

Oroginal paper

## THE ANTIOXIDANT CAROTENOID CONSTITUENT FROM MARINE MACRO ALGAE

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### ABSTRACT

Most of the potentially harmful effects for the human body is free radicals. A free radical is any chemical species that has one or more unpaired electrons. Many free radicals are unstable and highly reactive. Harmful effects of free radicals for the human body are their chemical reactivity can damage all types of cellular macromolecules and the chain effects.

Nutrition plays a key role in maintaining the body's enzymatic defenses against free radicals. It is believed to play an important role in cellular defenses against oxidative damage. Our work on new bioactive compounds from marine macro algae has led to the isolation, characterization, and antioxidant assays of semi polar extract from 5 different macro algae (2 green algae and 3 brown algae) and structure elucidation. By using a *in vitro* antioxidant assays DPPH radical scavenging (1, 1-diphenyl-2-picrylhydrazyl), the etil acetate extract of macro algae was separated by sephadex C-8 with eluent EtOAc/MeOH the fraction analyzed through LCMS 2010A. These studies of marine macro algae offered important new insight that the chlorophyll fractions from brown algae have better inhibition activity against free radicals than green algae did.

**Key Words:** Free radicals, antioxidant, DPPH, karotenoid, KCKT-LCMS

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## INTRODUCTION

### Oxidant and Free Radicals

Free radicals and other reactive oxygen species which generated in living organism are considered as the main cause for many pathological conditions for their oxidative properties which actively act as oxygen donor to other compounds in living organism (Lillian, 1995). Some reactive oxygen species are free radicals. Most of

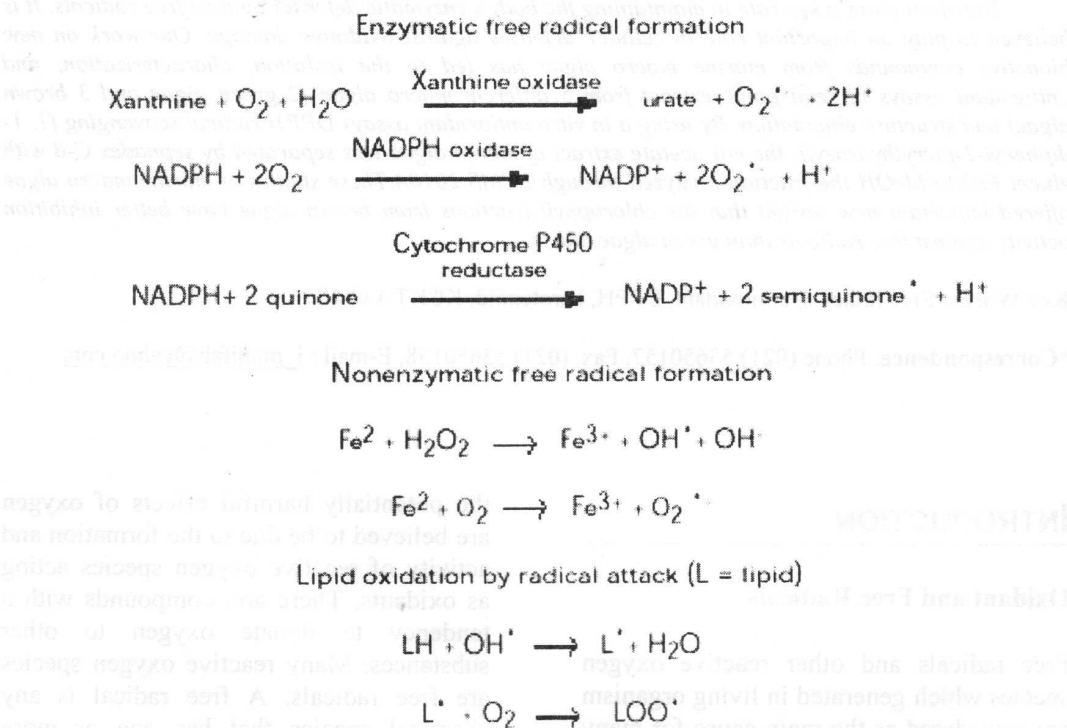
the potentially harmful effects of oxygen are believed to be due to the formation and activity of reactive oxygen species acting as oxidants, There are, compounds with a tendency to donate oxygen to other substances. Many reactive oxygen species are free radicals. A free radical is any chemical species that has one or more unpaired electrons. Many free radicals are unstable and highly reactive. Some of the free radicals and other important oxidants found in living organisms are shown in **Table 1**.

**Table 1.** Some important reactive oxygen species in living organisms

Free radicals	Non radicals
Hydroxyl radical OH .	Hydrogen peroxide H <sub>2</sub> O <sub>2</sub>
Superoxide radical O <sub>2</sub> .-Nitric oxide radical NO .	Singlet oxygen 1 O <sub>2</sub>
Lipid peroxy radical LOO .	Hypochlorous acid HOCl
	Ozone (O <sub>3</sub> )

Reactive oxygen species are produced continuously in the human body as a consequence of normal metabolic processes. Some reactions that lead to free radical formation are shown in Fig.1. If free radicals are not inactivated, their chemical reactivity can damage all types of

cellular macromolecules, including proteins, carbohydrates, lipids, and nucleic acids. Fig.1 shows some of the reactions involved in free radical attacks on lipids. Fig. 2 illustrates some of the types of damage that can result from the actions of free radicals.



**Fig. 1.** Examples of free radical reactions

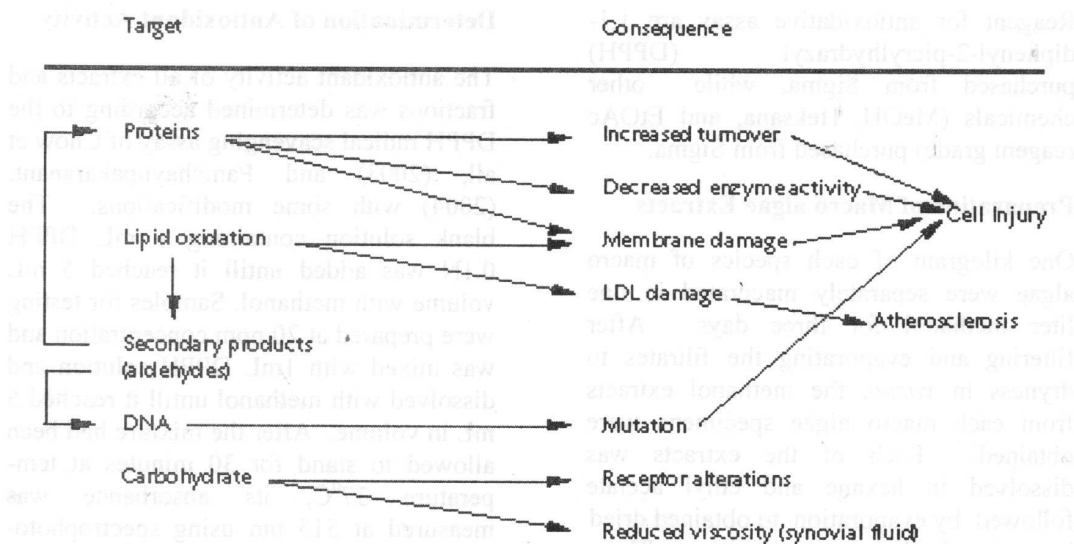


Fig. 2. Free radical damage

Several of these effects have been implicated in the causation of degenerative diseases and will be described in more detail later in this monograph. For example, destructive effects on proteins may play a role in the causation of cataracts, effects on DNA are involved in cancer causation, and effects on lipids apparently contribute to the causation of atherosclerosis. Free radicals and other reactive oxygen species in the human body are derived either from normal, essential metabolic processes or from external sources (Lillian, 1995 dan Panichayupakaranant, 2004).

### Protective System against Free Radicals

Nutrition plays a key role in maintaining the body's enzymatic defences against free radicals. Several essential minerals including selenium, copper, manganese and zinc are involved in the structure or catalytic activity of these enzymes. If the supply of these minerals is inadequate, enzymatic defences may be impaired. (Svilaas, et al., 2004). A second line of defence is small-molecular-weight

compounds which act as antioxidants; that is, they react with oxidising chemicals, reducing their capacity for damaging effects. Some, such as glutathione, ubiquinol and uric acid, are produced by normal metabolism. Ubiquinol is the only known fat-soluble antioxidant synthesised by animal cells. It is believed to play an important role in cellular defence against oxidative damage (Lillian, 1995).

This research is focused on exploration of marine natural product, especially for marine macro algae as a source of abundant natural life from Indonesia which potentially is one source of antioxidant to defenses from harmful of free radicals.

## MATERIALS AND METHODS

### Preparation of Macro algae

*Ulva fasciata*, *Caulerpa racemosa*, *Padina australis*, *Sargassum* sp, dan *Turbinaria deccurens* were collected on April, 15<sup>th</sup> 2005, from Krakal sea, Yogyakarta.

Reagent for antioxidative assay are 1,1-diphenyl-2-picrylhydrazyl (DPPH) purchased from Sigma, while other chemicals (MeOH, Heksana, and EtOAc reagent grade) purchased from Sigma.

### Preparation of Macro algae Extracts

One kilogram of each species of macro algae were separately macerated in one liter methanol for three days. After filtering and evaporating the filtrates to dryness in *vacuo*, the methanol extracts from each macro algae specimens were obtained. Each of the extracts was dissolved in hexane and ethyl acetate followed by evaporation to obtained dried hexane and ethyl acetate fraction. These semi-polar extract were used for anti oxidative assay.

$$\% \text{ Inhibition} = \frac{\text{Absorbance}_{\text{standard}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{standard}}} \times 100 \%$$

The compounds with antioxidant activity will be considered if the inhibition percentage of the solution is the same or higher as 50%.

### Purification of Antioxidative Compounds

The semi-polar fraction of ethyl acetate from each macro algae was subjected to Sephadex C-8 column with ethyl acetate/methanol solvent system to separate carotenoid compounds.

### HPLC condition for Carotenoids compounds separation in Green and Brown Macro algae

The most highly anti oxidative propesty compounds were identified using the High Performance Liquid Chromatography (HPLC) Shimadzu LCMS 2010A, with

### Determination of Antioxidant Activity

The antioxidant activity of all extracts and fractions was determined according to the DPPH radical scavenging assay of Chow et al, (2003) and Panichayupakaranant, (2004) with some modifications. The blank solution containing 1 mL DPPH 0.1N was added until it reached 5 mL volume with methanol. Samples for testing were prepared at 20 ppm concentration and was mixed with 1mL DPPH solution and dissolved with methanol until it reached 5 mL in volume. After the mixture had been allowed to stand for 30 minutes at temperature 37°C, its absorbance was measured at 515 nm using spectrophotometer UV-Visible Lambda 25 Perkin Elmer. The capability to scavange DPPH radicals was calculated by the following equation:

Princeton Omni C-18, 150 x 2 mm, 3 u, 100A column, with 100% acetonitril solvent system, and 5 µL injection volume. This HPLC system used Photo Diode Array detection with UV- Visible detector at 200- 800 nm.

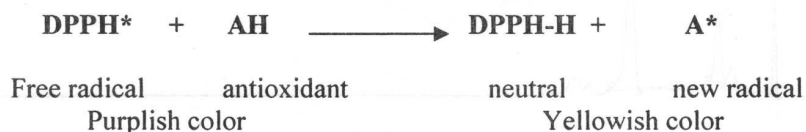
## RESULTS AND DISCUSSION

Endogenous free radicals such as superoxide, nitric oxide and hydroxyl free radicals are produced in the human body everyday. In addition, oxidant by-products of normal metabolism cause extensive damage to DNA, proteins, and lipids constituting a major contribution to aging and also to degenerative diseases of aging such as cancer, cardiovascular disease, brain dysfunction and cataracts. To prevent

or delay the oxidation process, addition of antioxidants to foods is the most extensively used method. Although synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyl-anisole (BHA), and tertbutylhydroquinone (TBHQ) have been commonly used as antioxidants in food for years, their safety has long been questioned. This has led to an increasing interest in natural antioxidants (Panichayupakaranant, 2004).

It has been reported that the antioxidant activities of compounds containing hydroxyl, polyhydroxyl, or carbonyl chains are due to the free radical chain formation mechanism. The free radicals are being neutralized with the unpaired electron from the hydroxyl chains

located at the active compounds using the proton donation mechanism (Oke and Hamburger, 2002; Hall and Cuppet, 1997). According to the reaction, the quantitative measurement of the antioxidative activities is due to the decreasing of the purplish visible color of DPPH compounds when it encounters proton-radicals scavengers. The purple color of DPPH solution fades rapidly when mixed with antioxidant compounds, because of concentration and also the inhibition ability of the antioxidant compounds towards the free radicals. The decreasing intensity of the purplish color of DPPH will show correlation with the antioxidant activities of the tested sample; it was calculated by the following equation (Januari, 2004):



The antioxidant activities toward each sample were focused on the semipolar extracts, hypothesized containing the pigment compounds. The preliminary separation was using ethyl acetate and further purified using Sephadex C8 column chromatography eluted by ethyl acetate – methanol to obtained the carotenoid fractions.

#### Antioxidant from Green and Brown Macroalgae

Although a wide variety of antioxidants in food may contribute to disease prevention, the bulk of the research to date has focused on three antioxidants which are of special interest because they are essential nutrients

or precursors of nutrients and are present in significant amounts in body fluids – vitamin E, vitamin C and carotenoids. The carotenoids are a group of red, orange and yellow pigments found in plant foods, particularly fruits and vegetables, and in the tissues of animals which eat the plants. Carotenoids have antioxidant activity, and some, but not all, can act as precursors of vitamin A. Important dietary carotenoids include  $\beta$ -carotene,  $\alpha$ -carotene, lycopene, lutein, zeaxanthin and  $\beta$ -cryptoxanthin (Lillian, 1995; Schulz, 2005).

The result of the antioxidant assay from the carotenoid fraction obtained from two green macroalgae and three brown macroalgae are shown in Table 2.

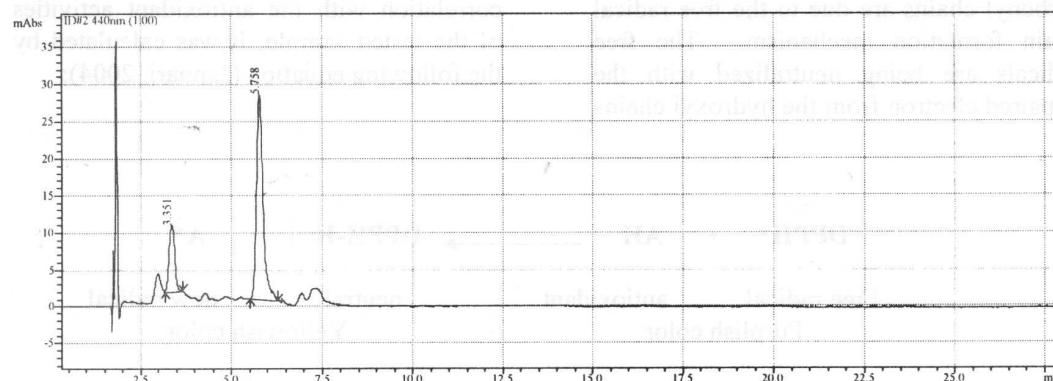
**Table 2.** The Free Radicals Scavenging Active Compounds from Carotenoid Fraction obtained from Green and Brown Algae.

Carotenoid Fractions		
Algae	515 nm	Inhibiton (%)
<i>Ulva Fasciata</i>	2.0186	39.04
<i>Caulerpa Racemosa</i>	2.2577	31.82
<i>Padina Australis</i>	1.4603	55.90
<i>Sargassum sp.</i>	1.0636	67.88
<i>Turbinaria Deccurens</i>	0.7166	78.36
Blank DPPH + MeOH	3.31135	0.00

**Structure Elucidation of the Active Carotenoid Antioxidant**

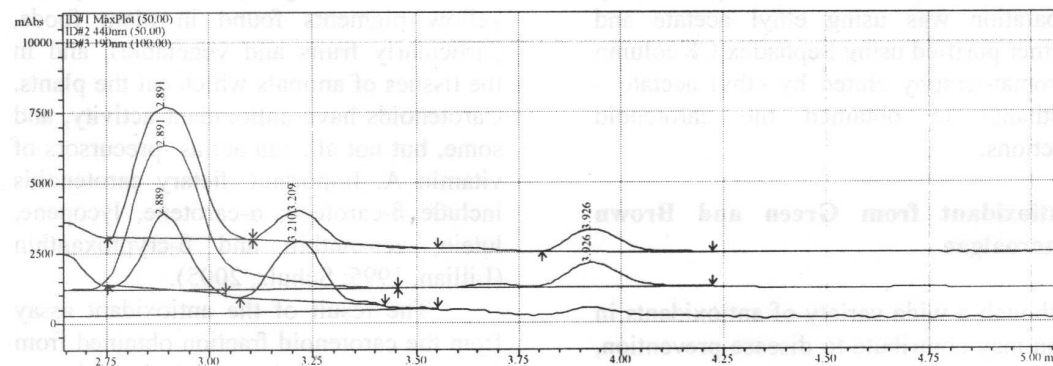
The carotenoid fraction separation of each sample shown by the following chromatogram

**Fig. 3a.** LCMS- Chromatogram of *Ulva fasciata*



Peak	Ret Time	Start Tm	End Tm	Area	Height	%Area	%Height	A/H
1	3.351	3.168	3.648	94,349	8,861	20.92939	24.54317	10.64
2	5.758	5.504	6.272	356,448	27,242	79.07061	75.45683	13.08

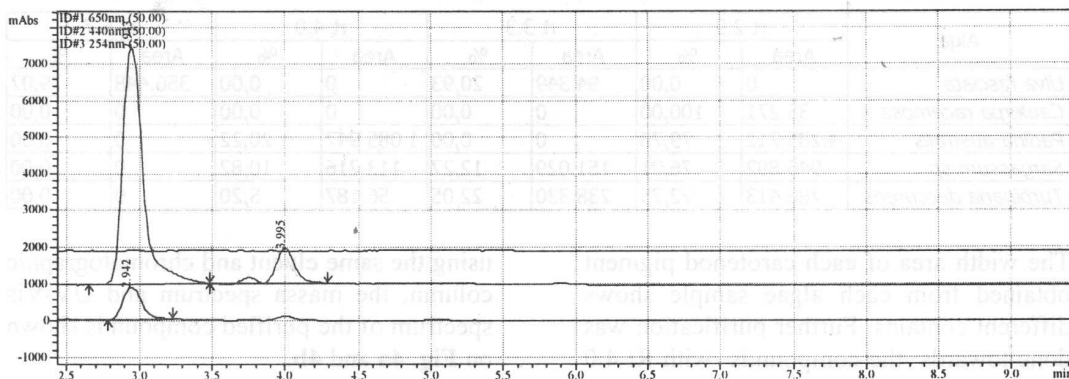
**Fig. 3b.** LCMS- Chromatogram of *Padina australis*



Peak	Ret Time	Start Tm	End Tm	Area	Height	%Area	%Height	A/H
1	2.891	2.752	3.104	946,892	92,535	76.91204	71.60982	10.23
2	3.209	3.104	3.552	151,029	21,191	12.2674	16.39904	7.12
3	3.926	3.808	4.224	133,216	15,495	10.82056	11.99114	8.59

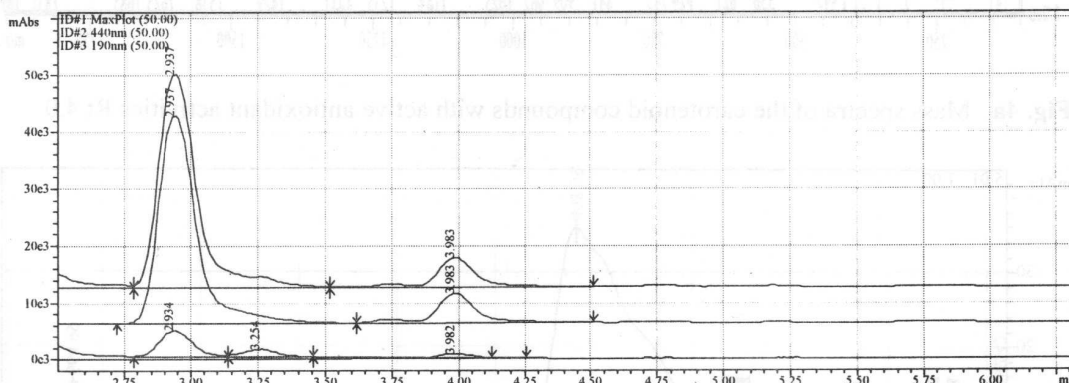


Fig. 3c. LCMS- Chromatogram of *Sargassum crossafolium*



Peak	Ret Time	Start Tm	End Tm	Area	Height	%Area	%Height	A/H
1	2.932	2.688	3.584	4,285,712	373,034	79.78377	83.86109	11.48
2	3.982	3.584	4.608	1,085,947	71,790	20.21623	16.13891	15.12

Fig. 3d. LCMS- Chromatogram of *Turbinaria deccurens*



Peak	Ret Time	Start Tm	End Tm	Area	Height	%Area	%Height	A/H
1	2.934	2.784	3.136	786,413	88,983	2.83809	5.82975	8.83
2	3.254	3.136	3.456	238,330	26,870	0.86011	1.76038	8.86
3	3.982	3.456	4.128	56,187	12,963	0.20277	0.84925	4.33

The carotenoid compounds obtained from each algae samples could be divided into four groups. Table 3 shows data from each isolate obtained from samples included the

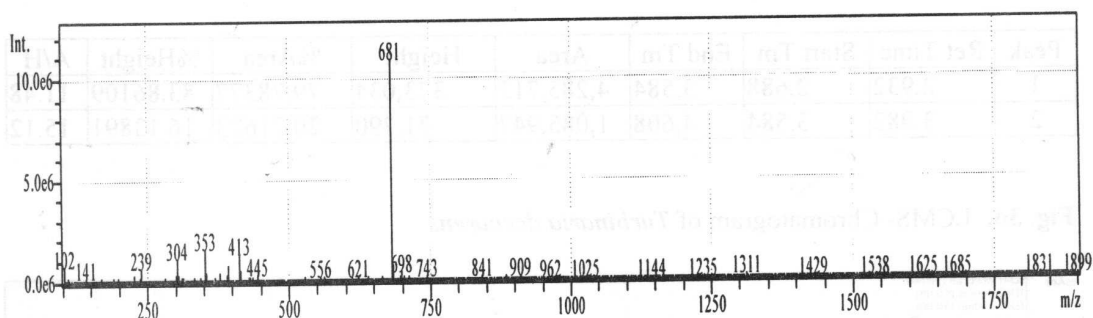
area width and the percentage of the chromatogram of the antioxidant compounds

**Table 3.** Chromatogram area and percentage of carotenoid fraction obtained from Green and Brown Algae.

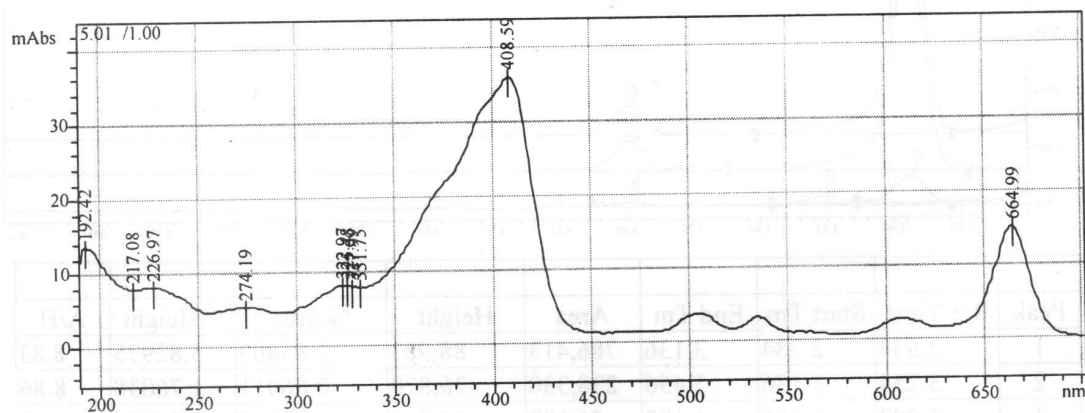
Alga	rt 2.9		rt 3.3		rt 4.0		rt 5.7	
	Area	%	Area	%	Area	%	Area	%
<i>Ulva fasciata</i>	0	0,00	94.349	20,93	0	0,00	356.448	79,07
<i>Caulerpa racemosa</i>	35.271	100,00	0	0,00	0	0,00	0	0,00
<i>Padina australis</i>	4.285.712	79,78	0	0,00	1.085.947	20,22	0	0,00
<i>Sargassum sp</i>	946.892	76,91	151.029	12,27	113.216	10,82	0	0,00
<i>Turbinaria deccurens</i>	786.413	72,75	238.330	22,05	56.187	5,20	0	0,00

The width area of each carotenoid pigment obtained from each algae sample shows different contains. Further purification was done towards the compounds with Rt 4.0

using the same eluent and chromatographic column, the mass spectrum and UV-Vis spectrum of the purified compounds shown on **Fig. 4a** and **4b**.



**Fig. 4a.** Mass spectra of the carotenoid compounds with active antioxidant activities Rt 4.0



**Fig. 4b.** UV – Vis Spectrophotometric spectra of the carotenoid compounds with active antioxidant activities Rt 4.0

On the basis of those spectroscopic data referring to the Wiley7 electronic database, Shimadzu electronic database of mass

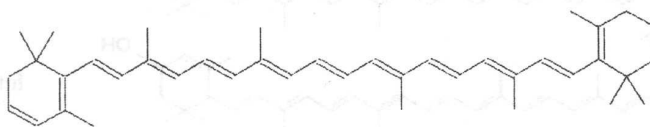
spectrum (2004), the result of active compound structure elucidation can be seen in **Table 4**.



**Table 4.** Active Antioxidant Carotenoid obtained from macroalgae

<b>rt 4.0*</b>	<b>2,3-diacetoxy-b-b-carotene-4,4'-dione</b>
Serapan maksimum UV-Vis	437-331-201
Puncak Spektrum Massa	680
Rumus Molekul	C44H56O6 (Wiley7)
Alga Produsen	sargassum, padina, turbinaria

This new active carotenoid compound can be grouped into xanthophyll compounds, with following structure:



**Fig. 5.** The Molecule structure of active antioxidant carotenoid compound obtained from macroalgae

Carotenoid occurring in plants are usually C 40 tetraterpenoids built from eight of C5 isoprenoid units and belong to the class of hydrocarbons (Carotenes) or their oxygenated derivatives (xanthophylls). Their distinctive characteristic is a long central chain with a conjugated double-bond system, which is a light-absorbing chromophore responsible for yellow, orange, or red color of these compounds (Delia, 2001; Hyoungshin Park, et. al., 2002). More than 400 carotenoids have been found in higher plants, algae, and bacteria of which carotene and lutein are uncounted most frequently. The carotenoid color from plants is a precursor for pigmentation in marine animals, egg, yolks, and fat globules and serves as a source for vitamin A for mammals (Delia, 2001; Masaya Kato, et. al., 2004). The chemical structure of carotenoids can be seen on **Fig. 6**.

Determination of the total carotenoid content at macroalgae, through the visible absorption at the max of the

principal carotenoid, although still done and attractive for its simplicity, yields insufficient information and is considered inadequate except as an estimate of the total pigment content. This type of work has given way to the determination of individual carotenoids because of their differing physicochemical properties and bioactivities.

The main problem in carotenoid analysis arises from their instability. Thus, whatever the analytic method chosen, precautionary measures to avoid formation of artifacts and quantitative losses should be standard practice in the laboratory. These include completion of the analysis within the shortest possible time, exclusion of oxygen, protection from light, avoiding high temperature, avoiding contact with acid, and use of high purity solvents that are free from harmful impurities. Oxygen, especially in combination with light and heat, is highly destructive.

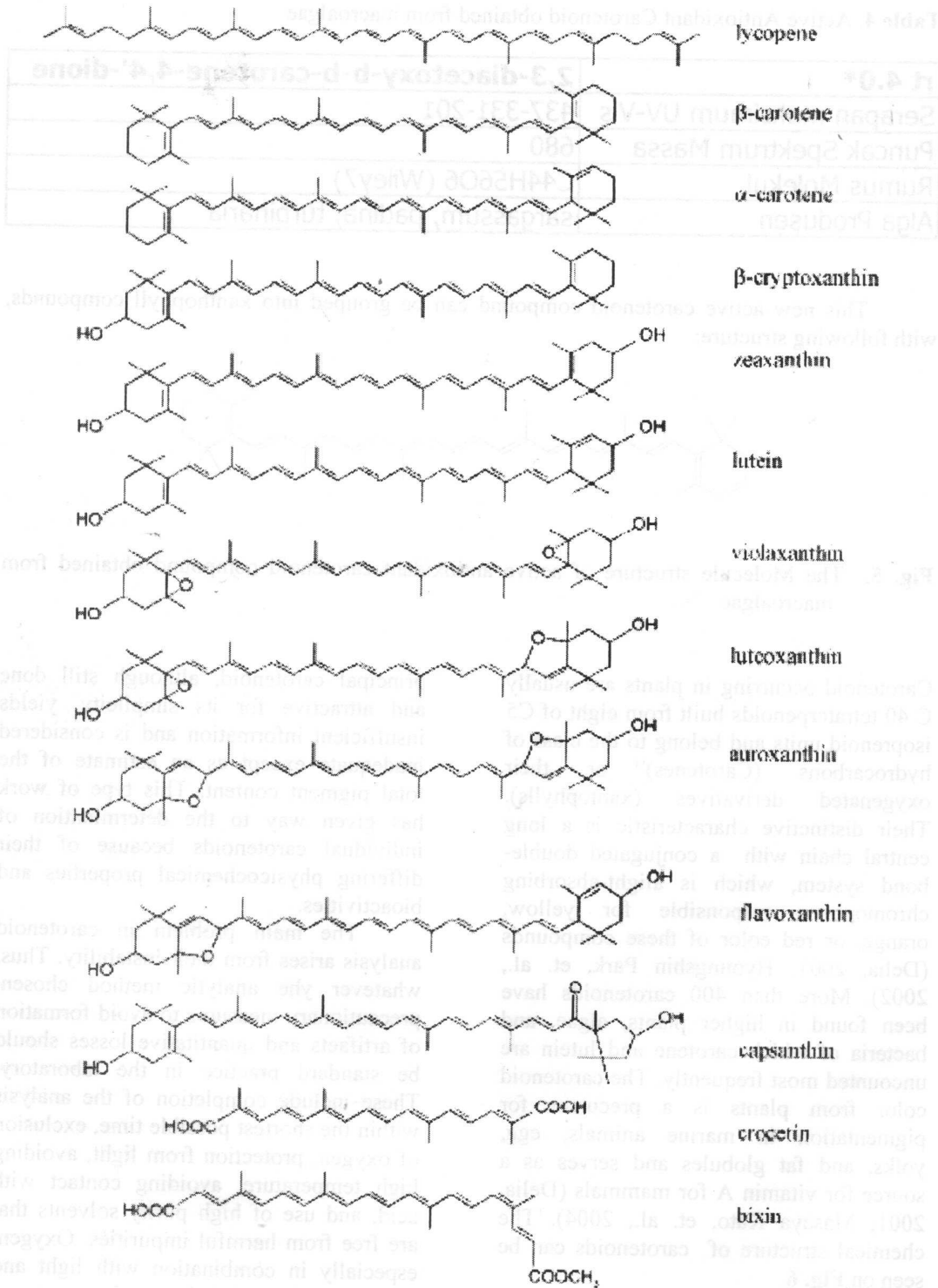


Fig. 6. The chemical structure of carotenoids

## CONCLUSION

In this research, we investigated the antioxidant contains from macro algae, a marine organism which has antioxidant as radical scavenger towards the free radicals. Some active compounds, such as carotenoid fraction found as xanthophylls obtained from green and brown macro algae proved to actively scavenging free radicals of DPPH solution. In the future, it should need to do the clinical study to determine the pharmacological active dose to prevent from toxicity of new formed carcinogenic effect from the overdose in using this new active antioxidant compound from marine macro algae.

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