophyta	DCM	222.4	4.45
8	*Sarconema filiforme*	Rhodophyta	DCM	142.4	2.85
9	*Dictyopteris acrostichoides*	Phaeophyta	DCM	662	13.24
10	*Rosenvingea orientalis*	Phaeophyta	DCM	213	4.26
11	*Padina boergesenii*	Phaeophyta	DCM	144.4	2,89
12	*Wrangelia tanegana*	Rhodophyta	DCM	361.3	7.23
13	*Sargassum prismaticum*	Phaeophyta	DCM	238.6	4.77
14	*Dictyopteris acrostichoides*	Phaeophyta	EtOAc	328.4	6.57
15	*Padina boergesenii*	Phaeophyta	EtOAc	282.6	5.65

Table 4. Cytotoxicity of organic extracts of marine algae collected from South Sulawesi coast against *Artemia salina* nauplii.

No	Algae species	Class	Extracts	LC_{50} value	Cytotoxicity Levels
1	*Rosenvingea orientalis*	Phaeophyta	n-Hexan	<62.5 µg/mL	High Cytotoxicity
2	*Wrangelia tanegana*	Rhodophyta	n-Hexan	<62.5 µg/mL	High Cytotoxicity
3	*Padina boergesenii*	Phaeophyta	n-Hexan	<62.5 µg/mL	High Cytotoxicity
4	*Codium dwarkense*	Chlorophyta,	n-Hexan	<62.5 µg/mL	High Cytotoxicity
5	*Sargassum prismaticum*	Phaeophyta	n-Hexan	<62.5 µg/mL	High Cytotoxicity
6	*Dictyopteris acrostichoides*	Phaeophyta	n-Hexan	125-250 µg/mL	Intermediary Cytotoxicity
7	*Codium dwarkense*	Chlorophyta	DCM	62.5-125 µg/mL	Intermediary Cytotoxicity
8	*Sarconema filiforme*	Rhodophyta	DCM	>1000 µg/mL	No Cytotoxicity
9	*Dictyopteris acrostichoides*	Phaeophyta	DCM	<62.5 µg/mL	High Cytotoxicity
10	*Rosenvingea orientalis*	Phaeophyta	DCM	<62.5 µg/mL	High Cytotoxicity
11	*Padina boergesenii*	Phaeophyta	DCM	<62.5 µg/mL	High Cytotoxicity
12	*Wrangelia tanegana*	Rhodophyta	DCM	<62.5 µg/mL	High Cytotoxicity
13	*Sargassum prismaticum*	Phaeophyta	DCM	<62.5 µg/mL	High Cytotoxicity
14	*Dictyopteris acrostichoides*	Phaeophyta	EtOAc	<62.5 µg/mL	High Cytotoxicity
15	*Padina boergesenii*	Phaeophyta	EtOAc	<62.5 µg/mL	High Cytotoxicity

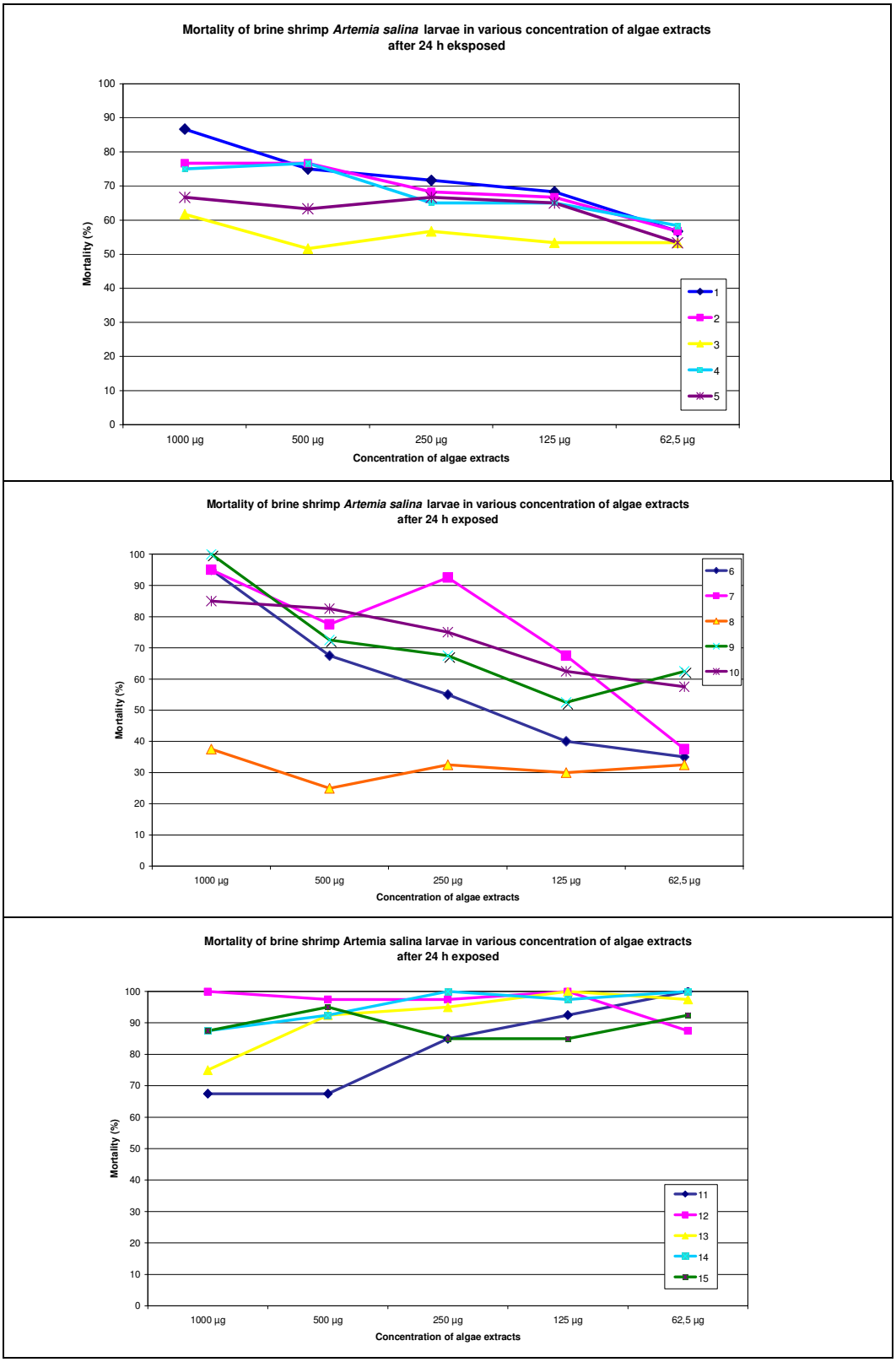

Figure 1. Graphic of percent mortality of *Artemia salina* nauplii after 24 h exposed in various concentrations of algae extracts (number on the graphic related to the number in Table 4).

Of six n-hexan extracts, only n-hexan extract of brown alga *Dictyopteris acrostichoides* had intermediary activity towards the nauplii of the brine shrimp *A. salina*

(LC_{50} value between 125-250 µg/mL), whereas others showed high cytotoxicity with LC_{50} below 62.5 µg/mL. Two dichloromethane extracts obtained from red alga *Sarconema filiforme* and green alga *Codium dwarkense* had no lethal effect (LC_{50} >1000 µg/mL) and intermediary cytotoxicity (LC_{50} occurred between 62.5 and 125 µg/mL) respectively. Ethyl acetate extracts of two brown algae *Dictyopteris acrostichoides* and *Padina boergesenii* showed a potent cytotoxic effect against *Artemia salina* nauplii (LC_{50} value below 62.5 µg/mL) (Table 4).

DISCUSSION

A number of studies have demonstrated that marine algae secondary metabolites from different geographical region had cytotoxic effects against *Artemia salina.* The algae, *Ulva fasciata* and *Hypnea musciformis* collected from India coast showed moderately cytotoxicity against brine shrimp (Selvin and Lipton, 2004). El-Baroty *et al.* (2007) demonstrated that the powdered red alga *Asparagopsis taxiformis* showed cytotoxic activities on *Daphnia magna.* In our study, almost all extracts from seven species of marine algae exhibited high and moderate cytotoxic activites against *Artema salina* nauplii.

In this study, the brown algae were established as a rich source of cytotoxic compounds since 10 of 14 potential extracts were obtained from these species. These results are comparable to the results of other cytotoxic study of algae, which five ethanolic extracts of brown algae species namely *Stoechospermum marginatum, Sargassum swartzii, Sargassum binderi, Spatoglossum asperum, Stokeyia indica* showed significant cytotoxicity on brine shrimp assay (Ara *et al*., 1999). From the other study with brown algae *Sargassum ringgoldianum* and *Porphyra yezoensis*, a potential antitumor activity to ehrlich carcinoma and meth A fibrosarcoma was detected (Noda *et al.,* 1989). Beside brown algae, many of the secondary metabolites produced by the marine red algae are well known for their cytotoxic property against cancer and tumor cells. As noted by Harada and Kamei (1997) that the extract of red alga, *Amphiroa zonata* exhibited strong cytotoxicity to human leukemic cell line.

In this study, from 15 non-polar extracts, almost all were effective against *Artemia salina* nauplii. This may be due to a non-polar structure of cytotoxic compound(s). Of all tested extracts, the hexan extracts performed high and moderate cytotoxic activities against the nauplii of *Artemia salina*. From the study of antitumor activity of 42 japanese seaweeds species against K562 human leukemia cells, significant cytocidal activity was found in hexane-extract from *Protomonostroma undulatum, Enteromorpha linza, Monostroma latissimum*, *Scytosiphon lomentaria, Hizikia fusiformis* and *Sargassum thunbergii*, while cytostatic activity was indicated in hexane-extract from *Colpomenia sinuosa, Ecklonia cava, Undaria pinnatifida* and *Sargassum muticum* and *Chondria crassicaulis*. The hexane-extract of *Ecklonia cava* (Phaeophyceae) had the highest antitumor activity which inhibited cell growth by 45, 92.1 and 96.4% at 10, 20 and 50 MU.g/ml, respectively. Hexane extract of *E. cava* also induced apoptosis of K562 cells (Kaori, 2002). This is also related with our study, where the hexane extracts of brown algae (Phaeophyceae) showed more potential than the other algae. The dichloromethane extracts showed also good potential against *Artemia* nauplii. Five of seven DCM-extracts exhibited high cytotoxic activity against *Artemia salina* nauplii (LC_{50}= <62.5 µg/mL). From the other study, the crude dichloromethane:chloroform extract of *Stypopodium zonale,* a Brazilian coastal seaweeds, showed good cytotoxic activity against the C32 cell line (Rocha *et al.,* 2006). In this study, all ethyl acetate extracts of brown algae showed high cytotoxicity against the nauplii of *Artemia* salina. This was also performed in the other studies, where the ethyl acetate extracts of *Gracilaria salicornia* and *Hypnea flagelliformis*, collected from Persian Gulf showed potent cytotoxic effect against *Artemia salina* nauplii with LC_{50} values of 3 and 4 µg/ml, respectively (Saeidnia *et al.,* 2009). The ethyl acetate extracts of *Sargassum thunbergii*

and *Dictyopteris divaricata* showed excellent cytotoxic activity against the HL-60 cell line. Furthermore, the *Sargassum thunbergii* extract also exhibited good cytotoxic activity against the HT-29 and B16F10 cell lines. These results suggest that *S. thunbergii* and *D. divaricata* have great potential value as food additives, medicinal supplements for patients with chronic diseases and preventive agents against cancer (Kim *et al.,* 2009).

Other than the extracts, many specific compounds also have been found in algae. Fucoidan, laminarin and terpenoids stated to possess anticancer, antitumor and antiproliferative properties (Smith, 2004). These cytotoxic compounds could be further explored as novel leads in cancer chemoprevention and complementary chemotherapy and necessitates further investigation (Vinayak *et al*., 2010). Fucoidan, a sulphated polysaccharides isolated from brown algae *Laminaria cichorioides,* exerts chemopreventive effects. It inhibits neoplastic cell transformation induced by epidermal growth factor or a tumor promoter (12-O-tetradecanoylphorbol-13-acetate) (Anonim, 2009). Beside fucoidan, other highly sulphated polysaccharide isolated from *Gracilaria dominguensis* inhibited the transplantation of Ehrlich ascites carcinoma in mice (Fernandez *et al.,* 1989). The polysaccharide DAEB was isolated and purified from *Enteromorpha* intestinalis and had potent antitumor activity which may be associated with its potent immunostimulating effect (Jiao *et al*., 2009). The *in vitro* and *in vivo* antitumor properties of a sulfated polysaccharide isolated from the alga *C. feldmannii* (Cf-PLS) has some interesting anticancer activity that could be associated with its immunostimulating properties (Lins *et al*., 2008).

Many species from genus *Sargassum,* such as *Sargassum micracanthum (Mori et* al., 2005), *Sargassum caryophyllum* (Tang *et al*., 2002) and *Sargassum tortile* (Numata *et al*., 1991) exhibited cytotoxic activity against cancer cell lines. The sulphated polysaccharides of *Sargassum* act as a potent anticancer agent (Dias *et al*., 2005). In our studies, one of genus *Sargassum* (Sargassaceae), *Sargassum prismaticum* showed high cytotoxic activity on *Artemia* nauplii.

CONCLUSION

High price of the medicine and difficult access to the pharmacy could be the reason for poor people in the coastal community to provide their drug with marine medicinal plant surround their area. As we know, culture location of algae or seaweeds is in the sub-tidal zone with 2 m depth. Like terrestrial plant, marine algae require sun light for their metabolisms and Indonesia is one of the tropical countries which obtain sun light through the year. This advantage makes the algae is easy to be cultivated in Indonesia regions (marine bioprospecting). With ±75% of marine area (5,8 million km2), ±81.000 km coast line and 17.500 big and small island, make the marine and fishery industries have good expectations. Sulawesi Selatan is one of the east Indonesia regions which has a potential area to develop the algae culture. Almost half of total national productions of algae commercial are come from South Sulawesi area. Therefore, the aim of the study is to explore the bioactive compounds of marine algae from South Sulawesi as marine bioprospecting resources. In conclusion, the results of this study showed that marine algae from South Sulawesi can be used as medicinal marine plant for anticancer drugs candidate.

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