Sterilization Protocol for the Sri Lankan Green Algae *Caulerpa racemosa* and Its Antioxidant Activity

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ABSTRACT

***Caulerpa racemosa*, which belongs to the family Caulerpaceae, is a rich source of bioactive secondary metabolites and functional ingredients. An investigation was carried out to evaluate the antioxidant activity of different solvent soluble fractions derived from 80% methanol extract of the Sri Lankan *Caulerpa racemosa*. For the evaluation, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl and alkyl radical scavenging potential was measured compared to the standard antioxidant ascorbic acid. The overall best fraction was the ethyl acetate fraction. The hexane, chloroform and aqueous fractions also reported the highest hydroxyl radical scavenging activity. Another experiment was conducted to develop an efficient sterilization protocol to obtain sterile callus and shoot culture of *Caulerpa racemosa*. Healthy and matured apical rhizome explants were selected, cleaned well and kept under continuous stream of running tap water for 15 min. Samples were subjected to sterilization using 0.5% sodium hypochlorite (10% commercial Clorox) for 1 and 5 min, 1% iodine (1% Betadine) for 1 and 5 min, antibiotic mixture (15 mg/L erythromycin and 30 mg/L penicillin) for 30 sec, 30 min and 24 h and fungicide (Captan 50%) for 3 min and cultured in both liquid and solid media. Results of liquid media showed that, exposure to antibiotic mixture for 30 min and 24 h without a final rinse with sterilized sea water, were better and significantly reduced the contamination of explants. Amongst the two, 30 min was better. The appropriate amount of agar to be used in solid media at pH 7.9 was 11 g/L.**

KEYWORDS: Antioxident activity, *Caulerpa racemosa*, *In vitro*, Rhizome, Surface sterilization

INTRODUCTION

Sri Lankan coastal waters are richly endowed with marine flora especially seaweeds, which have been identified by Boergesen (1936). Seaweeds are ecologically and economically significant living sources. Bioactivities of the Sri Lankan seaweeds have been studied briefly and only a few reports are available in the literature.

Caulerpa racemosa is siphonous green algae, belonging to genus Caulerpa, which includes about 85 species of tropical to subtropical green algae (Guiry and Guiry, 2007). It is commonly known as sea grapes (Madlener, 1977) and thrives in shallow marine habitats of Sri Lanka. A plant of *C. racemosa* consists of fronds with small densely packed grape-like clusters attached to long branching horizontal rhizomes. These rhizomes are anchored to the substrate by well developed, fine hair-like extensions known as rhizoids that play no role in absorbing mineral nutrients. Similar to other algae *C. racemosa* too reproduces through both sexual and vegetative methods.

Although *C. racemosa* is an invasive species in Mediterranean Sea (Verlaque *et al.*, 2003), *Caulerpa* is a common genus which is native to Sri Lanka (Jayasuriya, 1992). Though there are 340 species of seaweed growing in the coastal water of Sri Lanka, nearly 20 species are of commercial interest. Out of these seaweeds only two species of *Gracilaria* (Ceylon moss) are of commercial importance (Jayasuriya, 1992). In Sri Lankan perspective, people are unaware of the potential uses of *C. racemosa*.

It is noteworthy that many studies have discovered the agricultural and pharmaceutical potential of this seaweed. It is rich in chemical contents (protein, lipid, carbohydrate, fiber and ash), most of the essential amino acids, vitamins, macro (N, P, K, Na, Mg, Ca) and micro (I, Fe, Mn, Co, Zn, Cu, Mo, Bo, Pb, Cd, Sr) nutrients (Hong *et al.*, 2007). It is a widely used sea vegetable species in Pacific island communities (Ostraff, 2006). Xavier and Jesudass (2007) observed 100% seed germination on cluster bean in lower concentrations of *C. racemosa* liquid fertilizer. To date, few studies have been reported that *C. racemosa* has anticancer activity (Samarakoon *et al.*, 2014a), anti-inflammatory activity (Souto *et al.*, 2011), antibacterial activity against human pathogens such as *Vibrio cholera*, *Escherichia coli* (Radhika *et al.*, 2012), *Staphylococcus aureus* and *Proteus mirabilis* (Kathiraven *et al.*, 2015). Moreover, the derivatives caulerpin and caulerpinic acid exhibit different levels of larvicidal (mosquitocidal) activity (Yu *et al.*, 2014).

These derivatives may have the same significant lethality against most of the agricultural pests which share similar physiological aspect of mosquitoes. Samarakoon *et al.* (2014a) indicates that the crude methanolic extracts of *C. racemosa* showed significantly higher radical scavenging activity than other tested seaweeds against 2, 2-diphenyl-1-picrylhydrazyl (DPPH), alkyl and hydroxyl radicals. A part of present study also validates the fact that the antioxidant potential of *C. racemosa* is determined through different fractions derived from methanolic extract and reinforces the significance of tissue culturing of *C. racemosa.*

In recent years, the demand for *C. racemosa* is mounting due to the commercial importance of its secondary metabolites. This pioneering study of tissue culturing of *C. racemosa* which can prevent harvesting from wild could provide a continuous, reliable source of natural stock.

Tissue culture techniques for macro algal studies were started 40 years ago and in 1978 Chen and Taylor succeeded in tissue culturing of the red alga *Chorzdrus crispus*. The most critical step in tissue culturing is sterilization of explant for aseptic culture establishment. Seaweed surfaces are densely covered by a wide range of bacteria (Sieburth and Tootle, 1981), and their removal is more difficult than in higher plants (Polne-Fuller, 1988) due to their rapid proliferation characteristics (Enjalric *et al.*, 1998).

Hence, the objective of this study was to evaluate the antioxidant activity of different fractions of Sri Lankan *C. racemosa* derived from 80% methanol and develop a simple and least expensive method of sterilizing explants, using different types of sterilizing agents and varying their duration of exposure.

MATERIALS AND METHODS

Study Area

The study was carried out from January to May 2016 at the Industrial Technology Institute (ITI), Colombo - 07.

Seaweed Collection

Fresh marine *C. racemosa* were collected from Moragalla beach (6°26'44.5"N 79°59'05.5"E), Beruwela, Sri Lanka. Natural sea water was used for the transportation. A mother plant stock of *C. racemosa* was maintained in a tank at ITI.

Antioxidant Activities

Free radical scavenging capacity of organic solvent extractions obtained from *C. racemosa* was measured using electron spin resonance (ESR) spectrometer following a method described by Samarakoon *et al.* (2014b).

Nutrition Medium

Both liquid and solid f/2 media (Guillard and Ryther, 1962; Guillard, 1975) were used without $Na_2SiO_3.9H_2O$ to avoid precipitation. The pH of the media was 7.9. Five mililiters and 20 mL of media were taken for liquid and solid media respectively.

Aseptic Techniques

All glassware, vessels, and tools were disinfected. The process of sterilization and dissection of plant materials were carried out under sterile conditions in a lamina flow cabinet.

Surface Sterilization and Culture of Explant

Samples were cleaned well of epiphytes, debris, salt and sand manually and kept under continuous stream of running tap water for 15 min to remove iota dust. Sterility was maintained during the culture.

The sterilization was carried out with eight treatments and a control (T_1) without any sterilization procedure. They were 0.5% sodium hypochlorite (10% Clorox) for 1 min (T_2) and 5 min (T_3), 1% iodine (1% Betadine) for 1 min (T_4) and 5 min (T_5) antibiotic mixture (15 mg/L erythromycin and 30 mg/L Penicillin) for 30 sec (T_6), 30 min (T_7) and 24 h (T_8) and fungicide (Captan 50%) for 3 min (T_9). Except antibiotic and fungicide, others were then rinsed thoroughly three times with filtered, autoclaved sea water.

Apical rhizome part of *C. racemosa* was used as explant. These segments were trimmed to approximately 2 cm and 4 cm in size for liquid and solid media respectively. Explants were then cultured on these media and labelled properly. For each treatment, ten test tubes or petri dishes were used with one explant in each and the procedure was repeated twice. Liquid media were gently shaken. The growth room conditions were maintained at 23 °C, 60 µmol photon $m^{-2}s^{-1}$ with a photoperiod of 16 h day light and 8 h dark.

Data Recording and Analysis

The observations of sterilization procedure included the number of contaminated, survived, and dead cultures and recordings were carried out regularly. Mainly the changes in colour and appearance of explants and turbidity of the media for 14 days were considered to differentiate the healthy samples from contaminated and dead ones. The incubation period for solid media was one

week. Data were analysed statistically using maximum likelihood analysis of variance in SAS (9.2) statistical analysis package. Data of antioxidant activity were analysed statistically using one-way analysis of variance followed by Duncan's multiple range test (DMRT). Probability values of less than 0.05 were considered as significant.

RESULTS AND DISCUSSION

Samarakoon *et al.* (2014a) have reported that the methanolic extract of *C. racemosa* showed a significant average radical scavenging activity. Therefore, in this study, the antioxidant effects of different solvent soluble fractions derived from the 80% methonolic extracts were assessed, including DPPH, hydroxyl and alkyl radicals scavenging activity compared to the standard antioxidant ascorbic acid. Based on the identified radical scavenging properties, the best activity was reported in ethyl acetate fraction (CRME) among others and the determined IC_{50} values were 0.756±0.066 and 0.0921±0.007 mg/L against DPHH and alkyl radicals, respectively. The hydroxyl radical scavenging activity of CRME fraction also recorded a better IC_{50} value of 0.047±0.004 mg/L. CRMW, CRMC and CRMH fractions were indicated the highest hydroxyl radical scavenging activity with IC_{50} value 0.038±0.004, 0.039±0.0002, and 0.040±0.002 mg/L, respectively (Table 1). The results showed the potential of antioxidant activity of *C. racemosa*, and the necessity for culturing of this seaweed.

Liquid Media

During consecutive experiment trials, it was reported that there is less viability in response among the experimental tissue, if explants are placed together. So, each explant was placed in a separate test tube. The usage of both 10% Clorox for 10 min and 70% ethanol for 30 sec along with Clorox were not appropriate treatments. Consequently, the tender tissues of *C. racemosa* were damaged by these powerful phytotoxic sterilizing agents and the explants immediately lost their green colour due to the bleaching nature of sodium hypochlorite after a long exposure time. The test tube was quarterly filled with media because if the media was filled up to ¾th of the test-tube, then explants would have been submerged and consumed a lesser amount of dissolved O_2 and CO_2.

Results (Table 2) revealed that different treatments had significantly different effects on the contamination of growth media and death of explants. Survival rate was comparatetively lower (Figure 1).

Table 2. Effect of different sterilization methods on liquid culture of *Caulerpa racemosa* explant

Sterilization techniques	Frequency (out of 20 explants)	
	Contaminated	Dead
T_1	18^a	1^a
T_2	4^b	14^c
T_3	3^b	17^c
T_4	15^a	2^a
T_5	12^a	7^a
T_6	9^a	3^a
T_7	7^b	6^a
T_8	5^b	9^b
T_9	9^a	4^a

Control (T_1), Clorox for 1 min (T_2) and 5 min (T3), Betadine for 1 min (T_4) and 5 min (T_5), Antibiotic mixture for 30 sec (T_6), 30 min (T_7) and 24 h (T_8), and Captan for 3 min (T_9). Means followed by the same letter in each column are not significantly different at 0.05 levels.

Effect on contaminated cultures

No significant differences were observed among T_4, T_5, T_6, T_9 and control, where the highest percentage of contamination was recorded, which ranged from 45 to 90%. Treatments T_2, T_3, T_7 and T_8 showed the lowest contamination percentage ranged from 15 to 35%, which did not share any significant difference (Table 2 and Figure 1).

Effect on dead cultures

No significant differences were observed between control, T_4, T_5, T_6, T_7 and T_9, which recorded the lowest death percentage, ranged from 5 to 35%. Forty five percent of explant death was recorded in T_8 which is higher than control and lower than T_2. Highest death percentage ranged from 70 to 85% was recorded in T_2 and T_3 (Table 2 and Figure 1).

Table 1. Radical scavenging activities of different solvent extraction from *Caulerpa racemosa* [a]

Samples	CRM	CRMH	CRMC	CRME	CRMW	AA
IC_{50} values (mg mL^{-1}) [b]						
DPHH	0.915±0.057	>2	1.376±0.043	0.756 ±0.066	1.066 ±0.061	0.0035±0.003
Hydroxyl	0.182±0.002	0.040±0.002	0.039±0.0002	0.047±0.004	0.038±0.004	0.0033±0.005
Alkyl	0.319±0.014	0.326±0.043	0.222±0.0129	0.0921±0.007	0.122±0.048	0.0123±0.006

[a] *The values of IC_{50} were determined by triplicate individual experiments. Values are mean±SD of three determinations.* [b] *The concentration of sample required to scavenge 50% of the radical scavenging activity. Caulerpa racemosa (CR); Methanol extract (CRM); Hexane extract (CRMH); Chloroform extract (CRMC); Ethyl acetate extract (CRME); Aqueous extract (CRMW); Ascorbic acid (AA)*

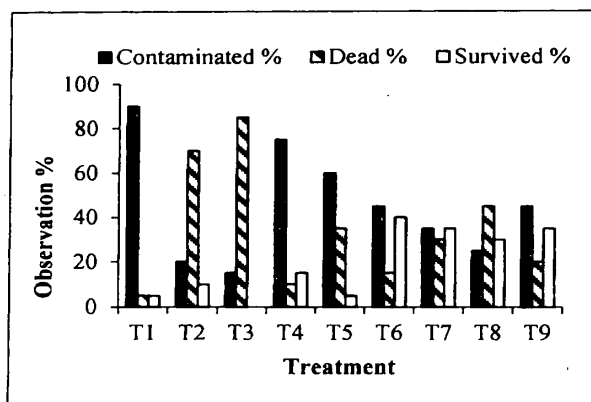

Figure 1. Effect of sterilization on liquid culture *Control (T_1), Clorox for 1 min (T_2) and 5 min (T3), Betadine for 1 min (T_4) and 5 min (T_5), Antibiotic mixture for 30 sec (T_6), 30 min (T_7) and 24 h (T_8), and Captan for 3 min (T_9).*

Surface sterilization with T_2 and T_3 showed lowest contamination but highest level of explant mortality. This could be because of the plant tissue death at high concentration of 0.5% NaOCl as stated by Ervin and Wetzel (2002). Betadine showed lowest mortality but it failed to inhibit the microorganisms from cultured explants. So, it was ineffective as a surface sterilizer as it had low concentration and short exposure time. Explants treated with the fungicide showed tip damage.

Significant differences were observed in antibiotic treatments at different periods of exposure. Treatment T_6 was inefficient because the contamination rate was not significant to the culture without any sterilization protocol. Both T_7 and T_8 showed the least culture contamination and minimum explant death, where T_7 showed the lower values.

Solid Media

Exact amount of agar 11 g/L to be used was found out by examining different amounts of agar 8, 9, 10, 11 and 12 g/L for the same pH level 7.9.

After sterilization (not including T_9) almost all solid media cultures of *C. racemosa* were shown growth of epiphytic bacteria and fungus, except 70% of T_2 and 30% of T_3, which were dead, indicating an unsuccessful surface sterilization. It agreed with the fact that calli of Chlorophyta developed regularly from isolated cells or protoplasts cultured on agar medium, but rarely from mature sections of intact tissue (Gusev *et al.*, 1987). It is indeed a great challenge to produce sterile *in vitro* explants using marine seaweed as a direct source. Because a failure in preventing pathogen had led to the extinction of the desired algal species in culture through competition and nullified the experimental work.

Size of the explant and endogenous microorganisms, which is difficult to eradicate, might be the main cause for these results because the petri dishes kept without explants were observed uncontaminated. Secondly, the microorganisms existing were favoured by the solid medium containing sugar. So, the total population of them in the medium can quickly increase during the culture. Under such conditions, sugar and other nutrients in the medium were consumed mostly by microorganisms, and partly by the plants *in vitro* and then the microorganisms may produce some toxic substances in the medium. Finally, the microorganisms may start growing quickly within cultures, competing with the cultured plants, resulting in the death or degradation of plants *in vitro* (Aitken-Christie *et al.*, 2013). Therefore, using medullary tissue culture method without sugar would be a better solution. Future researches can use this information, as a foundation to their studies in order to develop a complete tissue culture method for callus and shoot development of *Caulerpa racemosa.*

CONCLUSIONS

Different organic solvent extracts of *C. racemosa* showed the potentiality of free radical scavenging activities in ESR method. Reduction of bacterial contamination and mortality were best accomplished by stirring the explants of *C. racemosa* for 30 min in the antibiotic solution and culture directly without a last rinse with sterile seawater. However, the results revealed that treatment T_7 and T_8 have performed better than other treatments.

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