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Estimation of chlorophyll content in local *Caulerpa* seaweeds using acetone, DMSO and Methanol

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Abstract

Being photosynthetic organisms, seaweeds are comprised of different pigments which give rise to their coloration and thus classification into either being brown (Phaeophyceae), green (Chlorophyceae), or red (Rhodophyceae) seaweeds. Over the years, research regarding macroalgal pigments has grown due to the biological activities of these pigments. Thus, several methods have been developed for the estimation of macroalgal pigments. This study aims to conduct an estimation of pigment specifically the chlorophyll content from local Caulerpa seaweeds using acetone, DMSO, and methanol as solvents for extraction, to compare the amount of pigments present in Caulerpa seaweeds, as well as compare the extraction efficiency of the solvents. Fresh seaweeds were purchased from local markets in the Philippines during October 2016. The pigments were extracted using the three solvents and extracts were analyzed using UV-VIS spectrophotometry at wavelength maxima of the pigments. Estimation of chlorophyll content was made using the methods of Porra (1989), and Barnes et al. (1991). Comparisons were made between the chlorophyll content obtained from the different seaweed species with regard to the solvent of extraction. Results of the study indicate variations in chlorophyll content of the different Caulerpa seaweeds caused by the solvents used for extraction.

Keywords: Estimation, chlorophyll, macroalgae, *Caulerpa.*

Introduction

Marinemacrobenthic algae or seaweeds are photosynthetic in nature, with pigments making up 50% of their major productivity. These pigments have light-harvesting and photoprotective properties and are vital in the classification and biodiversity studies that make them valuable sources of food produce and industrial materials. The pigments usually found in seaweeds are chlorophylls, carotenoids, and phycobiliproteins. Based on these dominant pigments are the classification into green algae (Chlorophyta), brown algae (Phaeophyta), and red algae (Rhodophyta)[1]. Natural pigments found in seaweeds have also received attention due to findings that seaweeds exhibit beneficial biological activities such as anticancer, antioxidant, anti-inflammatory, anti-obesity, and neuroprotective activities[2]. Because of the many biological activities attributed to pigments from macroalgae, researchers have taken interest in the estimation of pigments through spectrophotometric analysis. Among the solvents used in extracting pigments are 80% acetone, DMSO, and methanol.

Seaweeds are known for their different commercial values, ranging from vitamin sources to raw materials for production. However, with their uses in the field of natural products, the totality of seaweeds in terms of biological components is maximized and to the best of the researchers' knowledge, the pigments are discarded since researchers are more interested with other properties like antioxidant properties and lipid content. The results of this research may contribute as basis for similar researches in the country involving pigment estimation as well as in giving importance to the different applications of pigments.

Chlorophylls are photosynthetic pigments that are hydrophobic in nature and are found in the chloroplasts of cells. These pigments are responsible for giving the distinct green color that we observe in seaweeds. The green color attributed to chlorophyll is usually the unabsorbed radiation that is reflected from the absorbed visible light in the wavelength range 400-500 nm (blue) and 600-700nm (red).

In addition, chlorophylls contain a porphyrin ring around which electrons can transfer freely, giving the ring the ability to gain or lose electrons easily and thus providing energized electrons to other molecules present. This process allows chlorophyll to capture energy from the sun[3].

There are different types of chlorophyll depending on the chemical substituents; chlorophyll *a* and b differ only in one substituent, with a methyl substituent for chlorphyll *a* whereas chlorophyll *b* has an aldehyde group. The presence of chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) gives macroalgae and other higher plants their green color. Chlorophyll extraction is often accomplished with the use of generally non-polar solvents with few polar groups like acetone[4].

This study aims to conduct an estimation of pigment specifically the chlorophyll content from local Caulerpa seaweeds using acetone, DMSO, and methanol as solvents for extraction, to compare the amount of pigments present in Caulerpa seaweeds, as well as compare the extraction efficiency of the solvents.

Materials and methods

Collection of seaweed samples: *C. lentillifera* from Region IV-B (composing of provinces Mindoro, Marinduque, Romblon, and Palawan - MIMAROPA) and Region VII (Cebu) and *C. racemosa* from Region IV-B (MIMAROPA) were bought at Pasig City Mega Market and Trabajo Market during October 2016. The seaweeds were immediately refrigerated upon purchase. The samples were also brought to the National Museum of the Philippines for authentication.

Sample preparation: The seaweed samples were thoroughly washed with tap water and contaminants such as small pieces of plastic and seashells were hand-picked and removed. The samples were rinsed with distilled water for final washing. Samples were then air-dried, packed in ziplock bags, and placed in the freezer before freeze-drying. After freeze-drying, the samples were cut into small pieces and macerated with the use of mortar and pestle to reduce to small pieces.

Pigment extraction: The pigments from the prepared samples were extracted according to the specific methods per solvent based on the modified methods of Sumanta et al.[5] and Hiscox and Israelstam[6].

Extraction with 80% Acetone and Methanol[5]: Freeze-dried local seaweed was collected and ground with the use of mortar and pestle and was further cut into smaller pieces. 50mg of the sample was weighed and subsequently transferred into a 15-mL centrifuge tube and 5mL of 80% acetone was added to the sample. The homogenate mixture was centrifuged at 4°C and 3000rpm for 15 minutes. The supernatant was collected, and the pellet was re-extracted with 5mL of 80% acetone and subject to centrifugation with the same initial parameters. The resulting extracts were pooled for pigment quantification. 0.5mL from the pooled extracts was diluted with 4.5mL of the solvent. Extraction for each seaweed sample per batch was performed in triplicate and was analyzed using the Hitachi U-2900. The same procedure was followed for methanol extraction.

Extraction with DMSO[6]: Freeze-dried local seaweed was collected and ground with the use of mortar and pestle and was further cut into smaller pieces. 50mg of the sample was weighed in test tubes and 2mL of DMSO was added to the samples. The test tubes were incubated at 60°C for 20 minutes in a water bath. The supernatant was decanted and another 3mL of DMSO was added to the residue and incubated at 60°C for 20 minutes. No degradation was observed during incubation. The resulting extracts were then pooled. From the pooled extracts, 0.5mL was diluted with 4.5mL of the solvent. This was performed in triplicate and was analyzed using the Hitachi U-2900 UV-V is spectrophotometer.

Estimation of Chlorophyll: Chlorophyll estimation was performed based on the methods of Porra[5] and Barnes et al.[6]. The chlorophyll *a*, chlorophyll *b*, and total chlorophyll (chl *a*+*b*) content were estimated. Absorbance of each extract was measured using the spectrophotometer at respective wavelengths required by each method. The simultaneous equations provided by Porra[7] were used for the measurement of chlorophylls extracted with acetone and methanol while the equations of Barnes et al.[8] were used for the DMSO extracts (Table-1). Table-1 shows equations (1) to (9) for chlorophyll measurements.

Table-1: Simultaneous equations of Porra[7] and Barnes et al.[8] for the determination of chlorophyll concentrations.

Porra's equations for Chl concentrations (µg/mL)		
	In acetone	In methanol
[Chl *a*]	$12.25A^{663.6} - 2.55\,A^{646.6}$	$16.29\,A^{665.2} - 8.54\,A^{652.0}$
[Chl *b*]	$20.31A^{646.6} - 4.91\,A^{663.6}$	$30.66A^{652.0} - 13.58\,A^{665.2}$
[Chl *a*+*b*]	$17.76\,A^{646.6} + 7.34\,A^{663.6}$	$22.12A^{652.0} + 2.71\,A^{665.2}$
Equations of Barnes et al. for Chl concentrations (µg/mL)		
	In DMSO	
[Chl *a*]	$14.85A^{664.9} - 5.14\,A^{648.2}$	
[Chl *b*]	$25.48\,A^{648.2} - 7.36\,A^{664.9}$	
[Chl *a*+*b*]	$7.49\,A^{664.9} + 20.34\,A^{648.2}$	

Statistical analyses: All measurements were performed in triplicate and the values for arithmetic mean and standard deviation were computed. Two-way Analysis of Variance (Two-way ANOVA) followed by Tukey's multiple comparisons test was applied for the chlorophyll extracts of each species to determine if there are significant differences in chlorophyll concentration with respect to the solvent used for extraction and with respect to. All statistical analyses were performed with a p-value of 0.05 using Prism version 6.0 for Mac OS X.

Results and discussion

The discussion of results as follows is done per chlorophyll pigment, with comparisons between the species as well as comparisons between the solvents.

For ease of writing, each seaweed sample has been assigned an acronym as follows: LC for *C. lentillifera* (Cebu), LM for *C. lentillifera* (MIMAROPA), and RM for *C. racemosa*.

The efficiency of the solvents used was determined on the basis of pigment content attained for a certain seaweed sample. However, it must be kept in mind that the efficiency may be linked to the method with which extraction was done. The efficiency of methanol and acetone can be compared directly in this study since the same method of extraction[3] was used. However, a different method was used[4] with DMSO. As such, if the pigment content in a DMSO extract was found to be higher than that of either the acetone or methanol extract, the comparison of efficiency is assumed to be in relation to the extraction method used with the solvent.

Chlorophyll *a* content: Among the solvents, extraction with DMSO yielded the highest Chl a content (5.8539±0.3580), specifically for C. racemosa (Figure-1). On the other hand, LC (4.0705±0.1897) and LM (5.7626±0.7285) seaweeds had the highest Chl a content with the use of acetone compared to their DMSO and methanol counterparts. The methanol extracts showed the lowest amount of Chl a for all seaweeds with chl *a* values of 2.2422±0.2905 for LC 2.4927±0.0525 for LM, and 3.0151±0.4273 for RM.

Two-way ANOVA revealed that the effect of the solvent used on the total variation of the chl *a* contents was 59.68% ($p<0.0001$) while the effect of the species on the total variation was 14.28% ($p=0.0019$). The interaction of both the solvent and species factors accounted for 11.81% of the total variation ($p=0.0221$).

Tukey's test showed that among the DMSO extracts, only LC and RM had significant differences with each other with an adjusted p value of 0.0012 (<0.05). For the acetone extracts, only LC and LM had significant differences (adjusted p value of 0.0095). None of the methanol extracts showed significant differences with each other in terms of chl *a* content.

Comparison of the solvents using Tukey's test also showed significant variations in terms of chl *a* content. Among the LC samples, statistical differences were seen in DMSO vs. methanol ($p=0.0268$) and acetone vs. methanol ($p=0.0053$). For the LM samples, there were also significant differences in DMSO vs. methanol ($p=0.0007$) and acetone vs. methanol ($p< 0.0001$). All RM samples were significantly different from one another with all adjusted p values being less than 0.05 for comparing the three solvents with one another.

Chlorophyll *b* content: In each of the solvents, the Chl b conent of each sample was found to be lower than that of Chl a. The highest Chl b content for each seaweed species were found in methanol extracts, with chl b values of 4.4809±1.2882 for LC, 4.8358±0.1618 for LM, and 3.8465±0.7027 for RM. The Chl b values seem to be lowest in DMSO (LC: 2.4053±0.4419; LM: 2.7558±0.2353; RM: 2.9159) and of average value in 80% acetone (LC: 3.3248±0.1221; LM: 3.9524±0.4741; RM: 3.4289±1.1454).

Analysis of the data using 2-way ANOVA showed that only the solvent factor, with a p value of 0.0002, had a significant effect on the total variation of the chl *b* content. Both the effect of the species and its interaction with the effect of the solvent on the chl *b* content had no significant effect on the total variation.

Using Tukey's multiple comparisons test, it was determined that among the DMSO extracts, none of the *Caulerpa* seaweeds had significant differences in terms of chl *b* content. The same results were found for both acetone and methanol extracts. Again, using Tukey's test, it was found that among the LC samples, only DMSO and methanol varied with each other significantly ($p=0.0035$). Likewise, only DMSO and methanol varied significantly in terms of chl *b* content in LM samples with $p=0.0035$. None of the RM samples had significant differences with one another.

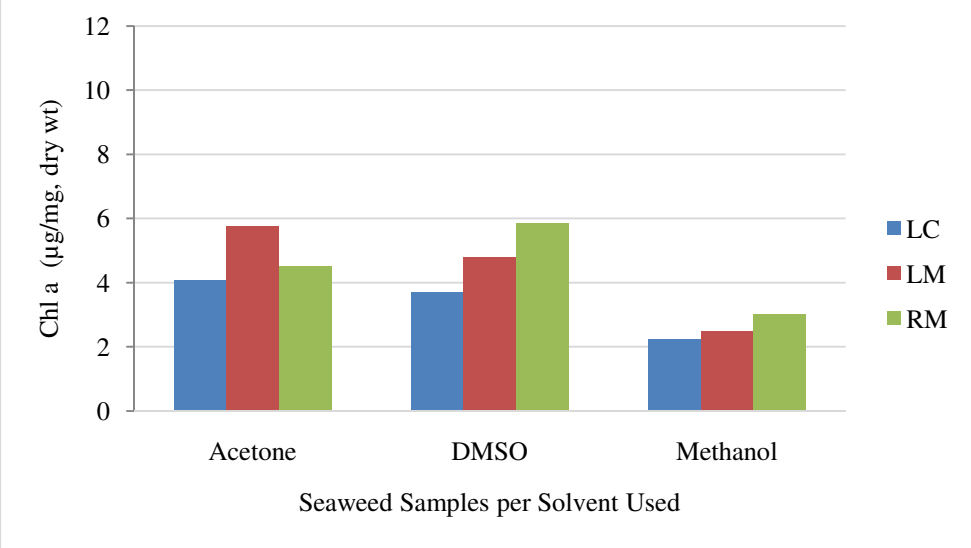

Figure-1: Comparison of Chl *a* content.

Chlorophyll *a+b* content: The total Chl content (a+b) showed variability among the three solvents. The highest total chlorophyll content was obtained from the 80% acetone extract of LM (9.7150±1.2025) while the lowest was seen in LC extracted with DMSO (6.0918±0.8633).

Two-way ANOVA showed that there is no significant effect of the solvent (p=0.0804) nor of the species (p=0.0526) on the total variation of the chl *a+b* content.

In comparing the effect of species on the chl *a+b* content, Tukey's test showed that only the DMSO extracts of LC and RM showed significant differences with each other (p=0.0390).

Tukey's test also showed that the effect of the solvent used had no significant effect on the chl *a+b* content of any of the samples.

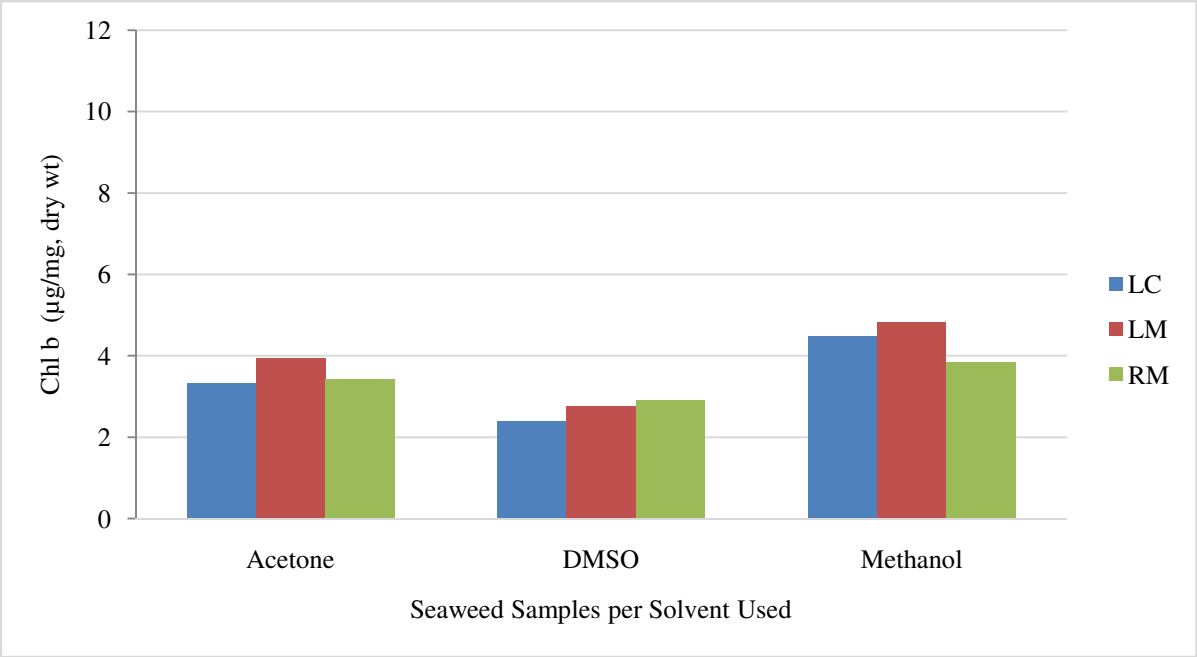

Figure-2: Comparison of Chl *b* content.

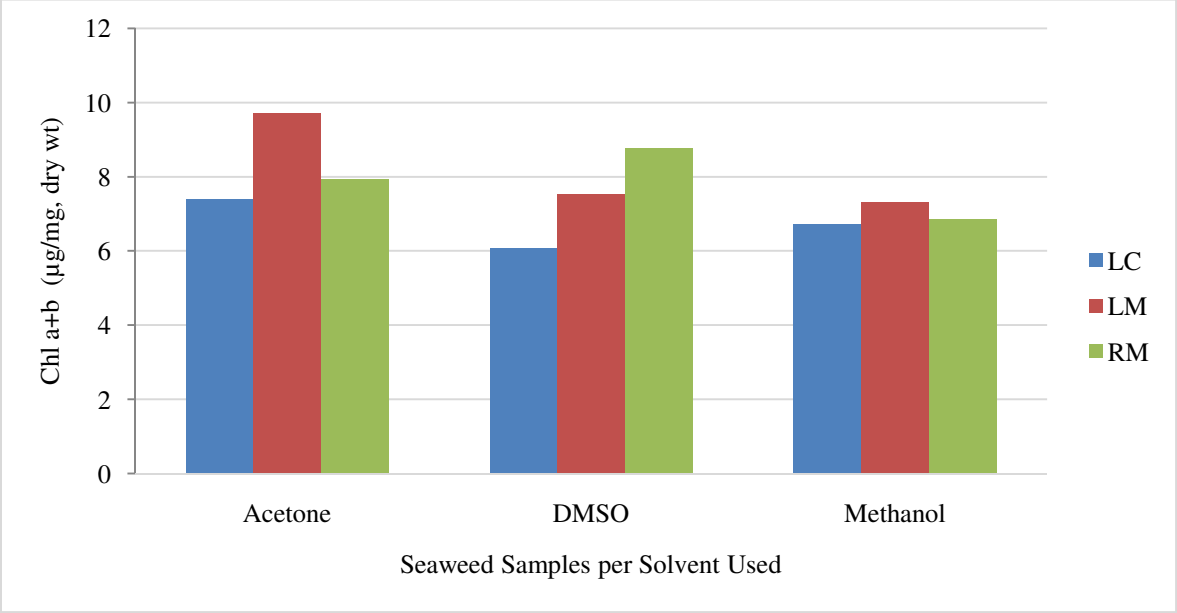

Figure-3: Comparison of Chl *a+b* content.

Conclusion

The study has provided estimations of the chlorophyll content of *C. lentillifera* (Cebu), *C. lentillifera* (MIMAROPA), and *C. racemosa*, harvested during October 2016, using 80% acetone, DMSO, and methanol as the solvents for extraction. Among the samples estimated, the chlorophyll *a* levels were higher than those of chlorophyll b. Furthermore, based on the results and data gathered and the subsequent statistical analyses performed, the greatest significant differences were observed in comparing the solvent effects on the chlorophyll *a* content of the samples. DMSO was most effective in extracting the highest amount of chl *a*, closely followed by acetone. Minimal variances were seen in the solvent effects on the chl *b* content, with only the solvent used having a significant effect on the total variation. Lastly, no significant variations among solvents were observed in the total chlorophyll content, suggesting that all three solvents were suitable for use in estimating the total chlorophyll content. Minimal instances of variation were also observed in comparing the different *Caulerpa* seaweeds, suggesting that the seaweeds had comparable chlorophyll content and that the location and species of the seaweeds had minimal effect on the chlorophyll content.

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