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Comparative Study of Agarophytes - *Gracilaria edulis* and *Gelidiella acerosa* as Biostimulant and Application of Agar for Water-holding in Soil and Plant Growth Promotion

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ABSTRACT

Background: Seaweeds and its derivatives are extensively used as biostimulants in horticulture and agriculture as a replacement for chemical fertilizers. *G. edulis* and *G. acerosa* are easily cultivable and economically important seaweeds. They are a rich source of phytohormones, amino acids, antibiotics, vitamins, micro, macro elements and agar. Such natural products have great demand and been commercialized these days to promote sustainable agriculture. Dried and finely powdered algal biomass is used directly as a biostimulant. Algal polysaccharides such as agar can be an innovative alternative to synthetic polymers used in horticulture as they contain active biostimulant compounds and also reported to hold water in the soil that aids plant growth with minimum water consumption than usually required. *A. aritis* being one of the most consumed leafy vegetables throughout the world can be harvested indoors with added nutrients and minimal water utilization.

Methods: The field trial is a comparative evaluation of the two selected species of agarophytes for promoting plant (*A. aritis*) growth and the extracted agar tested for germination tests, bio-stimulatory property under water stress. Growth parameters were recorded after three weeks. The agarophytes were also qualitatively screened for phytochemicals and WD-XRF analysis.

Result: The present work will be a supplementary contribution for assessing agarophytes with biostimulant properties and the characteristic agar gels that expand plant tolerance to abiotic stresses, thus constituting an alternative to synthetic plant protection products.

Key words: Agar, Biostimulant, *G. acerosa, G. edulis*, Water holding capacity.

INTRODUCTION

Seaweeds are eukaryotic organisms thriving in marine environment and are called as macro algae. Marine algae are mass producers of biologically active natural products that have been used as food, fodder, biofertilizer, medicine and valuable raw materials in industries for the production of agar, alginate, carrageenan (Kolanjinathan *et al.,* 2014).

Global demand for food production has led to the increased application of chemical fertilizers for enhancing crop production but the toxic chemicals can cause health problems if consumed (Hansra, 1993). This scenario sets a challenge to develop eco-friendly innovative methods that increase agricultural yields, with minimum input (Tilman *et al.,* 2002; Foley *et al.,* 2011) by adopting sustainable production practices that could reduce or substitute the use of chemical inputs like chemical fertilizers and pesticides with natural or biological substances. The development of natural substances promoting plant growth called plant biostimulants and water-holding polymer gels has received increased attention (Hicham, 2016; Salim, 2019).

A biostimulant is an organic material, when used in small quantities can enhance the plant growth and development such that the response cannot be assigned to the application of traditional plant nutrients. The use of macro algae as agricultural biostimulants on crop plants has several benefits such as enhanced rooting, higher crop and fruit yields, freezing, drought and salt tolerance, enhanced photosynthetic activity and resistance to microbial pathogens (Sharma *et al.,* 2014).

Macro-algae contains important plant growth hormones like auxins, abscisic acid, cytokinins, gibberellins, trace elements, vitamins, amino acids, antibiotics, micro and macronutrients. Cytokinins are one of the main active ingredients in seaweed extracts used as plant biostimulant (Booth, 1965; Tarakhovskaya *et al*., 2007). The nutrients and trace elements can be readily absorbed by plants and

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protect the plants from deficiency diseases. The carbohydrates and other organic matter present in the seaweeds promote plant growth and improve the moisture retaining capacity (Crouch and Van Staden, 1993).

Gracilaria edulis and *Gelidiella acerosa* are the members of red algae with high commercial value, found in the sub tidal areas in many parts of India and other countries (Sharma *et al.,* 2017). Various species of red algae are used as food and as sources of important hydrocolloids: agar and carrageenan. These are water soluble carbohydrates used to thicken gels, jellies of varying degree of firmness, to form water soluble films and to stabilize some products like ice cream for retaining a smooth texture (Kaladharan *et al.,*1998, McHugh, 2003). The commercial value of seaweed is judged by their agar content and gel quality (Wei-Kang Lee *et al.,* 2016).

Agar is a gelatinous polysaccharide present in the cell wall of many red algal species. It is used as a gelling, thickening and stabilizing agent. Agars are usually composed of repeating agarobiose units alternating between 3- linked β- D-galactopyranosyl (G) and 4-linked 3,6-anhydro-α- L- galactopyranosyl (LA) units. It has good gelling power in aqueous environment, can hold water, retains moisture, rich in nutrients and often used in orchid nurseries and plant tissue culture studies to cultivate plants (McHugh, 1987).

Seaweed extracts, its purified compounds like polysaccharides laminarin, alginates, carrageenans and their derivatives can be used as biostimulant (Craigie, 2011; Khan *et al.,* 2009). They can be applied on soils, in hydroponic solutions or as foliar treatments. In soils, their polysaccharides contribute to gel formation, water retention and soil aeration (Patrick du Jardin, 2015). Algal polysaccharides are biologically active compounds with several potential applications (Kulshreshtha *et al.,* 2008). These properties of polysaccharides suggest that agar can be used to promote plant growth with minimal use of water in a nutrient rich environment.

The objective of the present study is to ascertain the comparative study on the use of agarophytes 6 *G. acerosa* and *G. edulis* as biostimulant and application of agar in water-holding capacity and promotion of plant growth and evaluation of potential species by qualitative phytochemical screening and WD 6 XRF analysis.

MATERIALS AND METHODS

Sample collection

The red algae 6 *G. edulis* and *G. acerosa* were collected from the coast of Kilakarai (Lat. 9.2343°N, Lon. 78.7836°E), Gulf of Mannar, Tamilnadu, India. The collected seaweeds were washed with fresh water to remove salts and other impurities, sundried, powdered and stored.

Qualitative phytochemical screening

Qualitative phytochemical screening of the aqueous extracts of the seaweeds was carried out as per the standard methods (Harborne, 1998; Khandenwel, 2002).

Detection of alkaloids
Mayer's test

The extract was treated with Mayer's reagent and observed for the formation of cream coloured precipitate.

Detection of phenolics
Lead acetate test

A fraction of the extract was treated with 10% lead acetate solution and observed for the formation of white precipitate.

Detection of flavonoids
Sulphuric acid (H_2SO_4) test

A fraction of the extract was treated with concentrated H_2SO_4 and observed for the formation of orange colour.

Detection of coumarin

1 ml of 10% NaOH was added to 10 ml of the seaweed extract. Formation of yellow colour indicates the presence of coumarins.

Detection of terpenoids

Chloroform (2 ml) and concentrated H_2SO_4 was added to 0.5 ml of extract. Formation of red-brown colour at the interface indicates the presence of terpenoids.

Detection of quinone

Concentrated H_2SO_4 (1ml) was added to 1 ml of seaweed extract. Formation of red colour indicates the presence of quinones.

Detection of anthraquinones

Few drops of 2% HCl were added to 0.5ml of seaweed extract. Appearance of red colour precipitate indicates the presence of anthraquinones.

Detection of tannins
Ferric chloride test

The extract (5 mg) was dissolved in 5ml of distilled water and few drops of neutral 5% $FeCl_3$ solution were added. Formation of blue green colour indicates the presence of tannins.

Detection of phlobatannins

Few drops of 10% ammonium solution were added to 0.5 ml of seaweed extract. Appearance of pink colour precipitate indicates the presence of phlobatannins.

Detection of carbohydrates
Benedict's test

To 0.5 ml of extract, 0.5 ml of Benedict's reagent was added. The mixture is heated on a boiling water bath for 2 minutes. A red precipitate indicates the presence of sugar.

Detection of glycosides
Legal's test

Extracts were treated with sodium nitropruside in pyridine and sodium hydroxide. Formation of pink to blood red colour indicates the presence of glycosides.

Detection of cardiac glycosides

Glacial acetic acid (2 ml) and few drops of 5% ferric chloride were added to 0.5% of the extract. This was under layered with 1 ml of concentrated H_2SO_4. Formation of brown ring at the interface indicates the presence of cardiac glycosides.

Detection of proteins

The extract was dissolved in 10ml of distilled water and filtered by Whatmann No.1 filter paper and the filtrate was subjected to tests for proteins and amino acids.

Millon's test

Few drops of Millon's reagent were added to 2 ml of the filtrate. A white precipitate indicates the presence of proteins.

Detection of amino acids
Ninhydrin test

Two drops of ninhydrin solution was added to 2 ml of the filtrate. A characteristic purple colour indicates the presence of amino acids.

Detection of steroids and phytosteroids

0.5ml of seaweed extract was treated with equal volume of chloroform and few drops of concentrated H_2SO_4. Appearance of brown ring indicated the presence of steroids and bluish brown ring indicates the presence of phytoseteroids.

Detection of acids

0.5ml of the seaweed extract was treated with sodium bicarbonate solution. Formation of effervescence indicates the presence of acids.

Detection of saponins
Froth test

Extracts were diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

Detection of gums and mucilages

2.5 ml of the extract was added to 5 ml of absolute alcohol, stirred and filtered. The precipitate was air dried and examined for its swelling properties.

Agarophytes as biostimulant
Seed pretreatment

The algal powder: water was boiled in water bath at 60°C for 30 minutes. The extract was used to soak the spinach seeds (*Amaranthus aritis*) to promote germination and fast sprouting of the seeds.

Plant culture

In the present study, polythene bags were used for raising the crops. They were kept in the net house to prevent damages caused by birds, rats, squirrels and other animals. The plants were grown by two methods using *G. edulis* and *G. acerosa*.

Direct mixing with soil

The dried algae powder was mixed with soil in the ratio of 1:100 (10 g in 1 kg of soil) and 2:100 (20 g in 1 kg soil) labeled as A_L, A_H.

Contact placement

The seaweed powder was spread on the furrow in the proportion of 0.5g and 1 g in 1 kg of soil. The seeds were sown and covered with soil B_L, B_H.

Aqueous extract of the seaweed was used as a starter solution. Controls consisted of the soil without algae powder and seeds without pretreatment and starter solution.

Extraction of agar and use in plant growth

The washed seaweeds were soaked overnight, treated with HCl, washed, cooked in the digester. The agar gel was collected and freeze-dried (Kaliaperumal and Uthirasivan, 2001). The various steps involved in the processing of seaweed for agar extraction is given in the Fig 1.

The extracted agar was used to check its effect on the growth of plants with minimal use of water. A preliminary study was carried out in a cell culture plate. The extracted agar gel was mixed with soil in different concentration (2%, 4%, 6%, 8% and 10%) and labeled as A, B, C, D, E and Control. Five seeds were sown into each well. Control consisted of soil with seeds. About 200µl of water was sprayed into each well and observed for growth. The same procedure was followed for growing plants in transparent cups by spraying 2 ml of water for the soil: agar mixture and control.

WD-XRF analysis

Wavelength Dispersive X-ray Fluorescence Spectroscopy (WD-XRF) analysis was performed at CECRI - Karaikudi to analyze the micro and macro elements present in *G. edulis* and *G. acerosa* respectively.

RESULTS AND DISCUSSION

Qualitative phytochemical screening

Qualitative phytochemical screening indicated the presence of carbohydrates, proteins, alkaloids, flavanoids, cardiac glycosides, coumarins, quinones, anthraquinone and terpenoids, in *G. edulis* and *G. acerosa* which added to its potentiality as a bioactive principle. The results are summarized in the Table 1.

Agarophytes as biostimulant

The use of seaweeds in horticulture and agriculture has increased in recent years (Dhargalkar and Pereira, 2005). Seaweeds as biostimulant are preferred because of the presence of N, P, potash content, trace elements and metabolites similar to plant growth regulators. Seaweed was reported to be superior to chemical fertilizer because of high level of organic matter which aids in retaining moisture and minerals in upper soil level available to roots (Wallenkemp, 1955). The high fiber content acts as a soil conditioner, while the mineral content is a biostimulant.

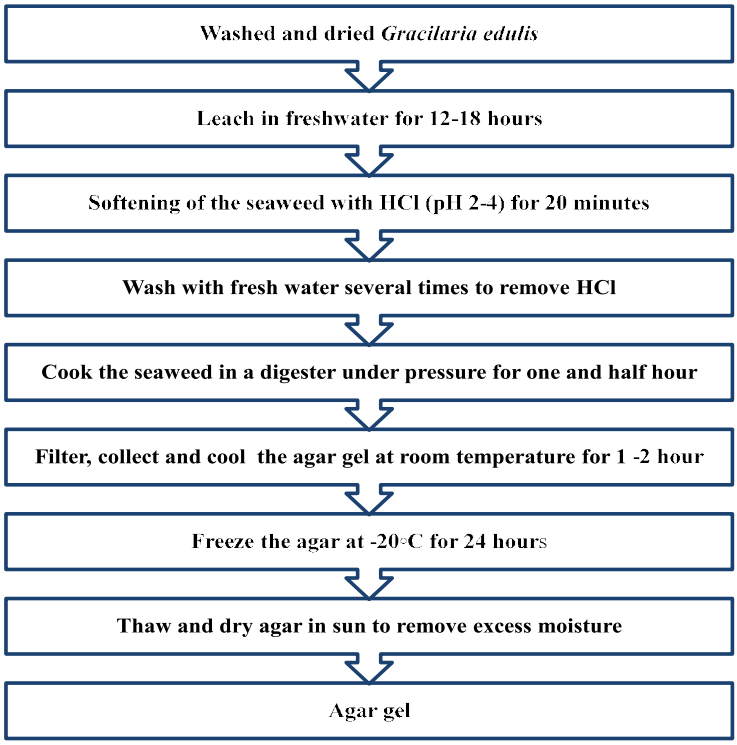


Fig 1: Steps involved in the extraction of agar.

Table 1: Qualitative phytochemical screening of *G. edulis* and *G. acerosa*.

Phytochemicals	*G. edulis*	*G. acerosa*
Alkaloid	+	+
Phenolics	++	+
Flavanoids	++	+
Coumarin	-	-
Terpenoid	++	+
Quinone	+	++
Anthraquinone	+	++
Tannin	-	+
Phlobatannin	-	-
Carbohydrate	+	+
Glycoside	+	+
Cardiac glycoside	++	+
Protein	+	+
Aminoacids	+	+
Steroid and phytosteroid	+	+
Acid	++	++
Saponin	++	+
Gums and mucilages	+	+

(+) Present, (-) Absent.

Among the two methods, plants grown by contact placement showed increased growth than the seaweed mixed directly with soil. This may due to the fact that in direct mixing of the seaweed with soil, there is a slow release of nutrients into the soil and the soil gets fertilized over time. But in contact placement, the roots of the plant are in vicinity of the seaweed where the nutrients are readily absorbed by the roots promoting fast growth of the plants thereby fertilizing the soil. *G. edulis* enhanced plant growth than *G. acerosa*. The results are given in the Table 2.

Use of agar for plant growth

The bioactive polysaccharides and oligosaccharides derived from seaweeds aids in plant growth stimulation and plant defense (Vera *et al.,* 2011; Gonzalez *et al.,* 2013). Algal extracts protect plants from pathogens, insects and from abiotic stress such as drought, frost and salinity etc. Marine macro algal polysaccharides can trigger signaling cascades that activate plant defense response and resistance to infection and diseases. Algal polysaccharides act as chelating agents by binding cations of trace elements, a large number of chemical groups, especially carboxyl residues. These hydrocolloids possess good gelling and water-binding capacity. This property is directly related to a degree of sulfatation (Michalak *et al*., 2017).

Agar gel extracted from *G. acerosa* was checked for water holding capacity and promotion of plant growth. This method can be effective when facing water scarcity and arid areas. Good growth of plants was observed even in low concentrations (2% and 4%) of agar mixed with soil with very minimal water requirement (200 µl) in cell culture plate. There was no growth of seeds in control well as the seeds were unable to germinate under water stress. Plants grown in transparent cups also showed similar result with good growth at low concentrations of agar (2% and 4%). After 10

Table 2: Growth parameters observed after 3 weeks using agarophytes as biostimulant.

Growth parameters	*G. edulis*				*G. acerosa*				Control (C)
	Soil + seaweed mix		Contact placement		Soil + seaweed mix		Contact placement		
	A_L	A_H	B_L	B_H	A_L	A_H	B_L	B_H	
No. of leaves	6	6	7	7	5	6	6	7	6
Shoot length (cm)	5	6.8	7.2	8	4	6	7	7.2	9.5
Root length (cm)	5.8	5.5	9	10	7.5	3.5	9	9.5	9
Leaf length (cm)	2.2	2.5	4	5.5	1.5	2.2	3.5	3.5	3
Leaf width (cm)	1.2	1.8	2.8	3.2	1	1.5	2.5	2.8	2.2

A_L, A_H- Agarophyte directly mixed with soil in low and high concentration.

B_L, B_H- Agarophyte applied by contact placement in low and high concentration.

C- Control.



Fig 2: Use of agar for plant growth in cell culture plate.

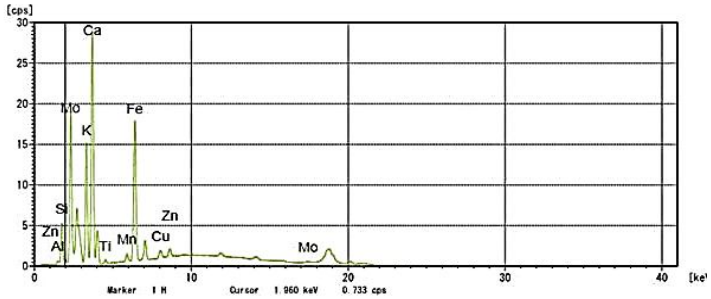


Fig 3: Use of agar for plant growth in transparent cups.

days, the baby plants in A and B remained fresh and healthy with the minimal water requirement but the baby plants in control cannot survive which proves that agar can be effectively used to grow plants under water stress in a nutrient rich environment. The results are shown in Fig 2 and 3. This method will help in improving the water and soil management in agriculture and horticulture. It was also observed that there was contraction of the agar gel over time (as observed in D, E and F). This may be due to the photodegradation process and temperature-relative humidity fluctuations that caused a reduction in the agar molecular size and a decrease in the number of sulfate groups. These changes alter the gel crystallinity, causing contraction that leads to formation of micro-fractures and embrittlement of the agar gel (Freile-Pelegrin *et al.*, 2006).

WD-XRF analysis

The WD-XRF analysis of the agarophytes- *G. edulis* and *G. acerosa* revealed the presence of various micro and macro elements such as Al, Si, K, Ca, Ti, Mn, Fe, Cu, Zn, Mo and O in *G. edulis* and Al, Si, S, K, Ca, Ti, Mn, Fe, Sr, O in *G. acerosa* in appreciable level. Among the elements estimated, Ca, Mo, Fe, K, Si was found to be dominant in *G. edulis* and Ca, Sr, Si, and Fe was found to be abundant in *G. acerosa.* The results of the WD-XRF analysis are shown in the Fig 4 and 5. For optimal growth conditions, plants need about 16 minerals whereas Nitrogen (N), Phosphorous (P) and Potassium (K) are used by plants in large quantities. The macro and micro elements determine the health of plants. Other constituents that promote plant growth include micro- and macronutrients, sterols, N-containing compounds like betaines, and hormones (Craigie, 2011; Khan *et al.,* 2009). The micro and macro elements such as Ca, Mo, Fe, K, Si, Zn, Mn, Al, Ti, Cu and O are present in *G. edulis* which are plant essential nutrients that acted as a biostimulant which is absorbed by the plants and enhanced their growth when compared with control group. By this method, *A. aritis* can be harvested even indoors and at much faster rate with added nutrients from agarophytes.

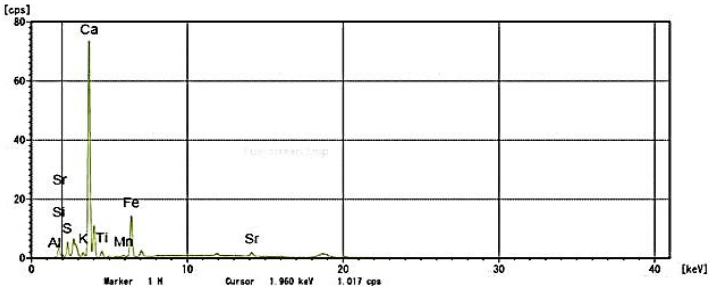


Live time : 60 s Processing Time : P2
XGT Dia. : 100 μm X-ray tube vol. : 50 kV
Current : 1.000 mA
X-ray Filter : Nonexistence Cell : Nonexistence

Quant. Corr. : Standard-less

Elem.	Line	Mass [%]	2sigma [%]	Atomic [%]	Intensity [cps/mA]	Formula	Mass [%]	Molecule [%]
13 Al	K	3.891	0.365	3.583	7.29	Al2O3	7.352	4.904
14 Si	K	15.103	0.366	13.358	68.13	SiO2	32.309	36.568
19 K	K	12.582	0.255	7.995	225.56	K2O	15.156	10.943
20 Ca	K	25.007	0.403	15.501	424.11	CaO	34.990	42.434
22 Ti	K	0.412	0.070	0.214	7.83	TiO2	0.687	0.585
25 Mn	K	0.403	0.048	0.182	16.48	MnO2	0.638	0.499
26 Fe	K	5.719	0.117	2.544	297.13	Fe2O3	8.176	3.482
29 Cu	K	0.285	0.037	0.111	20.28	CuO	0.356	0.305
30 Zn	K	0.269	0.037	0.102	20.94	ZnO	0.334	0.279
42 Mo	K	0.000	0.020	0.000	0.01	MoO3	0.000	0.000
8 O		36.329	0.560	56.410				

Fig 4: Elements present in *G. edulis* as analyzed by WD 6 XRF.



Live time : 60 s Processing Time : P2
XGT Dia. : 100 μm X-ray tube vol. : 50 kV
Current : 1.000 mA
X-ray Filter : Nonexistence Cell : Nonexistence

Quant. Corr. : Standard-less

Elem.	Line	Mass [%]	2sigma [%]	Atomic [%]	Intensity [cps/mA]	Formula	Mass [%]	Molecule [%]
13 Al	K	1.607	0.264	1.502	3.54	Al2O3	3.037	1.866
14 Si	K	9.449	0.293	8.482	53.52	SiO2	20.214	21.068
16 S	K	2.885	0.098	2.269	69.25	SO3	7.204	5.636
19 K	K	1.001	0.068	0.646	24.44	K2O	1.206	0.802
20 Ca	K	41.998	0.431	26.423	1140.89	CaO	58.764	65.627
22 Ti	K	1.625	0.086	0.855	32.98	TiO2	2.711	2.125
25 Mn	K	0.212	0.036	0.097	9.18	MnO2	0.335	0.241
26 Fe	K	4.322	0.086	1.951	238.99	Fe2O3	6.179	2.423
38 Sr	K	0.297	0.026	0.085	29.18	SrO	0.351	0.212
8 O		36.604	0.472	57.689				

Fig 5: Elements present in *G. acerosa* as analyzed by WD 6 XRF.

SUMMARY AND CONCLUSION

Marine algae are the richest source of a number of novel bioactive compounds. Red algae that are used for the extraction of agar are called agarophytes. There are many species of agarophytes but *G. edulis* and *G. acerosa* are the two most commercially important high quality-agar yielding and easily cultivable species. The qualitative phytochemical screening and elemental analysis showed that these two agarophytes are rich in nutrients such as proteins, carbohydrates, other essential nutrients, micro and macro elements. These agarophytes are used as a biostimulant to enhance the crop production without the use of chemical fertilizers.

The agar extracted from agarophytes is tested to promote plant growth and water holding capacity in soil under water stress. The water holding capacity of the agar in soil was good which retained the moisture but the agar gel contracted over time which suggests the need to apply agar periodically. This natural polysaccharide can be engineered in future to absorb and hold significant amounts of water suitable to be applicable in horticulture and agriculture. Environmental factors also play an important role in the growth of plants which have to be optimized. The comparative study on the use of agarophytes revealed that *G. edulis* and its agar can be best utilized as a biostimulant in promoting plant growth under water stress and also can be used to grow *A. aritis* indoors.

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