ANTIOXIDANT ACTIVITY OF

Sargassum crassifolium and *Padina australis*

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Antioxidant Activity of Seaweeds from Panay

Abstract

Antioxidants are compounds that inhibit the activities of radicals, compounds that degrade cellular constituents, causing severe illnesses known to man, and aging. Synthetic antioxidants are available, but safety concerns have been identified with use of them. Natural antioxidants have the advantage of being generally free from chemical contamination. It has been recently discovered that seaweeds are a source of natural antioxidants. Thus, this study aims to determine the antioxidant activity of seaweeds collected from coastal areas around Panay and obtain extracts. Once the extracts are obtained, DPPH assay will be conducted on them, and compare the antioxidant activity of extracts with that of BHT, a synthetic antioxidant.

Seaweeds are collected from Nogas Island in Anini-y, Antique. The seaweeds are freeze-dried before extraction. Crude extracts were first obtained, and then the antioxidant activity was determined using the DPPH assay. Final extraction was done using three different solvents, namely petroleum ether, ethyl acetate and butanol. After the extracts were obtained using the solvents, DPPH assay was again conducted to determine the antioxidant activity of the extracts. The antioxidants activities were then compared with that of BHT.

The mean scavenging activity of *Sargassum crassifolium* obtained using different extracts are as follows: petroleum ether, 0.882 ± 0.065; ethyl acetate, 0.589 ± 0.017; butanol, 0.947 ± 0.049. For *Padina australis*: petroleum ether, 0.920 ± 0.036; ethyl acetate, 0.849 ± 0.076; butanol, 0.744 ± 0.025. The mean scavenging activity of BHT solutions is 0.865 ± 0.041. Butanol extracts of *S. crassifolium* showed the greatest scavenging activity. Using One-Way ANOVA, it is found out that only the scavenging activity of ethyl acetate extracts of *S. crassifolium* showed significant difference when compared with BHT. In general, seaweed extracts showed significant difference when compared with BHT.

With the results that were obtained, seaweed extracts *Sargassum crassifolium* and *Padina australis* can compete with synthetic antioxidants in terms of efficiency. This information may be useful for researchers who wish to know the seaweed species that could be studied on. Such studies can be conducted to exactly identify and isolate the compounds that are responsible for the seaweeds' antioxidant activity.

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CHAPTER 1

INTRODUCTION

A. Background of the Study

Seaweeds have been the subject of the recent studies because of their new found use: a source of antioxidants. Antioxidants are bioactive materials that slow down oxidation processes caused by metabolism of oxygen in any system, including our body system. Oxidation processes produce metabolites that degrade cellular constituents in our body, which may induce serious illnesses known to us humans, including artherosclerosis, rheumatoid arthritis, some neurological disorders as well as aging. Antioxidants help in preventing these illnesses by diverting the target sites of the metabolites, oxidizing themselves instead of the cellular constituents (http://en.wikipedia.org/wiki/Antioxidants). Thus, antioxidants are very much in demand lately. During the last decade or so, only synthetic antioxidants have been available, but safety concerns have been identified with these synthetic antioxidants (SJ Heo, EJ Park, KW Lee, YJ Jeon, 2004). So, interest and attention have been turned to natural antioxidants. Their use has the advantage over synthetic ones, being considered safe because of absence of chemical contamination. These natural antioxidants have been found to be present in almost all photosynthesizing plants, including seaweeds, due to exposure to light and high oxygen concentrations, which lead to production of free radicals and other oxidizing agents. However, these plants seldom suffer harmful effects of the oxidizing agents due to the protective antioxidative mechanisms and compounds of their cells (SJ Heo, EJ Park, KW Lee, YJ Jeon, 2004).

In this study, crude extracts were obtained using chloroform-methanol extraction and ethanol extraction. The extracts from green, brown and red algae, specifically *Padina*

australis, *Sargassum crassifolium* and *Acanthophora spicifera*, were assayed using the DPPH free radical scavenging activity to assess its potential antioxidant activity. The antioxidant activity was determined by measuring the absorbance of the DPPH solution by the extracts using a spectrophotometer.

B. Statement of the Problem

Do the extracts of *Sargassum crassifolium* and *Padina australis* have antioxidant activities using the 2,2-diphenyl-1-pricrylhydrazyl (DPPH) assay?

C. Objectives of the Study

This study aimed to:

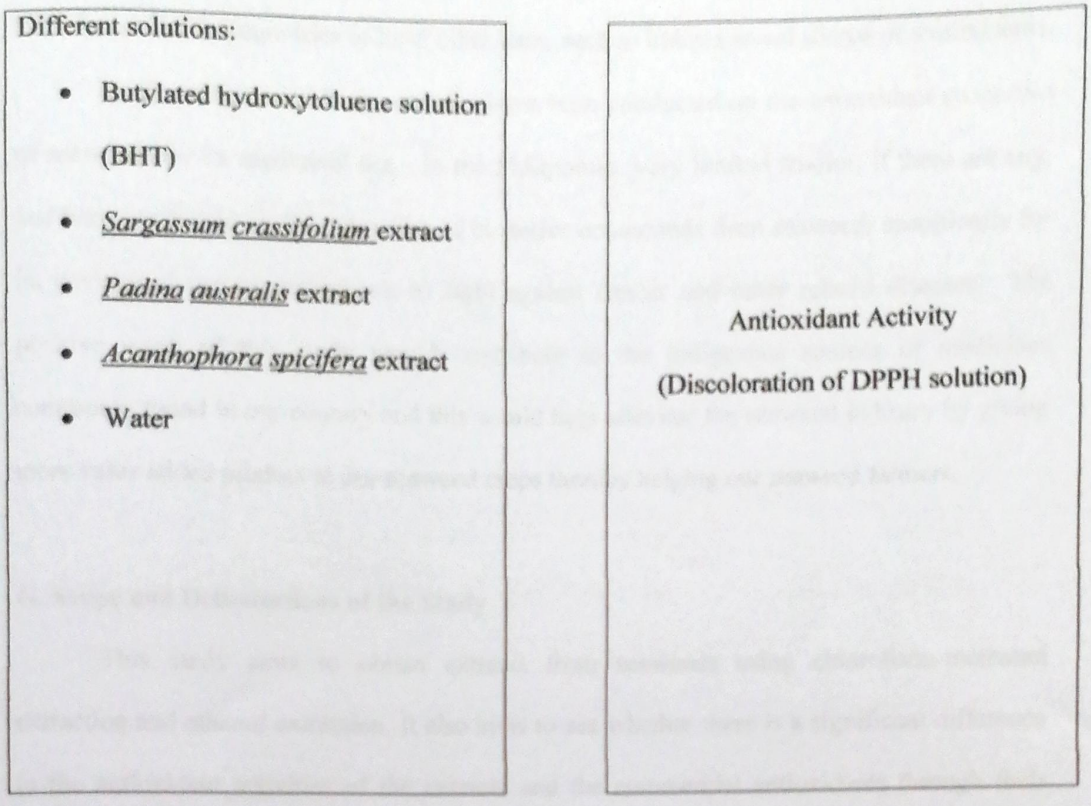
1. Determine if the seaweed extracts obtained from local seaweeds show antioxidant activities using DPPH free radical scavenging activity;
2. Compare the antioxidant activities of the seaweed extracts;
3. Compare the antioxidant activities of the seaweed extracts with the antioxidant activities of the synthetic antioxidant.

D. Hypotheses of the Study

1. *Sargassum crassifolium* and *Padina australis* will exhibit antioxidant activity;
2. The antioxidant activity of *S. crassifolium* will be greater than that of *P. australis*;
3. The antioxidant activity of the extracts will be greater than that of BHT.

E. Research Paradigm

DPPH Free radical scavenging activity:



F. Significance of the Study

Seaweeds are very abundant in the Philippines. They are used in many ways and have been found in other countries to have other uses, such as being a novel source of antioxidants.

Many studies outside the country have been conducted on the antioxidant properties of seaweeds for its medicinal use. In the Philippines, very limited studies, if there are any, had been conducted on the extraction of bioactive compounds from seaweeds specifically for its envisioned use as antioxidant to fight against cancer and other related diseases. The positive result of this study would contribute to the indigenous sources of medicines commonly found in our country and this would help alleviate the seaweed industry by giving more value added product to our seaweed crops thereby helping our seaweed farmers.

G. Scope and Delimitations of the Study

This study aims to obtain extracts from seaweeds using chloroform-methanol extraction and ethanol extraction. It also aims to see whether there is a significant difference in the antioxidant activities of the extracts and the commercial antioxidants through their percentage inhibition results.

The chloroform-methanol and ethanol extraction is used because it is the procedure that is described in detail and is feasible in the light of the researcher's capability and of the present situation. Thus, this study will use chloroform-methanol and ethanol extraction. The solvents chloroform, methanol and ethanol are commonly used solvents in extracting organic compounds from biological samples because of the polar nature of these solvents which extracts organic compounds present in these materials. Other extraction methods mentioned in the reference materials, such as the hot-water extraction, yielded a lower percentage of organic compounds.

To determine the difference of the commercial antioxidants and extracts, DPPH free radical scavenging activity were used. Other scavenging assays were also mentioned in the article by Heo et al, such as the superoxide anion scavenging activity, hydroxyl radical scavenging activity and hydrogen peroxide scavenging activity but due to the complexity of these methods, they will not be used.

H. Definition of Terms

To have a better understanding of the study, the following terms were defined:

Antioxidant- an inhibitor, such as ascorbic acid, effective in preventing oxidation by a molecule or oxygen (McGraw-Hill Dictionary of Scientific and Technical Terms, Fifth Edition, 1994)

Decant- to mechanically dewater a wet solid by pouring off liquid without disturbing the underlying sediment or precipitate (McGraw-Hill Dictionary of Scientific and Technical Terms, Fifth Edition, 1994)

Free radical- diatomic or a polyatomic molecule which possesses an unpaired electron (McGraw-Hill Dictionary of Scientific and Technical Terms, Fifth Edition, 1994)

In this study, free radical refers to DPPH (2,2-diphenyl-1-pricrylhydrazyl)

Holdfast- basal attachment organ of an alga

Oxidation process- a chemical reaction in which a compound or a radical loses electrons (McGraw-Hill Dictionary of Scientific and Technical Terms, Fifth Edition, 1994)

Seaweed- any marine plant, usually algae (McGraw-Hill Dictionary of Scientific and Technical Terms, Fifth Edition, 1994)

In this study, seaweed refers to *Sargassum crassifolium*, *Padina australis* and *Acanthophora*

Spectrophotometry- a procedure to measure photometrically the wavelength of radiant energy (visible light, ultraviolet light, x-rays) absorbed by a sample under analysis (McGraw-Hill Dictionary of Scientific and Technical Terms, Fifth Edition, 1994)

Stipe- stem-like, usually basal part of the thallus above the holdfast

Thallus- a vegetative plant body without an easy distinction between true leaves, stems and roots

Tidal flats- a marshy, sandy or muddy nearly horizontal coastal flatlands which is alternately covered and exposed as the tide rises and falls (McGraw-Hill Dictionary of Scientific and Technical Terms, Fifth Edition, 1994)

Vesicles- bladder-like cell filled with air present in most algae

CHAPTER II

Review of Related Literature

This chapter provides information on the different components of this study. Those components include the test subject, methods and principles, and the studies related to this study. The most important sections in this chapter are **Antioxidants**, **DPPH Free Radical Scavenging Activity**, **DPPH as Standard assay**, **Radicals and Oxidants**, and **Bioactive Compounds in Seaweeds.**

A. Seaweeds of Genus *Sargassum*

Seaweeds of the genus *Sargassum* are generally brown or dark green in color and usually have gas-filled bladders. They are usually found throughout tropical areas and often the most obvious macrophyte near the shores. Most of the species are attached to corals, rocks or shells in moderately exposed or sheltered rocky or pebble areas while others are floating in bodies of seawater (http://www.surialink.com/HANDBOOK/Genera/browns/Sargassum/Sargassum.htm).

A.1 *Sargassum crassifolium*

Known as common Pacific Sargassum, *S. crassifolium* can grow up to 350 mm. it is attached to substrate by a disc shaped holdfast. Its thallus (wholed body, undifferentiated into true leaves stems and roots) is yellowish brown. Its short primary stipe (stem-like part) gives rise to branches at irregular alternate arrangement. The blades are elliptical with coarsely dentated margin. It is commonly found in upper intetidal zone exposed to strong water movement. It is a source of algin (emulsifying, stabilizing and gelling agents in food, juices

and other products) and used as fertilizer and fodder locally (AQ Hurtado, Ma. RJ Luhan, NG Guanzon, jr., 2006).

B. *Padina australis*

Padina australis is a flat seaweed with an in-rolled margin that splits into narrow fan-shaped sections. It is light brown in color and slightly calcified. Attached by a single holdfast, it can be from 5 to 20 cm tall and is found in deep tide pools and on reef flats. It is found mostly on reef flats and tide pools and in places with plenty of sunlight and shallow water

C. Antioxidants

Antioxidants are substances or nutrients in our food which can prevent or slow oxidative damage to our body. When our body cells use oxygen, they naturally produce radicals as by- products which can cause oxidative damage. Antioxidants act as scavengers and hence prevent and repair damage done by these radicals, health problems such as heart disease, macular degeneration, diabetes and cancer are all contributed by oxidative damage.

Naturally, almost all photosynthesizing plants including seaweeds contain antioxidants in form of pigments that neutralize the harmful effects of being exposed to a combination of light and high oxygen concentrations. In other plants, antioxidants exist as vitamins such as vitamins A, C and E, and compounds such as betacarotene and as phytochemicals (SJ Heo, EJ Park, YJ Jeon, 2004).

Antioxidants are also made synthetically and are available to the public to maintain the shelf life of several food stuffs. Examples of synthetic antioxidants are BHA (butylated hydroxyanisol), BHT (butylated hydroxytoluene) and TBHQ (tert-butyl hydroquinone) (SJ Heo, EJ Park, YJ Jeon, 2004).

D. DPPH Free radical scavenging activity

Reactive oxygen species scavenging assays, including DPPH free radical scavenging activity, report the antioxidant activities of compounds extracted and/or isolated in plants (SJ Heo, EJ Park, YJ Jeon, 2004). This particular method uses the stable free radical DPPH or 2,2-diphenyl-1-pricrylhydrazyl to show the inhibitory effects of the compounds on the activities of the free radical. A compound to be tested for its antioxidant properties is mixed with a DPPH solution and the percentage inhibition of the compound on the DPPH solution is visible through the discoloration of the solution. The percentage inhibition were analyzed using a spectrophotometer. Compared to other scavenging assays such as the superoxide anion scavenging activity and hydroxyl radical scavenging activity, DPPH free radical scavenging activity is relatively simple. The superoxide anion activity makes use of a series of recording of the absorbance of the compound at regular intervals for a given period of time.

E. Freeze-drying

Freeze-drying, or lyophilization, is the sublimation or removal of water content from a frozen material. The dehydration process takes place in a vacuum where the material is solidly frozen during the process. The fundamental steps are: (1) freezing, which provides the necessary condition for low temperature drying; (2) sublimation, the vaporization of the solvent on the material without passing through the liquid phase; (3) heating, to accelerate the sublimation of the material; (4) condensation, the removal of the vaporized solvent from the vacuum chamber by converting it into solid, completing the separation process (http://inventors.about.com/library/inventors/blfrdrfood.htm).

F. DPPH as Standard Assay

The free scavenging activity or the DPPH assay makes use of the stable free radical 2,2-diphenyl-1-pricrylhydrazyl to determine the scavenging activity of natural as well as artificial substances. The structure of DPPH contains an odd electron, which is signified by purple color of the DPPH solution. When a substance is mixed with the DPPH solution, the odd electron is paired with a hydrogen atom from the substance, if the substance contains antioxidant compounds. For each electron that is paired with a hydrogen atom from the substance, the color changes from purple to yellow. The absorbance or the color change can be easily monitored using a spectrophotometer at 520 nm (TT Guan, M Whiteman, 2008).

G. Radicals and oxidants

Radicals are chemicals species with one ore more unpaired electrons. Several processes in the body produce radical by products, such as the reduction of molecular oxygen in the mitochondria during cell respiration, degradation of fatty acids and phagocytosis. The free radicals and oxidants can trigger lipid peroxidation as well as oxidation of cells and DNA, causing extensive damage to body cells. Oxidative damage resulting from the imbalance of oxidizing species and natural antioxidants has been hypothesized as the major contributor to aging and cause of severe diseases such as cancer, Alzheimer's disease, Parkinson's disease and also cardiovascular disorders (TT Guan, M Whiteman, 2008).

H. Extraction methods and Principles

Chloroform-methanol extractions are suitable for water-soluble and organic soluble metabolites. Chloroform-methanol mixtures, commonly 2:1 by volume, are often used to extract compounds from organic materials. In some cases, the mixture is not polar enough to extract the polar compounds present in the organic material. Therefore, it is recommended to

use a 1:1 mixture of chloroform and methanol in a solution. Another alternate is the sequential extraction using chloroform and methanol.

I. Bioactive compounds in Seaweeds

Phenolic compounds have recently received significant attention among various antioxidants and many studies have been performed to identify natural antioxidative phenols with pharmacological activity. Similar to the study of other marine organisms, the investigation of biologically active metabolites of marine algal origin has significantly increased in the last three decades. Examples of the compounds are the bromophenols (Li K, Li XM, Ji NY, Wang BG, 2007).

Potential antioxidant compounds in seaweeds were identified as some pigments (fucoxanthin, astaxanthin, carotenoid, etc) and polyphenols (phenolic acid, flavonoid, tannin, etc). Those compounds are widely distributed in plants or seaweeds and are known to exhibit higher antioxidant activities (SJ Heo, EJ Park, YJ Jeon, 2004).

J. Summary of Related Researches

In a study by Nai-Yun Ji et al entitled "Terpenes and Polybromoindole from the Marine Red Alga *Laurencia decumbens* (Rhodomelaceae)", several compounds have been isolated from *L. decumbens*. The article about the study states that seaweeds from the genus *Laurencia* have been the subject of several intensive studies due to the presence of metabolites in them. The study used chloroform-methanol in obtaining crude extracts.

A study by Ke Li et al (Natural Bromophenols from the marine red alga *Polysiphonia urceolata*: Structural elucidation and DPPH radical scavenging activity) isolated phenolic compounds and showed that the compounds possessed potent DPPH radical scavenging activity. The information corresponded with the report (of Ke Li et al) that the acetonitrile

soluble fractions of the crude extracts from *P. urceolata* showed high DPPH radical scavenging activity and phenolic compounds were proposed to be responsible. The samples of *P. urceolata* were dried and extracted using ethanol and partitioned between water and acetonitrile, then divided into fractions. The acetonitrile soluble fractions that were subjected to DPPH radical scavenging activity were compared to BHT (butylated hydroxytoluene), a commercial antioxidant, and found to be more potent than BHT.

A study from Cheju National University in Korea entitled "Antioxidant Activivties of Emzymatic Extracts from Brown Seaweeds" showed that enzymatic extracts exhibited significant antioxidant activities, some relatively higher than commercial antioxidants. The study used several methods including superoxide anion scavenging activity, hydroxyl radical scavenging activity, hydrogen peroxide scavenging activity and free radical scavenging activity (DPPH) to determine the inhibitory effects of the extracts on the radicals.

CHAPTER III

Methodology

A. Research Design

This study compared antioxidant activities of the crude extracts of the seaweeds of the genus *Sargassum* and *Laurencia* in Panay to the antioxidant activity of the commercial antioxidant BHT (butylated hydroxytoluene). However, the seaweed samples that were collected were that of *S. crassifolium*, *P. australis* and *A. spicifera* only. The seaweed samples were collected in Nogas Island in Antique. The samples were cleaned, identified and freeze-dried in preparation for the extraction process. To obtain extracts, the chloroform-methanol and ethanol extraction method was used. Before being partitioned, extracts were first subjected to DPPH free radical scavenging activity using a method mentioned in an article by Zhang et al to determine which of the crude extracts show antioxidant activity. Crude extracts were obtained again from the seaweed species that have shown antioxidant activity. The new crude extracts were partitioned between acetonitrile and water. The acetonitrile soluble layer was purified and divided into fractions through column chromatography. The fractions were subjected to the final DPPH assay.

B. Materials, Instruments and Equipment

The following reagents were used in this study:

BHT (butylated hydroxytoluene) will serve as the control in the free radical scavenging activity.

Chloroform-methanol ($CHCl_3$:CH_3OH) and ethanol (C_2H_5OH) were used to obtain crude seaweed extracts.

DPPH were used in the free radical scavenging activity

Petroleum ether, ethyl acetate and butanol were used in the final extraction process.

The following instruments and equipment were used in this study:

Reagent bottles (glass and plastic)

Glass funnel

Graduated cylinders

Stirring rod

Rotary evaporator

Spectrophotometer

Test tubes

Glass column

Separatory funnel

C. Description of the Source of Seaweed Samples

Seaweed samples were collected in tidal flats, several meters away from the shore in two to three-meter deep waters.

D. Procedure

D.1 Collection and Identification of Seaweed Samples

Seaweeds were collected in Nogas Island in Antique. They were collected during low tide from tidal flats in 2-3 meter waters by hand. Collected specimens were washed and cleaned with sea and freshwater to remove adhering debris. The samples were placed in a net bag labelled with the collection site. Sample specimens were sent to SEAFDEC for identification.

D.2 Treatment of Samples

Cleaned samples were store in a laboratory refrigerator for 8 hours prior to freeze-drying. The samples were taken out of the refrigerator and were freeze-dried for 8 hours. Freeze-dried samples were ground in preparation for extraction. The powdered samples were placed in separate plastic resealable bags labelled with its scientific name the source of the sample.

D.3 Preparation of Reagents

D.3.1 Chloroform-methanol solution

One-hundred fifty (150) millilitres of chloroform were poured in a 200 ml beaker. It was poured again in a graduated cylinder to have a more accurate measurement. It were then stored in a glass reagent bottle. One-hundred fifty (150) of methanol was poured in a beaker then into a graduated cylinder for a more accurate measurement. The two solutions

were mixed together to obtain a 300 ml 1:1 mixture of chloroform-methanol solution. The chloroform methanol solution was stored in a glass reagent bottle.

D.3.2 Ethanol solution

Three hundred (300) mL ethanol was poured in a 450 mL beaker. It was then poured in a graduated cylinder to be measured more accurately. From the graduated cylinder, the ethanol solution was transferred in a glass reagent bottle.

D.4 Extraction of crude extracts

One-hundred milligrams (100) of freeze-dried ground algae was extracted with 6 ml of methanol-chloroform mixture (CH_3OH:$CHCl_3$, 2:1, v/v) by ultrasonic wave for 55 minutes, then vortexed at 1900 rpm for 5 minutes. The mixture was left to stand in room temperature for 12 hours. After 12 hours, the mixture was decanted carefully. The liquid part was set aside. The residue was again mixed with 6 ml of methanol-chloroform mixture (CH_3OH:$CHCl_3$, 2:1, v/v) and sonicated for 55 minutes and vortexed at 1900 rpm for 5 minutes. The mixture was left again for 12 hours in room temperature. The liquid part from the previous extraction was combined with the new mixture. The combined solution was diluted to 25 ml with a 2:1 methanol: chloroform, obtaining the contraction 4 mg (seaweed dry weight) per ml solution of seaweed extract.

D.5 Free radical scavenging activity on crude extracts

The DPPH assay was done on crude extracts to determine which extracts will are to be subjected to the final DPPH assay. A 0.2 mL algal extract solution was mixed with ethanol of the same volume (0.2 mL). A 0.001 M DPPH/methanol solution (0.025 mL) was added to the solution. The extracts that showed antioxidant activity were partitioned between