Ethanol Extract of *Haliclona* sp. Improved Histological Grade of Mammary Adenocarcinoma in C3H Mice

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Ethanol Extract of *Haliclona* sp. Improved Histological Grade of Mammary Adenocarcinoma in C3H Mice

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Abstract. The sponge *Haliclona* sp. contains secondary metabolites belong to alkaloids which are cytotoxic to the human tumor. The following research was conducted to study the effect of a graded level of *Haliclona* sp. extract on mammary adenocarcinoma in C3H mice. *Haliclona* sp. was obtained from Bandengan water in Jepara, and the crude extract was prepared by maceration with ethanol. Fifteen C3H mice with an initial weight of 20-25 gram were assigned into control, H-1, and H-2 groups. Control, H1, and H2 groups each received the ethanol extract of 0, 0.15, and 1.5 mg per mouse per day respectively for two weeks. Cancer cells were introduced to all groups from a cancerous donor mouse. The donor cancer cells were injected into each mouse via left or right axilla and allowed to grow. The cancer mass was removed and processed for histological examination, and the cancer growth was determined according to Elston and Ellis criteria. The result showed that histological grade of cancer mass from control group was in grade 2 or differentiated moderately. The histological grade of cancer mass from H-1 and H-2 groups were in grade 1 or similar to a normal cell. Analyses of the data by Kruskal-Wallis showed a significant difference ($p<0,05$) between control and treated groups. No significant difference was found between H-1and H-2 groups. The results suggest the potential of active substances in the ethanol extract of *Haliclona* sp as an anti-cancer drug.
Keywords**:** tumor, mammae, bioactive, sea, marine, soft coral

1. Introduction

Breast cancer is the second cancer-related death in women. Breast cancer metastasis is the main cause of death in breast cancer patients post the eradication of the primary tumor that cancer can still occur in other areas of the body [1]. The sponge *Haliclona* sp. is abundant in nature however it has not been utilized for the source of the anticancer drug. The sponge contains secondary metabolites belong to alkaloids which are cytotoxic to human breast cancer cell lines in vitro. We had investigated the effect of subchronic administration (3 months) of the *Haliclona* sp. extracts on liver histopathology of Swiss mice and showed no significant difference to control groups which indicated its safe use via the oral route. *Haliclona* sp. extract has LD_{50} value >2000mg/kg and it is categorized as low toxicity [2].

However, to obtain an effective dosage of Haliclona sp extract against cancer cells efficacy data in the animal test is needed.

2. Material and methods

2.1. Extract of Haliclona sp.

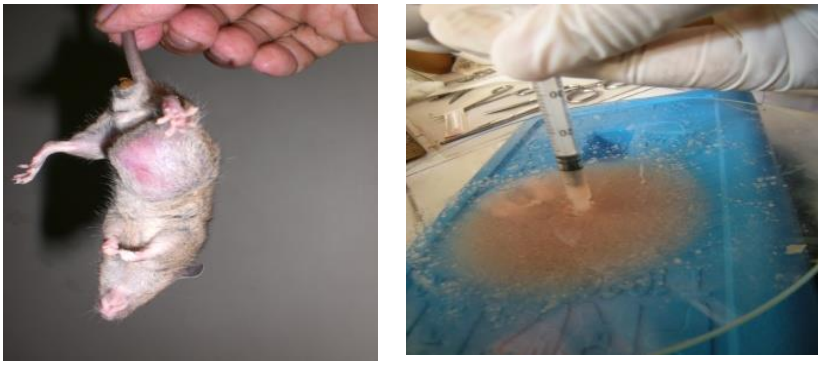
Sponge *Haliclona* sp. was sampled purposively form Bandengan water, Jepara Regency, Central Java Province, Indonesia. An amount of 1,350 kg was cut into smaller pieces, and soaked in ethanol and stood at room temperature for 24 hours. The solution was filtered using a paper filter, and the filtrate was dried by rotary evaporator under vacuum. The extraction process was repeated three times. The dry extracts were stored in the refrigerator until the further experiment [3].

2.2. Preparation of Hs extracts containing feed

Feed pellet for C3H mice used commercial feed (CP781) with 31 – 33 % protein, 3 - 5 % fat, 4 – 6 % crude fiber, 10 - 13 % ash, and 11 – 13 % moisture. The feed was mixed with the extract with three concentration 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 and stood until it was absorbed completely. The control pellet was mixed with 200 mL ethanol only. Then, the pellet was dried under vacum and heated at 40 ^{o}C to complete the drying process and stored in the freezer until use.

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 diluted in physiological salt solution, then ready to be transplanted into healthy C3H mice [4].



a. b.

Figure 1. Donor mice with cancer lumps (a), and the cancer cells in physiological salt (b).

2.4. Haliclona sp. extracts administration to adenocarcinoma bearing C3H mice

Mice were adapted for seven days to the lab environment. The feed pellet and drinking water were provided in *ad libitum* that provided daily. The experiment was designed as "*The Post Test Only Control Group Design"* with one control and three treatment [5]. Fifteen mice with an initial weight of 20-25 gram were randomly allocated into: control group (C), receiving 0.15 mg extracts (H-1), and receiving 1.5 mg extracts (H-2) per mouse per day for two weeks. The cancer cells were introduced to all groups from a cancerous donor mouse. The 0.2 mL donor cancer cells were injected into each mouse via left or right axilla and allowed to grow up to one month.

2.5. Histological score of tumor mass

The mice were terminated at the end of the experiment to surgically removed the tumor lump, and they were processed for histological preparation and *Hematoxylin-Eosin* staining. *Hematoxilyn* a nucleus blue, while *eosin* stains cytoplasm red [6].

Figure 2. Paraffin blocks of tumor tissue from experimental mice.

Histological examination of tumor mass was done by observation of cancer cells grade microscopically to determine structural abnormalities of the tissue. Observation under the microscope 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* in C3H mice are classified into the table 1.

Table 1. Classification of histological grades of *Adenocarcinoma Mammae* in C3H mice.

Features	Score
Tubule and gland formation:	
Majority of tumor (>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

Breast cancer cells are scored using Elston and Ellis (known as Nottingham Grading System) criteria [7]. The cancer was classified into three grades. The grade 1 (well differentiated) – the cancer cells look most like normal cells and are usually slow-growing with a 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. And, 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 base on Nottingham Grading System is determined from the tubular structure, the tubular structure, and mitotic count. The tubular structure observed the % tumor tissue which has a normal duct or tubular structure. The nuclear pleomorphism is base on an evaluation of the size and shape of the nucleus in the tumor cells. While, the mitotic counts is base on the presence of the number dividing cells, which represent the growth rate of the cancer cells.. 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.

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 $p<0,05$ the difference was significant when $p>0,05$ there was no significant difference [8].

3. Results and discussion

3.1. Cancer induction in C3H mice

The transplanted tumor in control mice which receive no sponge extracts appeared first (palpable) compared to mice receiving the sponge extracts. The tumor in control mice and the sponge extracts treated mice appeared eight days and 14 days respectively after transplantation. One month after tumor transplantation all mice had tumor lumps and they were terminated, and the tumor was surgically removed.

The sponge extract inhibited the tumor growth for six days in the experiment. The sponge is known contain the bioactive compounds belong to the renieramycins and mimosamycins that active againts the cancer cell [9,10].

3.2. Histological grade of tumor

During the administration of the extract, all mice were normal in their behavior, activity, and movement. The result in Figure 3 and Table 2 showed that histological grade of cancer mass from the control group was in grade 2 or differentiated moderately, from H-1 and H-2 groups were in grade 1 or similar to a normal cell. Analyses of the data by Kruskal-Wallis showed a significant difference ($p<0,05$) between control and treated groups. No significant difference was found between H-1and H-2 groups.

Table 2. Histological Grade of *Adenocarsinoma mammae* of C3H Mice.

	Treatment Groups		
Replicates	C	H1	H2
1	7,2	5,4	5,4
2	7,8	5	5,2
3	7,4	4,6	5,2
4	7	5,4	5
5	7,2	5,2	5,2
Average	7,32 [b]	5,12 [a]	5,2 [a]
SD	0,30	0,33	0,14
Significant $p<0,05$	(grade 2)	(grade 1)	(grade 1)

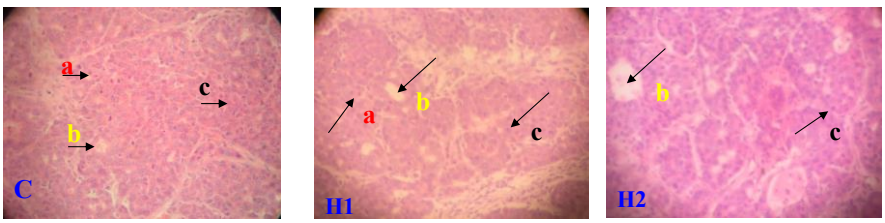


Figure 3. Observation of cancer tissue from C3H mice (400x magnification.
a = mitosis, cell division varies; b = tubulus, ductus;
c = pleomorfic nuclei, size and shape of nuclei

The results which showed that Hs extract could delay the appearance by six days suggested that the extracts can reduce the speed of tumor formation and hence has anticancer activity in vivo C3H mice. The sponge contains the bioactive compounds i.e. renieramycin, mitomycin and their derivates which have bioactivity as antileishmania, anticancer[11,12].

4. Conclusion

Ethanol extracts of *Haliclona* sp. improved the histological grade of mammary adenocarcinoma in C3H mice which suggest the potential of active substances in the ethanol extract of *Haliclona* sp. as an anti-cancer drug.

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