Production, Phytochemical Characterization and Acceptability of Wines from Green Seaweeds (*Halimeda macroloba, Caulerpa sertularioides* and *Caulerpa racemosa*)

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ABSTRACT

Seaweeds are tagged as the medical food of the 21st century due to its being rich in bioactive substances, minerals and vitamins. Seaweeds could be processed as wine and could be used as a medicine or a delicacy. The bioactive substances inherent to the seaweed and responsible for its various medicinal properties could be made available during the fermentation process. In this study, wines were produced from three (3) green seaweed species, namely: *Halimeda macroloba*, *Caulerpa sertularioides* and *Caulerpa racemosa* using three (3) kinds of sugar, characterized by its phytochemical profile, total phenolic content (TPC), total flavonoid content (TFC) and total antioxidant activity (TAA), and subjected to sensory evaluation for acceptability. Results show that wines produced from green seaweeds are rich in flavonoids, terpenoids, coumarin and betacyanin. TPC of wines ranged from 54 - 467 mg Gallic Acid Equivalent (GAE)/L. TFC ranged from 7 - 356 mg Cathechin Equivalent (CE)/L. TAA ranged from 97 - 558 mg Ascorbic Acid Equivalent (AAE)/L. In general, wines fermented using muscovado sugar have the highest TPC, TFC and TAA values. The general acceptability of seaweeds wines using a 5-point rating scale ranged from "liked moderately" to "liked very much".

Keywords: *Green seaweed wine, phytochemical profile, total antioxidant activity.*

1. INTRODUCTION

The term "Seaweed" refers to a diverse group of macrobenthic and photosynthetic plants found in the near-shore marine environment (https://www.biologyonline.com/dictionary/seaweed-d3). Seaweeds are classified into three major taxa based on their pigmentation [1]: Chlorophyceae (green seaweeds), Phaeophyta (brown seaweeds) and Rhodophyta (red seaweeds).

In Panay Island, Western Visayas, Philippines, a total of 112 seaweed species were identified [2]. Forty-one (41) of which belong to the green seaweeds, 20 species of brown and 51 species of red. Most of these seaweeds were used as human food, as animal feed, source of agar (red seaweeds), source of algin, simple sugars and sugar alcohol (brown seaweeds) and source of pigments. Some were used as medicines (*Caulerpa spp.*, *Gracilaria spp.*, *Acanthopora spp.*, *Dictyota spp.*). Seaweeds are utilized in the food industry, pharmacy and medicine, thus, are considered of significant economic importance [3]. In fact, seaweeds are tagged as the medical food of the 21st century due to its being rich in minerals, vitamins, trace elements and bioactive substances [4].

Caulerpa species, commonly known as seagrapes, are widely harvested and consumed in the Philippines [5]. Sea grapes are regarded as an economically important commodity and are abundant during the months of January to April [6]. Magdugo et al. [7] reported that *Caulerpa racemosa* have high amounts of proteins and the essential amino acids present were comparable to FAO/WHO requirements. *Caulerpa* species were found to be rich in essential minerals and trace elements [2, 8], exhibited promising antioxidant, reducing and antidiabetic activities [8], showed immunostimulatory activities [9, 10] and displayed potent antimicrobial activity and anti-inflammatory activity [11, 12].

Unlike the *Caulerpa* species, *Halimeda macroloba* is not utilized commercially although it was reported to contain growth regulators such as auxin, gibberellin and cytokinin [2]. Various studies have reported that *Halimeda macroloba* has a great potential as new antioxidant sources for human health [13, 14, 15] and also exhibited broad spectrum of antibacterial and antifungal activity [16, 17].

Seaweeds could be processed as wine and could be used as a medicine or a delicacy. In 2006, seaweed wine has hit the German market picking a price of US$28 a bottle (http://www.dw.com/en/seaweedwinehitsgermanysstores/a1993083). The bioactive substances inherent to the seaweed and responsible for its various medicinal properties could be made available during the fermentation process. During fermentation, these bioactive compounds are released into wine (aqueous ethanolic solution) making the polyphenols and bioactive components exposed, thereby increasing their bioavailability and free during their absorption [18]. According to Morina and Kongoli [19], wines rich in phenolic compounds were found to have powerful antioxidant activity. Antioxidants play a crucial role in the prevention of many diseases such as cancer, inhibiting tumor initiation and heart diseases. Furthermore, they reported that phenolic compounds play an important role in several sensory properties of wine such as color, flavor, astringency and hardness.

The production and characterization of wines from seaweeds collected in the Philippines has been reported. Lazado (2007, Unpublished Thesis) studied the physico-chemical properties and sensory attributes of wine produced from seaweed *Gracilaria verrucosa*. Wine was also being produced from sea grapes (*Caulerpa sp*.) (Intellectual Property Philippines Application Number 2/2017/000905) and from sea lettuce (*Ulva sp*.) (Intellectual Property Philippines Application Number 2/2018/000935). However, none of these studies reported on the phytochemical composition and antioxidant activity of the seaweed wines produced in which people are after for in drinking wines.

In view of this, a research project was initiated to investigate the suitability of pureed green seaweeds for wine production, and also to characterize the wine produced in terms of phytochemical composition and antioxidant activity. It had three objectives, namely: (1) to determine the right formulation for wine making with green seaweeds as raw material; (2) to quantify the phytochemicals present in produced wines and its antioxidant activity; and (3) to determine the acceptability of the produced wines. The laboratory experimental results obtained are presented in this paper.

2. METHODOLOGY

2.1 Collection of Seaweed Sample

The seaweed samples, except for *Caulerpa racemosa*, were collected at the coastal area of San Dionisio, in Northern Iloilo, Philippines. Seaweed samples were collected manually or by the use of scissor or knife. Each seaweed species was placed in a net bag, cleaned with seawater to remove extraneous materials and brought to the processing area. Upon arrival in the processing area, the seaweed samples were again cleaned with foreign materials, thoroughly washed with tap water until the final washing was clear and finally with distilled water. Samples of *Caulerpa racemosa* were purchased at a public market in Northern Iloilo. A representative sample of each species was set aside for preservation, identification and documentation. The seaweed samples were identified up to the species level based on the book of Hurtado et al. [2] and were confirmed by the Farming Systems and Aquatic Ecology Section, Aquaculture Department, Southeast Asian Fisheries Development Center in Tigbauan, Iloilo.

2.2 Wine Making/Fermentation

One kilogram of cleaned seaweed samples were homogenized separately using a blender machine to make a puree. Several treatments were conducted using different kinds of sugar. Sugar syrup was added to the seaweed puree solution until the brix content of 22% was reached. The mixture was heated to 60ºC – 80ºC, cooled to 40ºC and added with yeast. The seaweed-sugar-yeast solution was allowed to ferment until the sugar content of the liquid was $\leqslant$10%. The wine produced after about 4 weeks of fermentation was transferred into a large bottle for settling. The clear wine was decanted, bottled and stored away from direct sunlight.

2.3 Phytochemical Screening of Seaweed Extract

Sub-samples of pureed seaweeds were soaked in ethanol for 48 hrs and the extracts were concentrated using rotary evaporator. The concentrated extract was subjected to qualitative phytochemical analysis along with the seaweed wines produced following the procedure described by Guevara [20] and Sivagnanavelmurugan [21].

2.4 Determination of Total Phenol Content

The total phenol content of the seaweed wines was determined according to the method described by Waterhouse [22] using Gallic acid as a calibration standard. The color intensity was measured spectrophotometrically at 765 nm after the reaction with Folin-Ciocalteau phenol reagent. Results are expressed as Gallic acid equivalents (GAE, mg/L). All measurements were performed in triplicate.

2.5 Determination of Total Flavonoids

The total flavonoid content in seaweed wine samples was determined spectrophotometrically using the aluminum chloride assay [23]. The absorbance was measured at 510 nm and the concentration was expressed as catechin equivalent (CE, mg/L). The result in every assay was obtained from three parallel determinations.

2.6 Determination of Total Antioxidant Activity

The phosphomolybdate assay described by Prieto et al. [24] was modified and used to determine the total antioxidant activity (TAA) of the seaweed wines produced. In summary, 1 mL of molybdate reagent solution was added to 10 µL of wine samples. The vials were capped and incubated in a water bath at 95ºC for 90 min. The sample mixture was cooled to room temperature, and the absorbance was measured at 695 nm against a blank. Ascorbic acid was used to prepare a standard curve in the range 20-100 mg/L, and the total antioxidant activity was expressed in terms of milligram per liter ascorbic acid equivalent (AAE, mg/L). All measurements were triplicated.

2.7 Sensory Evaluation

All produced wines were subjected to sensory evaluation based on the method described by Roldan & Edica [25].

3. RESULTS AND DISCUSSION

3.1 Seaweed Samples

Three (3) green seaweed species were collected namely: *Halimeda macroloba* Decaisne, *Caulerpa sertularioides* (S.G. Gmelin) Howe and *Caulerpa racemosa* var. *laetevirens* (Montagne) Weber-van Bosse.

The seaweeds collected were abundant in the coastal areas of San Dionisio, Iloilo and in Estancia, Iloilo and of no economic importance to the community except for *Caulerpa racemosa* in which it is sold in the market for food as simple salad. Hurtado et al. [2] reported that *Caulerpa* species were used as human food, are source of amino acids, fatty acids, lipids and minerals. They are also found to lower blood pressure aside from having an antifungal property. On the other hand, *Halimeda macroloba* was reported to contain growth regulators such as auxin, gibberellin and cytokinin [2].

3.2 Wine Characteristics

Table 1 shows the physico-chemical characteristics of wines produced from green seaweeds. Wine color is affected by the sugar used during fermentation. The use of muscovado sugar resulted to a yellowish-dark brown wines, while the refined and washed sugars resulted to light yellow-colored wines. The pH values of wines produced ranged from 3.2 – 3.8 and the total acidity ranged from 0.097 – 1.034 g Tartaric Acid (TA)/ 100 mL. Sugar content after fermentation ranged from 3.0 – 4.0 ºBrix while the alcohol content of the wines produced ranged from 11.8 – 12.2 %.

Table 1. Physico-chemical characteristics of green seaweeds wines.

Wine	Physical Appearance	pH	Titrable Acidity (g TA/ 100 mL)	% Alcohol
GS1-R	Faint yellow, clear	3.4	1.034	12.1
GS1-W	Faint yellow, clear	3.8	0.766	12.0
GS1-M	Yellowish dark brown, turbid	3.6	0.779	12.0
GS2-R	Faint yellow, clear	3.2	0.378	11.9
GS2-W	Faint yellow, clear	3.3	0.323	12.1
GS2-M	Yellowish dark brown, turbid	3.3	0.401	12.0
GS3-R	Faint yellow, clear	3.6	0.165	11.8
GS3-W	Faint yellow, clear	3.7	0.097	11.9
GS3-M	Yellowish dark brown, turbid	3.3	0.533	12.2

Note: GS – green seaweed; 1 – *Halimeda sp.*; 2 – *Caulerpa sp1.*; 3 – *Caulerpa sp2*; R – Refined sugar, W – Washed sugar; M – Muscovado sugar

The pH of a wine is a measure of the strength and concentration of the dissociated acids present in the said medium. It plays a vital role in winemaking process. pH influences wine's microbiological stability, affects the equilibrium of tartrate salts, influences the solubility of proteins and ability to clarify, influences sensory attributes and affects color and oxidative and browning reactions [26, 27, 28]. A pH between 3.0 and 4.0 is optimal for most wines [26]. For grape wines, white wines may have pH values of 3.5 or lower while red wines may have higher values [27]. The pH of the seaweed wines produced are within the reported optimum pH values for most wines.

Titratable acidity (TA) is a measure of the total amount of hydrogen ions or the acid content of wine. TA is usually reported in units of tartaric acid, malic acid, or citric acid depending on the nature of fruit or plant material. TA analysis is one of the most basic analyses in a winery lab because fruits and other possible plant wine materials contain significant amounts of organic acids. The acid content impacts the taste, color, and microbial stability of the wine. Some regulatory bodies prescribe a minimum TA (like 0.45 grams tartaric /100 mL for red wine in the EU). A general range for TA in red wine is 0.6 to 0.8, for red wine must it is 0.7 to 0.9 (chateauhetsakais.com/total-acidity; Accessed date: April 11, 2019). The seaweed wines produced using the muscovado sugar have TA values within the reported prescribed TA for red wine. Generally, seaweed wines produced using refined and washed sugars have lower TA values (0.097 – 0.407 g /100 mL) except for *Halimeda* wines with TA values of 1.034 g/100 mL and 0.766 g/100 mL, respectively. The increase in TA values could be attributed to the seaweed sample itself wherein continues fermentation of wine occurs. Other possible reasons could be the high acetic acid concentration due to oxidation and the formation of Succinic acid as a by-product of alcoholic fermentation [29].

There is no direct or predictable relationship between pH and TA, and the same titratable acidity can be measured in different wines with either low pH or high pH. The pH is not correlated with the concentration of acids present, but is influenced by their ability to dissociate (The Australian Wine Research Institute, Accessed Date: April 10, 2019; https://www.awri.com.au/industry_support/winemaking_resources/frequently_asked_questions/acidity_and_ph/).

3.3 Phytochemical Profiles of Seaweed Ethanolic Extracts and Wines

The concentrated ethanolic extracts of seaweed samples were subjected to phytochemical screening for the presence of alkaloids, tannins, anthraquinones, glycosides, reducing sugar, saponins, flavonoids, phlobatanins, steroids, terpenoids, coumarin, emodins, anthocyanin and betacyaninas chemical constituents. The detailed results of all tests for phytochemical screening of each species are summarized in Table 2.

Table 2. Phytochemical constituents of seaweed ethanolic extracts.

Phytochemical Constituent	Seaweed Ethanolic Extract		
	GS1	GS2	GS3
Alkaloids	-	-	-
Tannins	-	-	-
Anthraquinones	-	-	-
Glycosides	-	-	-
Reducing Sugar	-	-	-
Saponins	-	-	-
Flavonoids	-	-	-
Phlobatanins	-	-	-
Steroids	-	-	-
Terpenoids	+	+	+
Coumarin	+	+	+
Emodins	-	-	-
Anthocyanin	-	-	-
Betacyanin	+	+	+

Note: GS – green seaweed; 1 – *Halimeda sp.*; 2 – *Caulerpa sp1.*; 3 – *Caulerpa sp2.*

Terpenoids, coumarin and betacyanin are common in all seaweed species studied. Terpenoids were reported to be present in *Caulerpa sp.* [30]. Coumarin have been characterized in green seaweeds by Perez-Rodriguez et al. [31] as reported by Stengel et al. [32]. Rajasekar et al. [33] reported that betacyanin was found to be present in green seaweeds. Other studies [34, 35, 36] found that *C. racemosa* possess alkaloids, flavonoids, glycosides, phenols, saponins and steroids, however, these secondary metabolites were not detected qualitatively in the ethanolic extract of our samples. The differences in results could be attributed to some factors like climatic condition, season, species, subspecies, harvest and the method used for extraction of compounds which greatly affects the chemical compositions of the extract [37].

Phytochemical screening of the generated seaweed wines was also conducted after fermentation. Results are presented in Tables 3. In general, the phytochemicals present in seaweeds are not retained in wines except for flavonoids, terpenoids, coumarin and betacyanin. Some phytochemicals were lost during the fermentation process and some were enhanced. The detection of chemical constituent not present in the ethanolic extract of the seaweed itself could be attributed to sugar used or to the fermentation process itself. Rathi [38] reported that during fermentation, bioactive compounds are released into wine (aqueous ethanolic solution) making the polyphenols and bioactive components exposed, thereby increasing their bioavailability.

Table 3. Phytochemical constituents of green seaweeds wines.

Phytochemical Constituent	Seaweed Wine								
	GS1-R	GS1-W	GS1-M	GS2-R	GS2-W	GS2-M	GS3-R	GS3-W	GS3-M
Alkaloids	-	-	-	-	-	-	-	-	-
Tannins	-	-	-	-	-	-	-	-	-
Anthraquinones	-	-	-	-	-	-	-	-	-
Glycosides	-	-	-	-	-	-	-	-	-
Reducing Sugar	-	-	-	-	-	-	-	-	-
Saponins	-	-	-	-	-	+	+	-	-
Flavonoids	+	+	+	+	+	+	+	+	+
Phlobatanins	-	-	-	-	-	-	-	-	-
Steroids	-	-	-	-	-	-	-	-	-
Terpenoids	+	+	+	+	+	+	+	+	+
Coumarin	+	+	+	+	+	+	-	+	-
Emodins	-	-	-	-	-	-	-	-	-
Anthocyanin	-	-	-	-	-	-	-	-	-
Betacyanin	+	+	+	+	+	+	+	+	+

Note: GS – green seaweed; 1 – *Halimeda sp.*; 2 – *Caulerpa sp1.*; 3 – *Caulerpa sp2.*
R – Refined sugar; W – Washed sugar; M – Muscovado sugar
- Not present; + Present

The phytochemical constituent flavonoids which are not detected in the ethanolic extracts of green seaweed samples were found present in the fermented solutions. Terpenoids, as well as the coumarins and betacyanins were retained in wines (Table 3) regardless of the sugar solution used.

The study of phytochemicals present in wines are essential. Phytochemicals have antioxidant or hormone-like effect which helps to fight against diseases like cancer, heart disease, diabetes, high blood pressure and preventing the formation of carcinogens on their target tissues [39].

3.4 Total Phenolic Content

The total phenolic content (TPC) of wines produced were determined quantitatively and the results are shown in Figure 1. TPC is expressed as milligram Gallic acid equivalent per liter (mg GAE/L). Phenolic compounds play an important role in several sensory properties of wine such as color, flavor, astringency and hardness [19].

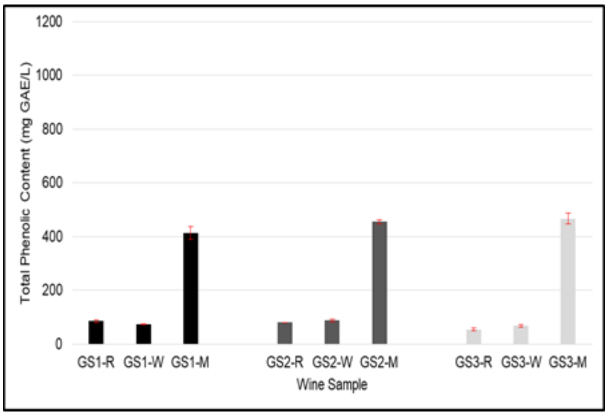

Figure 1. Total phenolic content of wines generated from fermentation of three (3) green seaweed species. Results are expressed as mean of three (3) replicates ± standard deviation which is indicated by an error bar.

The TPC value of the green seaweed wines is 54 – 467 mg GAE/L. In general, seaweed wines fermented using muscovado sugar have significantly higher TPC than wines produced from the two other sugar types.

The reported TPC values for Cabernet Sauvignon wines varied from 1130 to 2710 mg GAE/L and from 860 to 1657 mg GAE/L for the Merlot wines [40]. These values are higher compared to the TPC values determined for green seaweed wines (413 to 467 mg GAE/L) produced using muscovado sugar (considered as red wine). For white wines, the reported TPC values of grape white wines varied from 238 – 420 mg GAE/L [41]. The TPC values determined for green seaweed wines fermented with refined and washed sugars (considered as white wine) ranged from 54 – 87 mg GAE/L and are lower compared to the values reported for commercial grape white wines.

3.5 Total Flavonoid Content

The total flavonoid content (TFC) of each seaweed wine produced was determined. Total flavonoid concentration is expressed as milligram catechin equivalent per liter (mg CE/L). TFC values of green seaweed wines ranged from 7 mg CE/L to 356 mg CE/L (Figure 2) with wines fermented using muscovado sugar having the highest TFC values (294 – 356 mg CE/L). One green seaweed wine (GS1 – *Halimeda macroloba*) fermented using washed sugar has TFC value comparable to wines fermented using muscovado sugar (311 mg CE/L). The rest of the green seaweed wines fermented using refined and washed sugars have TFC values <100 mg CE/L.

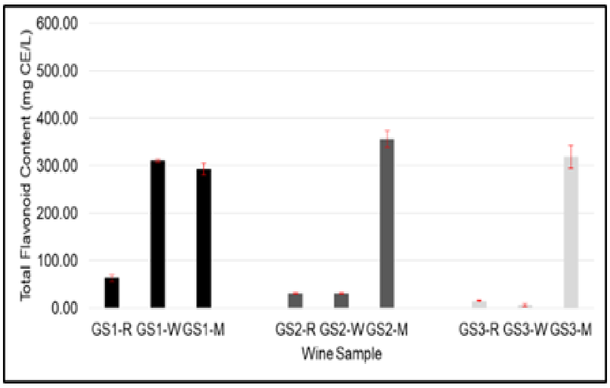

Figure 2. Total flavonoid content of wines generated from fermentation of three (3) green seaweed species. Results are expressed as mean of three (3) replicates ± standard deviation which is indicated by an error bar.

In general, green seaweed wines fermented with muscovado sugar (considered as red wine) have the highest TFC values (311 – 356 mg CE/L). The determined values are less compared to the TFC values reported [40] for grape wine Cabernet Sauvignon wines (860 – 2290 mg CE/L) and Merlot wines (661 – 1,375 mg CE/L). The TFC values of seaweed wines fermented using refined and washed sugars (considered as white wine) varied from 7 – 74.7 mg CE/L (n = 16) except for one wine sample (GS1-W) having TFC values of 311 mg CE/L. The reported TFC values for 10 Serbian white wines [41] varied from 45.30 – 81.32 mg CE/L.

3.6 Total Antioxidant Activity

The total antioxidant activity (TAA) of each seaweed wine produced was determined. TAA is expressed as milligram ascorbic acid equivalent per liter (mg AAE/L). The results are presented in Figure 3.

The total antioxidant activity (TAA) values of wines generated from green seaweed wines have TAA values of 97 – 558 mg AAE/L. The kind of sugar used during fermentation has also an effect in the antioxidant activity of the produced wines (Table 4). There exists a direct relationship between TAA and TPC. According to Morina and Kongoli [19], wines rich in phenolic compounds were found to have powerful antioxidant activity. Antioxidants play a crucial role in the prevention of many diseases such as cancer, inhibiting tumor initiation and heart diseases.

Previous study [42] showed that a high total antioxidant capacity (TAC) diet could reduce the risk of hypertension incidents among women, suggesting that promoting a diet naturally rich in antioxidants might help prevent the development of hypertension. Villaverde et al. [42] reported that the maximal effect of TAC for hypertension could be associated with a TAC intake around 5.0 mmol/day. Thus, drinking a glass of green seaweed wine (558 mg AAE/L; 300 mL) could contribute 19 % of the daily TAC intake. Furthermore, several studies showed that a diet with a high antioxidant capacity will exert its protective effects against type 2 diabetes [43], non-alcoholic fatty liver disease (NAFLD) in adults [44] and increase the antioxidant capacity of serum [45].

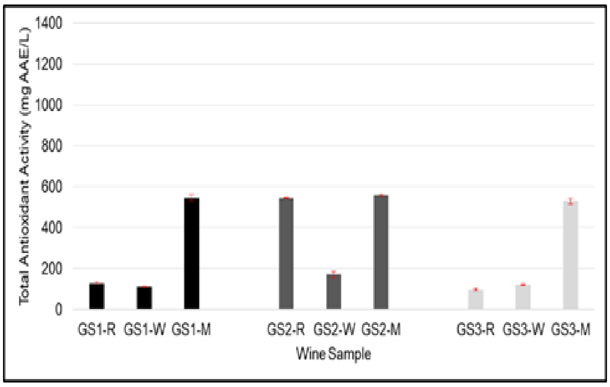

Figure 3. Total antioxidant activity of wines generated from fermentation of three (3) green seaweed species. Results are expressed as mean of three (3) replicates ± standard deviation which is indicated by an error bar.

In general, seaweed wines produced using muscovado sugar during fermentation have higher TAA values. In this case, the type of seaweed used as a material in wine making is a significant factor to the TAA of the wine.

Table 4. Range of total antioxidant activity determined in seaweed wines produced using different kinds of sugar during fermentation.

Kind of Sugar	Total Antioxidant Activity (mg AAE/L)
	GS Wines
Refined (R)	97 – 544
Washed (W)	110 – 171
Muscovado (M)	528 – 558

3.7 Sensory Evaluation

The generated seaweed wines were subjected to sensory evaluation using a 5-point rating scale. Scores of 4.3 – 5.0 was interpreted as "Liked Extremely", 3.5 – 4.2 as "Liked Very Much", 2.7 – 3.4 as "Liked Moderately", 1.9 – 2.6 as "Disliked Very Much" and 1.0 – 1.8 as "Disliked Extremely". The produced wines were evaluated based on color, aroma, flavor and general acceptability. Green seaweed wines fermented using muscovado sugar are liked very much while the wines fermented using washed and refined sugars are liked moderately (Table 5).

Table 5. General acceptability scores of seaweed wines produced.

Wine	Characteristics				Remark
	Color	Aroma	Flavor	General Acceptability	
GS1-R	3.7	3.3	3.3	3.5	Liked Very Much
GS1-W	3.6	3.4	2.9	3.3	Liked Moderately
GS1-M	4.0	3.7	3.3	3.9	Liked Very Much
GS2-R	3.4	3.4	3.0	3.2	Liked Moderately
GS2-W	3.4	3.4	2.9	3.3	Liked Moderately
GS2-M	3.9	3.8	3.5	3.8	Liked Very Much
GS3-R	3.7	3.5	2.9	3.3	Liked Moderately
GS3-W	3.7	3.7	3.3	3.5	Liked Very Much
GS3-M	4.1	4.0	3.5	3.9	Liked Very Much

4. CONCLUSION AND RECOMMENDATIONS

Seaweed wines fermented using muscovado sugar have the highest total phenolic content, total flavonoid content, total antioxidant activity and liked very much. The wines produced from the studied green seaweed species have the potential to be used as functional food for the prevention of degenerative diseases caused by oxidative stress, such as hypertension, type 2 diabetes, non-alcoholic fatty liver disease (NAFLD). Also, high dietary total antioxidant capacity increases the antioxidant capacity of serum indicating absorption of the antioxidants and an improved in vivo antioxidant defense status. Assessment of the potential health benefits and safety of wines produced from green seaweeds is recommended.

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