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Research Article

ANTIANGIOGENESIS AND ANTIBACTERIAL ACTIVITIES FROM AN INDONESIAN MARINE-DERIVED FUNGUS *Dactylaria* sp

Wilmar Maarisit[1,2]*, Dicki Huga Tangiono[2], Marstella Minelko[2], Tan Tjie Jan[2] Reinhard Pinontoan[2], Joke Luis Tombuku[3]

[1] Dept. of Pharmacy, Faculty of Science and Matematics, Indonesia Christian Univ. Tomohon, Jl. Talete II Kampus II Tomohon- North Sulawesi 95400
[2] Dept. of Biology, Faculty of Science and Mathematics, Univ. Harapan Tangerang Jl.MH. Thamrin Boulevard 1100 Lippo Karawaci, Tangerang-Banten 15811
[3] Dept. of Biology, Faculty of Science and Matematics, Indonesia Christian Univ. Tomohon 95400

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*Corresponding author
Wilmar Maarisit

Email :
wmaarisit@yahoo.com

ABSTRACT

Marine-derived fungi have been proven to be rich sources of chemically diverse natural products with a broad range of biological activities. The aim of this study was to determine the antibacterial and antiangiogenesis activities of marine derived fungi *Dactylaria sp*. Cultivation of the fungus *Dactylaria sp* (*strain* TID 24041021-1) was isolated from the marine invertebrate sponge. Culture of marine fungus was macerated with acetone and partitioned with ethyl acetate. The ethyl acetate extracts with 50, 100, and 200µg/mL concentrations, were assayed for their antiangiogenic activity by using chorioallantoic membrane *in vivo.* At the same time, ethyl acetate extracts at levels of 5, 10, 15, and 20mg/mL were assayed to pathogenic bacteria *Bacillus subtilis, Escherichia coli*, and *Staphylococcus aureus* using well diffusion method. The result of this study showed that ethyl acetate extract at concentration 50µg/mL could inhibit angiogenesis. The best antiangiogenic activity was showed at concentration of 200µg/mL ethyl acetate extract. Antibacterial activity from ethyl acetate extract inhibited the growth of *B. subtilis* (2.25-5mm), *E. coli* (0.63-3.50mm) and *S. aureus* (0-1.75mm) bacteria.

Key words: Marine sponge-derived fungi, antiangiogenesis activity, antibacterial activity

INTRODUCTION

Angiogenesis is a multistep process, leading to the formation of new blood vessels from the existing ones. It occurs during embryonic development, endometrial regulation, reproductive cycle, and wound healing. Angiogenesis also plays a critical role in many disease conditions like solid tumor progression, metastasis, diabetic retinopathy, arthritis, psoriasis, hemangiomas, and atherosclerosis (Folkman, 1995). New blood vessels are needed to supply oxygen and nutrition into tumor cells, so that tumor cells can keep growing, developing or even metastases (Hanahan, 1996). Based on this evidence, angiogenesis can be said act as main concept of tumors growth. Antiangiogenesis is inhibition of blood vessel growth, as a way of treating primary tumors and reducing their metastases (Folkman, 1971). Angiogenesis inhibitors are described as class 1 (specific and semi-specific) and class 2 (non-specific), depending on whether they inhibit proliferation and/or migration of endothelial cells only, or are also toxic for tumor cells (Voest, 1996).

Bacterial infection causes high rate of mortality in human population and aquaculture organisms. For an example, *B.subtilis* is responsible for causing food borne gastroenteritis. *E.coli, S. aureus* and *Ps.aeruginosa* cause diseases like mastitis, abortion and upper respiratory complications, while *Salmonella* sp. causes diarrhea and typhoid fever (Leven, 1987; Jawetz *et al*., 1985). Usage of antibiotics increased significantly due to heavy infections and the pathogenic bacteria becoming resistant to drugs is common due to indis-criminate use of antibiotics it becomes a greater problem of giving treatment against resistant pathogenic bacteria (Sieradzki *et al*., 1999). Decreased efficiency and resistance of pathogen to antibiotics has necessitated the development of new alternatives (Smith *et al*., 1994). Sponge-derived fungi are a rich source of new natural

products with a wide range of biological activities for example enzyme inhibitor, anticancer, antitumor, antiangiogenesis and antibacterial. The aim of this study is to evaluate antiangiogenesis and antibacterial activities from an Indonesian marine-derived fungus.

METHODOLOGY

Sampling collection

Marine invertebrate sponge was collected by snorkeling at -1~-5m depth from Tidung Island Kepulauan Seribu on April 2010. A specimen was deposited at the Laboratory of Microbiology, Faculty of Science and Mathematics Pelita Harapan University, Tangerang-Indonesia, under 5-10°C. Then, the specimen of marine-derived fungus was identified and isolated.

Isolation and culture conditions

Sponges were rinsed with sterile sea water, cut in small pieces, and put in PDB medium. 1mL of PDB was transferred to the 35ppm salinity PDA medium and incubated in 25°C for 7 days. Fungal isolates were obtained in pure cultures by single conidial transfer to 150 PDA plates at 25°C for 21 days.

Identification of fungi

The isolated fungi were identified to the genus level and to the species when possible on the basis of macro morphological and micro morphological characteristics using PDA medium, slide cultures and the most updated keys for identifications.

Macroscopic study

Colonies of marine fungi were cultivated on PDA medium at 25°C for 5 days. The following morphological characteristics were evaluated: colony growth (length and width), presence or absence of aerial mycelium, colony color, presence of wrinkles and furrows, pigment production etc.

Microscopic study

Fungal spores were cultivated on PDA in Glass microscopes slides at 25°C for 6 days. The germination and growth of mycelium was observed daily under a light microscope.

Extraction

The cultured plates (150 plates) were extracted with acetone three times and filtered. The extract was obtained after removing the solvent under reduced pressure on a rotary evaporator. The resulting residue was partitioned between ethyl acetate and water to give 300mg of ethyl acetate extract.

Antiangiogenesis assay

Antiangiogenesis activity was using *chick chorioallantoic membrane* (CAM) method. Fertilized chicken eggs were cleaned with a 70% alcohol solution and incubated at 37-38°C with approximately 60-75% humidity. After eight days chick embryos was exposed by making a window 20mm in the egg shell. The sterile paper discs were impregnated with extract solution (50, 100, dan 200µg/mL.) to achieve desired concentration and placed in CAM.

Blank paper disc of 6mm diameter were used as normal control, paper disc of *colostrum* (10µL) as positive controls and paper disc of *colostrum* (50µg/mL) + ethyl acetate (10µL) using for solvent control. The window was sealed and the eggs were reincubated. On day 13, the window is opened for observation. Angiogenic reaction around paper disc were calculated and scored 0, +1, +2 and +3. Score 0 is no reaction observed, CAM normal. Score +1 is weak antiangiogenic response, score +2 is middle antiangiogenic response, and score +3 is strong antiangiogenic response (Salamah *et al* 2009).

Antibacterial assay

In vitro anti-bacteria studies were carried out by the agar well diffusion method. The used indicator of inhibition activity was Gram-negative bacteria (*E. coli* ATCC 25922) and Gram-positive bacteria (*B. subtilis* ATCC 6633 and *S. aureus* ATCC 25923). All of the test microorganisms were obtained from the Laboratorium Pengembangan Teknologi Industri Agro dan Biomedika BPPT-Indonesia.

The Pathogen bacteria was transferred to 10ml Nutrient Broth (NB) and incubated for 24h at 35°C. The prepared culture was appropriately diluted to achieve an inoculums size of approximately 10^6cfu/mL. Then, 0.5mL

inoculums were added to 20mL melted Nutrient Agar (NA) cooled at 45°C. These were then poured into 90mm diameter Petri dishes and maintained for 3h at room temperature. A 6mm diameter wells were cut in the agar plate. The ethyl acetate extract were applied to the wells with the concentration 5, 10, 15, and 20mg/mL. Pure extraction solvent (Ethyl acetate) 50µL were used as solvent control and Amoxicillin 50µg/mL were used as positive control. The plates were incubated at 37°C for 24 hours and antimicrobial activities were determined by measuring the diameter of the inhibitory zones in millimeter.

Statistical analysis

The mean values were statistically analyzed using program SPSS (Statistical Package for the Social Sciences) *19.0 for windows* program by the general one-way analysis of variance (ANOVA) to find out the most effective marine fungi extract and the most sensitive test microorganisms.

RESULTS AND DISCUSSION

Identification of marine fungi

Fungi (TID.24041021-1) were identified in microscopic using light microscope. Hyphae are septate with conidiophores that are hyaline, erect, and sometimes knobby or bent at the point of conidial formation. Conidia (average 3.5 x 10µm) from on threadlike denticles, they are brownish, two celled, oval to tear shaped, and typically have a marked constriction at the central ceptum. A frill of the denticle often remains on the base of the conidium after detachment from the conidiophores. Young conidia may be round and single celled (Figure 1). Surface of colony marine fungi is woolly and dark olive-gray, reddish brown or gray-black. Reverse is dark; a red to brown pigment usually diffuses into the medium (Figure 2). From microscopic and macroscopic study, marine fungi (TID.24041021-1) similar with the fungi *Dactylaria sp.* (Larone, 2002).

Antiangiogenesis activity

On blank control (paper disc), there were a few new blood vessels growing to paper disc. It was caused by no colostrum containing angiogenesis given to normal control. Giving Calostrum® to CAM can stimulate new blood vessels forming. Calostrum® contains growth factor which can stimulate angiogenesis.

Activity test result of antiangiogenesis ethyl acetate extract showed that the higher ethyl acetate extract concentration, the higher angionesis inhibition occurring on colostrum CAM induction as indicating by new blood vessels amount reduction (Figure 3 and Table I).

Ethyl acetate extract with concentration 50µg/mL showed +2 on the inhibition of new blood vessels forming. It can be observed from the decreasing of the amount of blood vessels to paper disc compared with colostrum control +3 score. Ethyl acetate extract on 100µg/mL concentrations showed the same antiangiogenic activities as 50µg/mL concentrations, the given score is +2. Ethyl acetate extract of 200µg/mL showed the best antiangiogenesis activities with +3 score. It can be seen from the amount of blood vessels to paper disc is less than the amount of blood vessels of other treatments (Figure 3).

On solvent control (colostrums + ethyl acetate 10µL), the amount of blood vessels on CAM is less than of on angionenic control (colostrum 60µg/mL), but on angiogenesis activity, the amount of blood vessel is equal to positive control.

The main Growth factor in angiogenesis is basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). On tumor cell, secreted bFGF and VEGF will form new blood vessels to meet tumor's cells nutrition. It enables to occur metastasis on tumor cells. Crude extract ethyl acetate is antiangiogenic, unfortunately its inhibition mechanisms have not been known yet.

The inhibition mechanisms of blood vessels forming by antiangiogenic compounds can be done in two categories. Firstly, agents that blocked the activity of pro-angiogenic molecules, secondly, agents that directly affected endothelial cell function or survival (Ribati, 2010). Over 300 antiangiogenic molecules targeting different signaling pathways are being tested for their anticancer properties at preclinical and clinical stages. Although the results of the clinical trials are encouraging, the effects are modest. Clinical practice reveals that

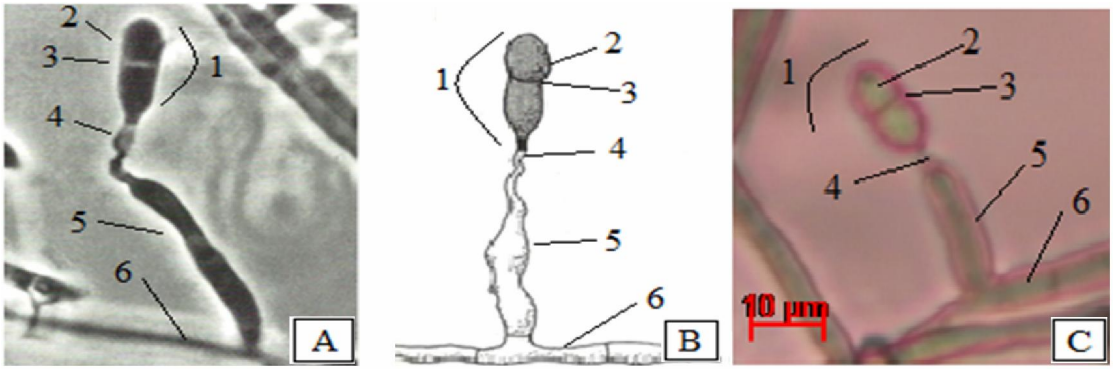

Figure 1. (1) Conidium; (2) Cell of fungi; (3) Septum; (4) Dentrikel; (5) Conidiophore; (6) Hifa. (A) Morphology of fungi *Dactylaria constricta* (Larone, 2002); (B) Illustration of fungi *Dactylaria constricta* (Larone, 2005); (C) Marine fungi (TID.24041021-1).

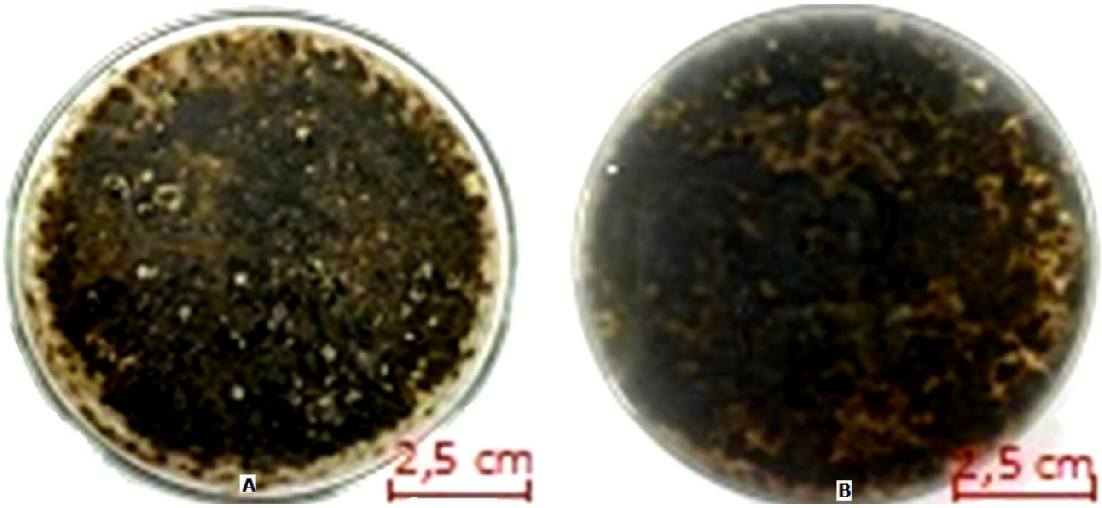

Figure 2. (a) Surface of colony marine fungi (TID.24041021-1) (b) Reverse of colony marine fungi (TID.24041021-1)

therapy with angiogenesis inhibitors does not prolong survival of cancer patients for more than months, because tumors elicit resistance (Ribatti, 2010).

Antibacterial activity

Ethyl acetate extract was tested its ability to inhibit pathogen *B. subtilis, E. coli*, dan *S. aureus* bacteria using well diffusion method. The used concentrations were 5, 10, 15, and 20mg/mL. The result of ethyl acetate extract inhibition toward pathogen bacteria can be observed on figure 4 based on figure 4 ethyl acetate extract has the first highest inhibition activity on *B. Subtilis* bacteria with inhibition diameter around 5mm on 20mg/mL concentration. The second highest inhibition activity was found on *E. coli* bacteria with inhibition diameter 3,50mm on 20mg/mL concentration. The lowest inhibition activity was on *S. aureus* bacteria with inhibition diameter around 1,75mm on 20mg/mL concentration. The tested solvent control did not show any inhibition activities on bacteria.

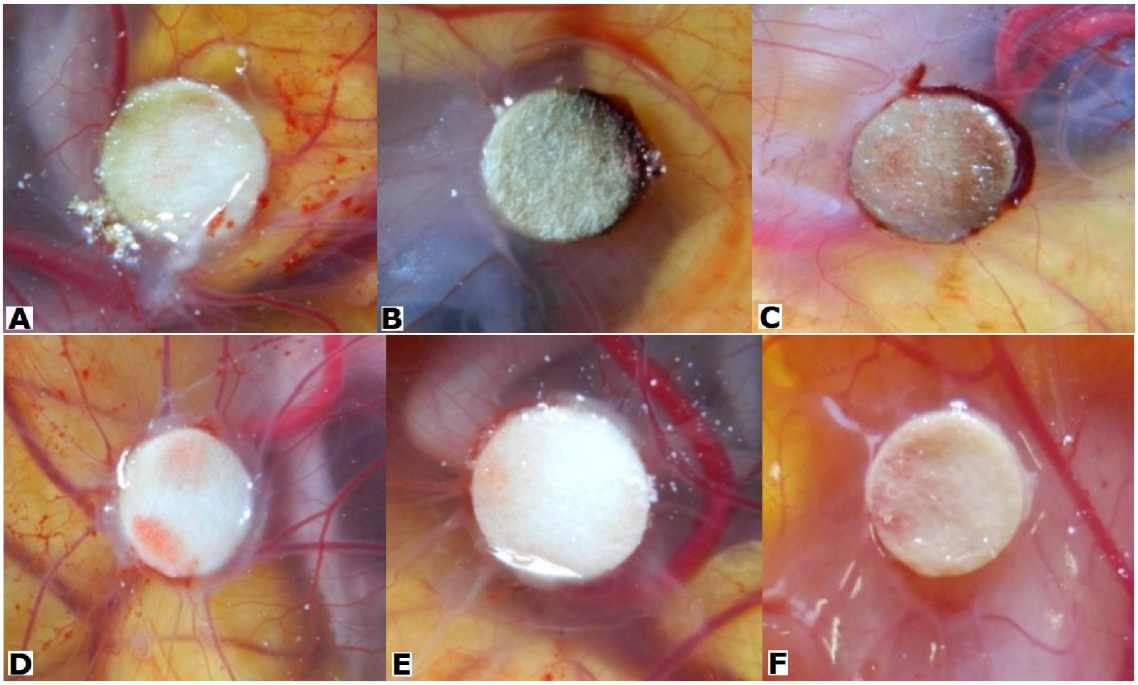

Figure 3. The results of antiangiogenesis test observation on CAM from ethyl acetate extract fungi TID.21-1
(a). Colostrum + ethyl acetate extract 50µg/mL (b). Colostrum + ethyl acetate extract 100µg/mL; (c). Colostrum + ethyl acetate extract 200µg/mL. (d). Positive control (colostrum 50µg/mL); (e). Solvent control (colostrum 50µg /mL + ethyl acetate 10µL), (f). Normal control (paper disc).

Table I. The amount of blood vessel on CAM.

Treatment	**Amount of Blood Vessels**	**Score**
Colostrum + ethyl acetate extract 50µg /mL	24.33	+2
Colostrum + ethyl acetate extract 100µg /mL	21.33	+2
Colostrum + ethyl acetate extract 200µg /mL	18.33	+3
Positive control (Colostrum 50µg /mL)	40	+1
solvent control (Colostrum 50µg /mL + Ethyl acetate 10µL)	37	+1
Normal control (Paper disc)	7	0

Angiogenic control 50µg/mL showed a high inhibition activity on *B. Subtilis* 14mm, *E. coli* 11,24mm, and *S. aureus* 13,00mm. Based on figure 4 can be seen there was any correlation between the increment extract concentrations which caused the inhibition bacteria increment growing. Crude extract of ethyl acetate fungi (TID.24041021-1) can inhibit three indicator pathogen bacteria; *B. subtilis, S. aureus* (Gram-positive bacteria), *E. coli* (Gram-negative bacteria). Generally, ethyl acetate extract has the possibility to have broad *spectrum* compounds in which effectively inhibit Gram-positive bacteria. Gram-negative bacteria were less sensitive than Gram-positive bacteria, which may be due to their differences in the cell wall composition (Ahmad and Beg, 2001).

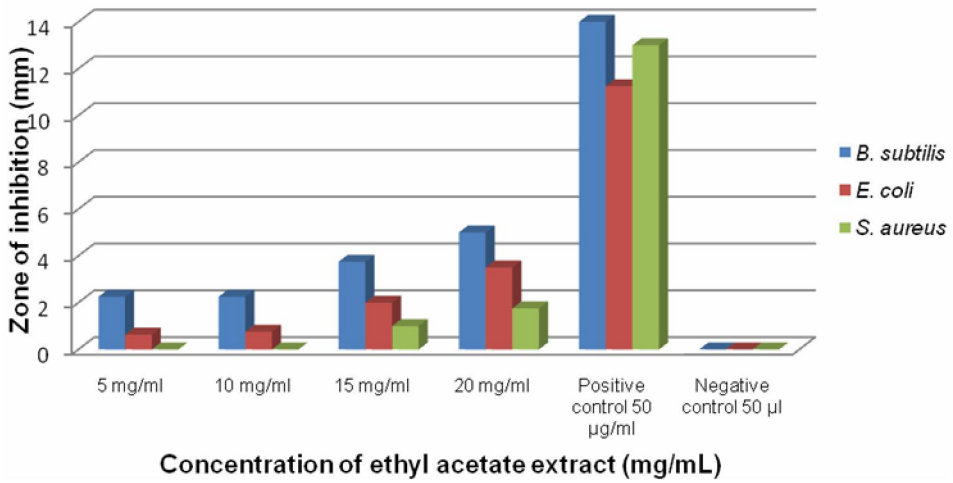

Figure 4. Antibacterial activity of ethyl acetate extract from marine-derived fungi (TID.24041021-1)

It was interesting to note that tested bacteria showed more sensitivity to the investigated fungi extracts. This clearly indicates that active compounds of these extracts interfere with composition of Gram-negative bacteria cell wall. Ebell (2010), reported that the *Isocyclocitrinol A* from marine sponge derived fungi *Penicillium citrinum* showed antibacterial activity. Capon *et al* (2007), reported that *diketopiperazines* isolated from a marine fungus *Penicillium* sp showed antibacterial (*Escherichia coli* and *Bacillus subtilis*), antifungal (*Septoria nodurun*) and antiparasitic (*Haemonchus contortus*) activities.

CONCLUSIONS

Ethyl acetate extract of marine fungi (strain TID.24041021-1) used in the present investigation showed better antiangiogenesis and antibacterial activities. They are potential sources of bioactive compounds and should be investigated for natural antiangiogenesis and antibiotics. But further study should be made to identify and purify these antiangiogenesis and antibacterial substances.

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