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The Ethanolic Extracts The Gorgonian *Isis hippuris* Inhibited the Induced Mammary Carcinoma Growth In C3H Mice

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Abstract. The gorgonian *Isis hippuris* contains secondary metabolites gorgosterol and hippuristanol which are capable of inhibiting cancer cells. However, *in vivo* test of the gorgonian *Isis hippuris* extract as the anticancer drug has not been conducted. The research to study of the effect of ethanolic extract of the gorgonian on the induced tumor growth in C3H mice. The *I. hippuris* was obtained from Karimunjawa water in Jepara. The extract was prepared by maceration using ethanol. A total 20, 10 moths old of C3H mice with an initial weight of 20-25 gram were assigned into control, Ih-1, Ih-2, and Ih-3 groups. Control, Ih-1, Ih-2, and Ih-3 groups each received 0, 0.15, 1.5, and 15 mg extract per mouse per day respectively for two weeks. Cancer cells were introduced to all groups from a donor cancer mouse by injection via left or right axilla and allowed to grow. The cancer mass was removed and processed for histological examination, and cancer growth was determined according to Elston and Ellis criteria. The result showed that histological grade of cancer mass from the control group was in grade 2 or differentiated moderately. The histological grade of cancer mass from Ih-1, Ih-2, and Ih-3 groups were in grade 1 (low grade) or similar to a normal cell. Statistical analysis by Kruskal-Wallis test showed a significant difference ($p<0,0$ between control and treated mice. Mann-Whitney test found no significant differences among Ih-1, Ih-2, and Ih-3 treated mice. The results indicated the potential of active substances in the ethanol extract of *I. hippuris* as an anti-cancer drug.

Keywords: tumor, mammae, bioactive, marine biota, soft coral

1. Introduction

Breast cancer is a major health problem and was among the top three most common malignancies globally in 2012 [1]. Surgery, chemotherapy, or radiation are standard therapies with varying success depending on the stage of the breast cancer. The cost of breast cancer therapy is high with side effects which often

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put the patients to give up treatment.The side effect of chemotherapy is due to less specificity of the drug which kills not only cancer cells but also normal cells. There is also breast cancer cells which develop resistance to the various drug and named as *Multidrug Resistance* (MDR) breast cancer cells [2]. The presence of MDR breast cancer cells is linked to breast cancer recurrence and prevent recovery. Breast cancer is a global burden, and various lines of approach have been made to reduce it. New drugs are constantly being sought and searched to improve specificity, efficacy, and reduce toxicity with the goal to improve prognosis and complete recovery [3].

There is a great diversity of marine organisms from which a large number of pharmacologically active compounds have been isolated. Some of the compounds have anticancer activity. Ascididemin, lamellarin-D, and kahalalide F are the examples of bioactive compounds from a marine source that inhibit the human MCF7 breast cancer cells[4].

Gorgonian *Isis hippuris* is one of the marine organisms that has potent as a source of anticancer compounds. In Indonesia, *I. hippuris* can be found in Jepara, Makasar and Flores waters, or in the islands of Karimunjawa and Krakatau. However, their potency has not been fully explored. Previous in vitro study on hippuristanol and gorgosterol have shown that they can inhibit cancer cells [5] [6] however in vivo study is still rare. Therefore the following study was carried out to examine the effect of *Isis hippuris*extracts on *adenocarcinoma mammae in vivo* C3H mice.

2. Material and Methods

2.1. Extract of I. hippuris

I. hippuris was collected purposively from Karimunjawa water, Jepara. An amount of 2,5 kg was cut into small pieces and extracted with ethanol at room temperature for 24 hours with three repetitions. The extract solution was filtered on the paper filter and concentrated with a rotary evaporator under vacuum. The extracts were transferred into vials and dried further using a vacuum pump. The extract was kept in the refrigerator until further experiment [7].

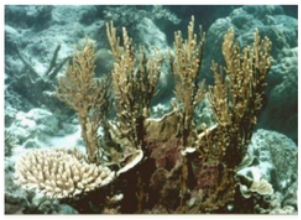

Figure 1. Isis Hippuris from Karimunjawa

2.2. Preparation of I. hippuris extracts containing Feed

The feed for C3H mice used commercial pellet feed (CP781) with 31 – 33 % protein, 3 - 5 % fat, 4 – 6 % crude fiber, 10 - 13 % ash, and 11 – 13 % moisture. The pellet was mixed with the extract with three concentrations, i.e., 0.00, 0.03, and 0.3 mg/g. The extracts were dissolved in ethanol 96% until homogenous, then, the pellet was mixed with the dissolved extract until absorbed completely. The control pellet was mixed with 200 mL ethanol only. Then, the pellet was dried under vacuum and heated at 40 ^{0}C to obtain a dry pellet.

2.3. Donor cancer cells

Cancer cells were obtained from donor mice. The cancer lumps were surgically removed from the donor mouse and transferred to sterile physiological salt containing petri dish. The tissue was disintegrated using a special scissor to release the cancer cells. These cells were ready to be transplanted into healthy C3H3 mice. Both cancer donor and experimental mice were obtained from Universitas Indonesia, Jakarta.

2.4. Isis hippuris (Ih) extracts administration to adenocarcinoma bearing C3H3 Mice

Mice were adapted for seven days to the lab environment, feed pellet and drinking water. The pellet and drinking water were provided everyday *ad libitum* [8]. The experiment was designed as "*The Post Test Only Control Group Design*" with one control and three treatment [9]. Fifteen mice with an initial weight of 20-25 gram were randomly allocated into control group (C), receiving 0.15 mg Ih extracts (Ih1), receiving 1.5 mg Ih extracts (Ih2), and 15 mg Ih extracts (Ih3) per mouse per day respectively for two weeks. Cancer cells were introduced to all groups from a cancerous donor mouse. The donor cancer cells of 0.2 mL were injected to each mouse via axilla and allowed to grow.

Figure 2. C3H mouse (left) and injection of donor cancer cells

2.5. Histological score of tumor mass

Mice were terminated at the end of the experiment to surgically remove the tumor lump, and they were processed for histological preparation and *Hematoxylin-Eosin* staining. *Hematoxylin* stains nucleus blue, while *eosin* stains cytoplasm pink [10]. Histological grade of tumor mass was determined by microscopic observation of cancer cells consisting of structural abnormalities of the tissue [9]. Microscopic observation was done using 400x magnification from 5 viewing fields, i.e., one in the middle and four in each corner. Histological grades of *Adenocarcinoma Mammae*of C3H mice are classified into the following scoring :

Features	*Score*
Tubule and gland formation:	
Majority of tumour (>75%)	*1*
Moderate degree (10-75%)	*2*
Little or none (<10%)	*3*
Nuclear pleomorphism:	
Small, regular uniform cells	*1*
Moderate increase in size and variability	*2*
Marked variation	*3*
Mitotic counts:	
Dependent on microscope field area	*1-3*

The Elston and Ellis (known as Nottingham Grading System) criteria were utilized for the breast cancer cells scoring that classified the cells into three classes/grade. The grade 1 (well differentiated): the cancer cells look most like normal cells and are usually slow-growing with score of 3-5. The grade 2 (moderately differentiated): the cancer cells look less like normal cells are growing faster with a score of 6-7. The grade 3 (poorly differentiated): the cancer cells look most changed and are usually fast-growing with a score of 8-9. The histological characteristic in Nottingham Grading Systems was determined base on the tubulus structure (% tumor tissue which has normal duct or tubular structure, nuclear pleomorphism (an evaluation of the size and shape of the nucleus in the tumor cells), mitotic counts (the number of divided cells, which indicated the growth rate of the tumor cells and divide). These characteristics were each scored 1 to 3 (1 for the best and 3 for the worst), then each score is

added to reach a score of 3 to 9. With this method, breast cancer is scored from 3 to 9 dan classified into one of the three histological grades [11].

2.6 Data Analyses

All data were analyzed qualitatively and quantitatively using tables, figures, or photos of histological examinations and compared to control. Data analyses were assisted using SPSS 16, and normality of the data was tested using Kolmogorov-Smirnov. When data were normally distributed, they were analyzed by ANOVA and tested further with Post Hoc. When data were not distributed normally, they were analyzed by nonparametric Kruskal-Wallis and Man Whitney tests. When $p<0.01$the difference was very significant, when $p<0.05$ there was a significant difference when $p>0.05$ there was no significant difference [12].

3. Results and Discussion

3.1. Cancer induction in C3H Mice

Cancer transplantation by injecting cancer cells donor via axilla was successful and shown by the presence of lump around axilla. There is a lymphatic system in axilla which transports the cancer cells into breast gland. The first lump was observed 19 days after transplantation. All transplanted mice were 100% successfully formed tumor lumps. After one-month extract administration, all mice were sacrificed and terminated to remove the tumor.

3.2. ObservationofTumor Histological Grade

Microscopic examination for histological grade according to Elston dan Ellis criteria are shown in Figure 3 and summarized in Table 1.

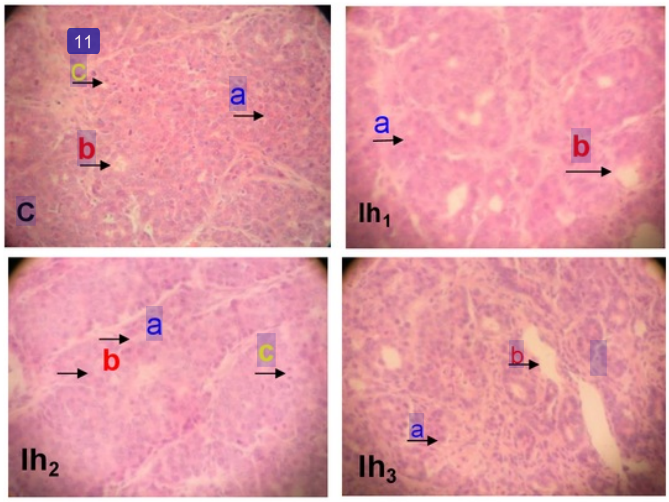

Figure 3. Observation of cancer tissue from C3H mice (400x magnification)
a = pleomorfic nuclei, size and shape of nuclei varies,b = tubulus/ ductus; c = mitosis, cell division.

Figure 3.Ih1 is in grade 1 with pleomorphic nucleus (2), tubular (2), dan mitosis (1) and the total score (5). Ih2 is in grade 2 with pleomorphic nucleus(3), tubular (2), dan mitosis (1), and the total score

(6). Figure3.Ih3 is in grade 1 with pleomorphic nucleus(2), tubular (2), dan mitosis (1), total score (5). C is in grade 3 with pleomorphic nucleus (3), tubular (3), dan mitosis (2), total score (8).Histological grade of each replicate sample (from five viewing fields) was averaged and displayed in Table 1. For statistical analyses, each score of a, b, and c (1 to 3) was used.

Table 1. The average of histological grade scores of*Adenocarsinoma mammae*of C3H mice from 5 viewing fields

Samples	C	Ih_1	Ih_2	Ih_3
1	7,2	5,4	5,4	5,4
2	7,8	4,6	4,4	5,6
3	7,4	6	5,4	5,4
4	7	4,6	5,2	5,4
5	7,2	5,2	4,6	4,6
Average	7,32	5,16	5	5,28
SD	0,30	0,59	0,47	0,39
Significa	b (grade 2)	a (grade 1)	a (grade 1)	a (grade 1)

On average Ih1, Ih2, and Ih3 showed that the cancer tissue cells had similarity to normal cells with good differentiation (grade 1). For control group without administration of *I. hippuris* extract the histological grade was 2 or 3 with a total score between 6 to 8. It indicates that *I. hippuris* extracts at a dosage of 0,15 mg/g, 1,5 mg/g, dan 15 mg/g per mouse can inhibit adenocarcinoma mammae cell mitosis and therefore reduced cancer growth.

Gorgonian *Isis hippuris* contain anticancer compounds especially belong to gorgosterol and hippuristanol groups. Gorgonian *Isis hippuris* from Okinawa water, Japan has been studied to contain gorgosterol and a new polyoxygenated steroid which showed antitumor and anticancer activity. Another compound from *I. hippuris* i.e. hippuristanol also showed potency as anticancer [13][14]. It also selectively inhibits *eukaryotic initiation factor* (eIF4A) which plays a role in the activity of RNA helicase [15]. It is possible that hippuristanol in the extracts may inhibit the activity of *eukaryotic initiation factor* 4A (eIF4A) which is found in RNA helicase. The eIF4A is a *prototypical* member of DEAD-box on RNA helicase. eIF4A functions to open the helical structure of mRNA using ATP [15][16]. Inhibition of translation on RNA prevent the formation protein, and therefore interphase in cell cycle can not proceed and consequently prevents mitosis.

The potency of *I. hippuris* extracts to inhibit and kill cancer cells has lent itself as the candidate of an anticancer drug from the ocean.

4. Conclusion

The ethanolic extract of *I. hippuris* inhibited the cancer cells growth and improved the histological score of mammary adenocarcinoma in C3H mice indicated. Therefore, the gorgonian has potency as an anti-cancer drug.

Acknowledgments

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