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Fucoxanthin Effects of Pure *Sargassum filipendula* Extract Toward HeLa Cell Damage

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Abstract: In this study, the cytoprotective effects of fucoxanthin were isolated from *Sargassum filipendula* towards HeLa cell damage which characterized DNA damage. The purpose of this study was to obtain fucoxanthin isolated from *S. filipendula* and to determine the damage effect of fucoxanthin isolated against HeLa cells by Tunnel method. Fucoxanthin was identified with qualitative methods refer to fucoxanthin R*f* 0.25 to 0.28. Fucoxanthin 97% purity was tested using the method of ESI LCMS. We obtained molecular weight of 658.77 m / z. Antioxidant activity in fucoxanthin of *S. filipendula* was assessed by DPPH for 1.0974 ± 0.0306 ppm with IC_{50} = 1.4174 ± 0.0126 ppm. While the test of cell apoptosis assay using the TUNEL method with the best dose anti cancer fucoxanthin of 50 ppm and 100 ppm indicates approaching 100% of apoptotic cells.
Keywords: antioxidant, apoptotic cells, Fucoxanthin, *S. filipendula*, TUNEL method.

Introduction

Seaweed that grows in tropical climates such as Indonesia receives more harmful ultraviolet radiation that can damage the cells, therefore, the active component of seaweed synthesizing a number of antioxidants [1]. A protective compound against ultra-violet rays has been found in species of brown algae known as flavonoids [2]. Flavonoids are secondary metabolites of seaweed from the low molecular weight fraction [3,4]. The ability of flavonoids as antioxidants can be estimated using the chemical properties of hydrogen flavonoid phenols which are hydrogen donor to a free radical scavenger [5]. The role of flavonoids as antioxidants depends on the molecular structure and the position of hydroxyl groups on the chemical structure to other activities of antioxidant and free radical scavenging activity [6].

Currently, public concern against oxidative damage from reactive oxygen species (ROS) and free radicals are very large, because it damage the biomolecules such as proteins, nucleid acid, lipids and other cell components [7,8]. ROS cause aging and stimulate lipid peroxidation as the main causes of food damage [9], and increasing the membrane lipid peroxidation thus damage the membrane by affecting cell death [10]. Damage to biomolecules due to ROS can cause a variety of chronic diseases such as cancer, coronary heart disease, cataract, aging muscular dystrophy and other neurological damage [11]. Therefore a lot of products are widely developed as antioxidant to reduce the damage caused by ROS.

The purpose of this study was to obtain fucoxanthin isolated from brown algae *Sargassum filipendula* and to determine the effect of different doses of isolated fucoxanthin towards HeLa cell damage.

Material And Methods

Fucoxanthin Extraction of *Sargassum filipendula*

Preparation to obtain fucoxanthin extracts of *Sargassum filipendula* explained as follows. The sample of *S. filipendula* was washed, cleaned, and cut into ± 1 cm pieces and weighed for 40 grams, then mashed with a mortar and given sufficient $CaCO_3$ which is used as a neutralizing agent. *Sargassum filipendula* extracted with DMSO (Dimethil sulfoxide) (1:10, w / v) for 20 min then filtered and labeled as X_1 (DMSO extract volume).

Fraction X_1 separated in a separating funnel with ethyl acetate (1/2 volume of extract X_1) and 0.5 M ammonium sulfate (NH_4 $(SO_4)_2$) (a volume of extract X_1). Phase partitioned on the results returned by the same solvent as above. Lower phase was color partitioned twice with 10 ml of diethyl ether and added with saturation salt and tap water to clarify the separation.

From the results of partition, the upper phase was collected and evaporated with a vacuum rotary evaporator at a temperature of 30°C and 100 rpm. Results of a rotary vacuum evaporator anhydrous Na_2SO_4 added to absorb water, and then dried with argon gas and dry pigment extracts obtained (X_1).

DMSO extract residue (X_1) re-extracted with acetone (1:10, w / v) for 10 minutes at a speed of 100 rpm. Results filtered with extraction filter paper. Above extract residue was re-extracted with acetone and added with distilled water (until the residue submerged) (1:10, w / v) for 10 minutes at 100 rpm. Extraction results filtered with filter paper and labeled X_2. Volume X_2 extract partitioned with hexane (1/3 volume X_2) and distilled water (1/4 volume X_2). Partitioned phase is over 2x with 75% methanol (1/9 volume X_2). The top phase partitioned again with 80% methanol (1/9 volume X_2). Phase of the results above are labeled as X_{2A} partition (hexane phase) and the lower phase fraction labeled X_{2B} (phase acetone-methanol-distilled water). Fraction X_{2A} dried with argon gas and pigment extracts obtained dry X_{2A}.

X_{2B} fraction (acetone-methanol-distilled water) was partitioned with diethyl ether (2/5 X_{2B} volume) and added saturation salt and water tap to clear the separation. The top phase of X^{2B} added anhydrous Na2SO4, then dried with argon gas and pigment extracts obtained dry X_{2B}. Bottle containing dry pigment extracts covered with aluminum foil and stored in a freezer.

The Making of 1,1-diphenyl -2-picrilhydrazyl (DPPH) Solution

Materials needed to make a solution of DPPH are DPPH powder and ethanol solvent. DPPH in 0.1 mM ethanol solution made by weighing 0.0039 grams total DPPH powder then dissolved in 100 mL of ethanol [12].

Free Radical Testing Procedure using DPPH [13]

Total of 2 mL sample were taken, added 2 mL DPPH, homogenized, and incubated for 30 min at room temperature. After 30 minutes, the absorbance was measured at a wavelength of 517 (Ai). We used solvent ethanol-water by 1: 1 (v / v). We also measured the absorbance of 2mL DPPH and 2mL mixture of water (Ac). Then the absorbance of the mixture was also measured compared to 2mL DPPH and 2mL ethanol-water (Aj). Repetition performed three times.

The first step of dissolving the antioxidant activity of the test sample (crude extract) with absorbance ≈ 1 in the solvent acetone was then made into concentration variations [12]. Sample solution consisted of 1 mL sample plus 4 mL of DPPH, while the blank formed of 4 mL of 95% methanol plus 1 mL of sample. Blank and samples incubated in the dark for 30 minutes. Then the absorbance was measured at a wavelength of 517 nm using a double beam spectrophotometer. The inhibitory activity was calculated using the formula:

$$\%\ inhibition = \frac{[\mathbf{DPPH}]_0 - [\mathbf{DPPH}]_s}{[\mathbf{DPPH}]_0} \times 100\%$$

Description:
[DPPH0] : initial DPPH concentration
[DPPHs] : remaining concentration of DPPH

Determination of the IC_{50} value [12]

Value of IC_{50} was determined by measuring the antioxidant activity (% inhibition) at different concentrations on the two series of the same sample. The first concentration series should have antioxidant activity below 50%, while the concentration of the second series must be above 50%. Furthermore, from the two values obtained in the standard curve which made up of linear equations obtained IC_{50}. IC_{50} values were then calculated by entering the number 50 to substitute the variable Y in the linear equation.

Cell HeLa Culture

This study used HeLa cells from the collection of Medical Faculty, University of Brawijaya, Malang. HeLa cells were grown in RPMI 1640 medium (Sigma) containing the complete fetal bovine serum (FBS) 10% (Gibco), 0.5% fungizon (Gibco), penicillin-streptomycin and 2% (Gibco). T47D cells were grown in DMEM medium (Gibco) containing complete 10% FBS (Gibco) and 2% penicillin-Streptomycin. Both cells were cultured using 25 mL flask (Nunc) in a CO_2 incubator at a temperature of 37°C and 5% CO_2 stream.

Apotosis HeLa cells by TUNEL method

Cell death through apoptosis is characterized by DNA fragmentation conditions at 200-250 bp and/or 30-50 kbp. Then internucleosomal DNA fragmentation on 180-200 bp will also occur. The character has been used to differentiate apoptotic cells from normal and necrotic cells. Apoptosis was detected using Apo_BrdU-IHCTM IN Situ DNA Assay Kit from BioVision Fragmentatior. Internucleosom DNA fragmentation is important parameter in apoptosis of mammalian cells. BioVision's Apo_BrdU-IHCTM Kit, i.e. TUNEL (terminal transferase dUTP Nick Deoxynucleotide End Labeling) assay two-color labeling to detect apoptotic DNA damage by immunohistochemistry. The parting of the single-stranded DNA strands can be detected enzymatically (enzymatic labeling) of 3-OH terminal groups on the free DNA fragments with terminal Deoxynucleotide transferase (TdT). Therefore, this painting is known by TdT-mediated d-UTP nick-end labeling (TUNEL).

TUNEL detects DNA fragmentation and corrupt the DNA strand (nicks) which indicates the occurrence of apoptosis molecular level. The morphology of the cells was positive nucleus was observed with 400x magnification. Condensed nucleus in the middle phase and apoptosis showed as brown color.

Results and Discussion

Pigment Composition

Pigment composition was analyzed with a crude extract of brown seaweed with TLC. Yellow pigment (β-carotene) (spot 1) had R*f* 0.94. This is consistent with previous research using the same mobile phase, namely 5% acetone: methanol 4%: 1% IPA in toluene, which has a yellow pigment R*f* range from 0.91 to 0.94 **[14,15]**. Added by other research, the mobile phase of nonpolar R*f* values obtained β-carotene between 0.8 to 1.0 **[16]**. The TLC system using the "normal phase", in which the stationary phase is more polar than the phase of the motion, thus the pigment will propagate nonpolar first **[17]**.

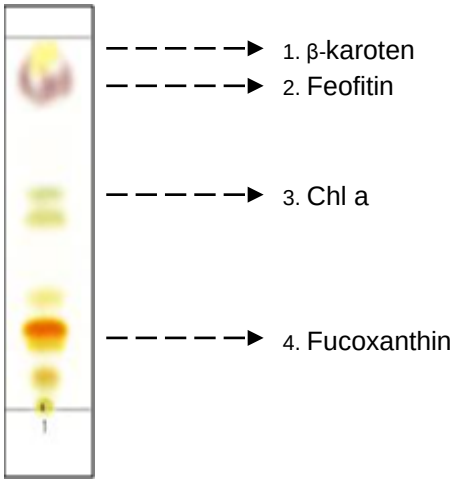

Figure 1. Separation of Pigment Pattern on TLC plates of *S. filipendula*

The data in Fig.1 shows that the first peak is the molecular weight fucoxanthin 658,77m / z, while the second peak is the molecular weight of 640.80 m / z, where the peak is assumed as fucoxanthin dehydro removal of water. The third peak 681, 23 m / z, when the analyzer is no addition of Na ions (H- ions attached to). The fourth peak is the Na atom there is an abundance of isotopes with a molecular weight 682,87m / z [18].

Fucoxanthin Detection

Prediction of the chemical shift values of compound 1 as reported by previous research [19] shows that pure fucoxanthin can be detected by HPLC and LCMS and obtained a molecular weight of 658.77 m / z as shown in Fig.2.

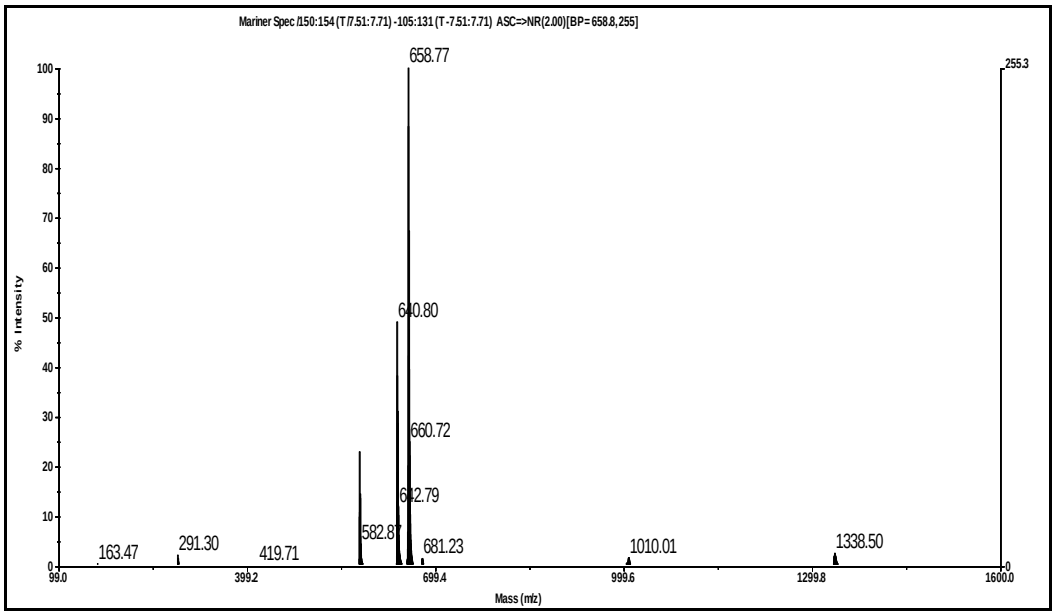

Figure 2. Fucoxanthin Analysis by LCMS

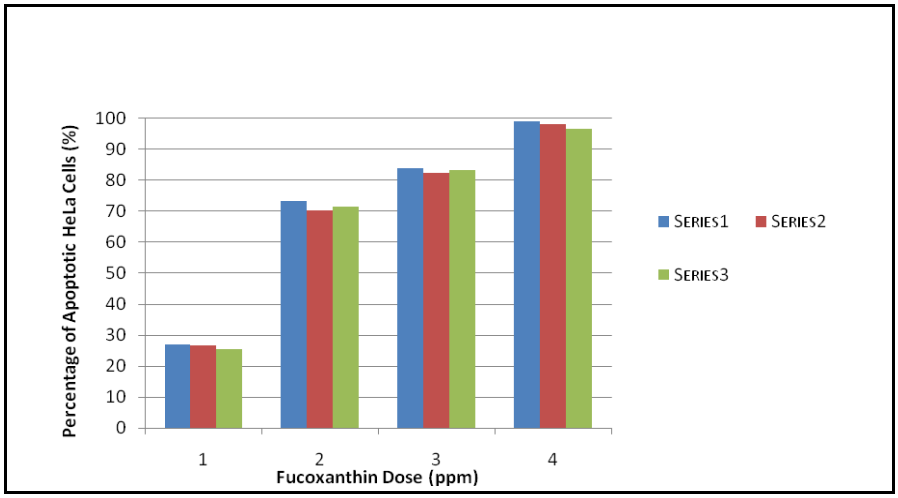

Figure 3. Fucoxanthin treatment 1 (12.5); 2 (25); 3 (50); and 4 (100) ppm with TUNEL Kits in three replication

Antioxidant Activity

IC_{50} value in the samples of *S. filipendula* is 1.0974 ± 0.0306 ppm. Previous study [20] mentioned that the smaller the IC_{50} value, the greater the antioxidant activity. Fucoxanthin which is the dominant pigment in brown algae has IC_{50} value of 1.4174 ± 0.0126 ppm. This value is relatively larger than β-carotene IC_{50} of only 1.0915 ± 0.0389 ppm. Thus, fucoxanthin has higher antioxidant activity than β-carotene.

Fig.3 shows the addition of higher concentrations of fucoxanthin towards the HeLa cells, i.e. the provision of 50 ppm and 100 ppm lead to almost 100% apoptosis. This is due to the blocking of DNA by TUNEL staining. The tendency of an increase in the percentage of apoptotic HeLa cells at doses of 3 (50 ppm)

showed that the fucoxanthin extract of *S. filipendula* led to an increase in apoptosis of HeLa cells. In this study, based on the mechanism of action of fucoxanthin, apoptosis was observed possibly through increased activity of Reactive Oxygen Species (ROS), which would cause the loss of mitochondrial membrane, the solution of the Bid, and cytochrome c release which lead to dysfunction mitochondrial pathway (intrinsic pathway), which then activates caspase and resulting in apoptosis (in research characterized by DNA fragmentation via TUNEL method).

The increased percentage of apoptotic HeLa cells dose of 50 ppm also assumed caused by the content of antioxidants (fucoxanthin) in the leaf extract of *S. filipendula* which inhibits free radical cells. As explained in early research [21], algae carotenoid extracts from *S. filipendula* prove fucoxanthin as the highest carotenoid in algae.

Fucoxanthin is a large number of antioxidant from brown algae obtained from the leaves of *S. filipendula* [22]. Fucoxanthin contains antioxidant compounds which defined as a substance in the lower levels that capable of inhibiting the oxidation rate [23]. The molecular target of these compounds can stop chain reaction of free radical formation [24] which can damage body tissue and cause degenerative diseases such as cancer [25].

Therefore, it can be said that at doses 2 (25 ppm), the fucoxanthin activity from the leaf extract of *S. filipendula* as pro-oxidant was more dominant, resulting in increased apoptosis of HeLa cells. However, at dose 3 (50 ppm) antioxidants activity in fucoxanthin began to increase, thus offsetting pro-oxidant activity in the fucoxanthin extracts from *S. filipendula*, resulting in an increase in the percentage of apoptotic HeLa cells.

Natural ingredients (nutrients) commonly contain pro-oxidants and antioxidants [26]. Initiation of programmed cell death in tumor cells by pro-oxidant activity can lead to loss of integrity cellular malignancy. Natural materials can act as reactive oxygen molecules, which produce pro-oxidant molecules. This molecule induces oxidative stress in premalignant cells, transformed cells, and impairs cellular signaling system which will modified the transcription factor, resulting in cell cycle and inhibited DNA fragmmentation, activation of the tumor suppressor, inhibiting the formation of new blood vessels, which in turn resulted in the shrink of malignant tumor cells. In contrast, antioxidant (reducing agent) which inhibits the production of free radicals or reactive oxygen in tumor cells can lead to the proliferation of transformed cells, the increase DNA repair, and make the cells more resistant to therapy [26].

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