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RESEARCH ARTICLE

Characterizing organic biostimulants from *Kappaphycus alvarezii* (Doty) and assessing their stimulant potential on Zea mays (maize)

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Abstract

Marine algae are rich in minerals and phytochemicals and are utilized in agriculture as biostimulants for plant growth after industrial extraction. Therefore, in the current study, red marine algae (RMA) were extracted using cow urine and water to produce suitable biostimulants for organic agriculture. The raw RMA and its extracts were characterized for nutrients and bioactive metabolites. The extracted RMA biostimulants were tested at different concentrations using maize species to optimize the most effective concentrations for growth promotion. Results showed that cow urine extracts (CUE) contained higher essential nutrients compared to aqueous extracts (AQE), with average extractions of 34 % primary nutrients, 31 % secondary nutrients, 10 % micronutrients and 5 % heavy metals in CUE and 3 %, 6 %, 6 % and 12 % in AQE respectively. Maize germination parameters were significantly higher at 2.5 % concentration for both CUE and AQE solutions and also performed well at 5 % concentration. Germination percentage, energy and index ranged from 25-35 %, 20-30 % and 0.6-0.9 % respectively, with higher values observed with CUE. Seedling parameters such as seedling vigor index, length and biomass were significantly higher with 7.5 % CUE and 5 % AQE. Similar results were observed for root traits, except for root tips, which were higher at 10 %. The study suggests that concentrations of 5.0-7.5 % for CUE and 2.5-5.0 % for AQE are optimal for enhancing maize germination and seedling growth. Furthermore, cow urine -based extract of red marine algae at 5 % and 7.5 % concentrations showed superior performance compared to aqueous extract in promoting plant growth. These findings warrant further validation under field conditions to assess their impact on crop production and quality.

Keywords

biostimulant extraction; cow urine; growth promoters; root growth; organic agriculture

Introduction

Changing lifestyles and consumer habits are contributing to increase in nutritional hunger worldwide, leading to an increasing demand for truly sustainable food production. Soil erosion, urbanization and climate change threaten the availability of arable land, impacting the efficiency of current farming methods. To improve the sustainability of agricultural systems, reducing reliance on external resources is essential. Biostimulants, which

promote plant growth and improve plant health and the nutritional quality of food produces are crucial in this effort (1). They are widely used in conventional and organic agriculture to boost plant growth, increase resistance to environmental stresses, including climate change and improve nutrient acquisition and source-sink partitioning efficiency (2).

The plant-based biostimulant derived from various marine algae (seaweed) contains plant growth-regulating compounds such as auxin, cytokinin and gibberellins, which promote root development, enhance nutrient uptake and increase plant vigor (2, 3). The presence of wide range of bioactive compounds of hormones, flavanoids, phenolics, coumarins, amino acids, vitamins, anthocyanins, benzoic derivatives etc. in various algae species have also been documented (4, 5). Marine algae are also rich in essential plant nutrients, including nitrogen, phosphorus, potassium and trace elements like magnesium, iron and calcium (6). Applying liquid extract of seaweed directly to plants ensure quick absorption of nutrients and growth promoting substances, enhancing photosynthesis, increases chlorophyll production and improves plant vigor (7).

Seaweed extracts have been reported to promote germination, enhance seedling vigor by increasing root size and density and protect plants such as tomato, cabbage and marigold from transplantation shock. Additionally, these extracts have positive effects on the plant height, leaf numbers, root width, root length and overall biomass in tomato plants (4, 8). In addition, the seaweed contains various bioactive compounds such as plant growth regulators, antioxidants, polysaccharides which benefit plants in multiple ways. These compounds enhance plant defenses against pest and diseases, improve stress tolerance (such as drought and temperature extremes) and promote overall plant health (9, 10). Hence, in organic farming, seaweed is highly valued and often used as a natural fertilizer and soil amendment.

Among the various species of seaweed, the red marine algae contain surplus minerals and phyto-chemicals to be used in agriculture as biostimulant for improving the plant growth and productivity. The use of organic solvents, heat and synthetic chemicals for producing the seaweed biostimulant are not suitable for organic and natural farming. These methods are more compatible with integrated and conventional agriculture and involve industrial process and significant investment to the farmer. Hence, the present study aims to characterize RMA and its extracts based on water and cow urine and evaluate their efficacy on seed viability, plant growth and root system development in maize plants.

Materials and Methods

Alga collection, processing and extraction

The *Kappaphycus alvarezii,* red marine alga (RMA) was collected from the Mandapam coastal waters, Ramanathapuram, India. Freshly picked seaweed (*K. alverzii*) was cleaned in seawater and transported to the lab in a portable cooler that was kept out of the sunlight. After careful washing with both fresh and double-distilled water, the epiphytes, necrotic sections, muds, dust and other detritus were eliminated and air-dried for a week. The dried RMA was oven dried at 60 ± 2 °C and ground into powder using mechanical grinder and stockpiled in an airtight container until analysis at room temperature.

The red seaweed was soaked in cow urine and de-ionized water for 72 h at a ratio of 1:50 (w/v) (10 g in 500 mL) for extracting the growth promoting compounds and nutrients efficiently. Cow urine of *Holstein Friesian* breed (fed with organically harvested hays and open pasture in certified organic field only) collected from dairy farm, Central farm unit, Tamil Nadu Agricultural University (TNAU), Coimbatore and de-ionized water were utilized for extraction. At the end of the soaking period, all extracts were filtered through a clean muslin cloth and centrifuged and decanted to obtain 100 % stock solution. It was stored in an airtight container for further use.

Phyto-chemicals profiling of red marine alga by GC-MS and LC-MS

One g of raw RMA was macerated in 50 mL of 95 % methanol - HPLC-grade (11) at 24 to 25 °C for approximately 72 h using rotary shaker at 160 rpm. The mixture was centrifuged at 4000 rpm for 10 min. The supernatants were dried under reduced pressure to evaporate and was filtered using Agilent - 0.2 µm nylon membrane filters and subjected to phyto-chemical analysis in GC-MS and LC-MS.

An Agilent GC-MS (Model- GC 7890A / MS5975C) equipped with EI triple axis detector was engaged for volatiles profiling. A standard non-polar DB-5 MS capillary column with a length of 30 m, an inner diameter of 0.25 mm and a film thickness of 0.25 µ of Agilent Co., USA, was used for separation. Helium was used as a carrier gas at flow rate of 1.0 mL/min. Then 1 mL of the sample was kept in a 2 mL screw top vial in an auto injector and 1 µL of the sample was injected. The oven temperature was programmed at 60˚C and the mass scan range (m/z) was 50 to 350 amu. The bioactive molecules were identified by comparing mass spectra with the NIST Mass spectra library (National Institute of Standards and Technology) and further information such as name, molecular weight and molecular formula were ascertained from the NIST, PubChem, Chemsphere and HMDB data bases. The each peak was integrated from the base to the top of the peak for assessing the mass intensity.

Also the metabolite profiling was assessed using a Shimadzu LC-MS-8040 (Shimadzu UFLC - LC-20 AD) with an electro spray ionization detector. Liquid chromatographic separation was performed on a reversed-phase C18 analytical column (with TMS end-capping) measuring 4.6 mm × 250 mm and a particle size of 5 µm (SHIMADZU). The column temperature was maintained at 35 °C and the total run time was 20 min for an injection sample volume of 10 µL, with an m/z range of 100-1000. Compounds were eluted using a mobile phase consisting of 0.1 % formic acid in water (A) and methanol (B) in gradient mode. The gradient started with 5 % B for 2 min, followed by a linear

increase to 90 % B over 10 min, a decrease to 5 % B over 15 min and finally maintaining 5 % B for 20 min. The flow rate was set at 0.2 mL/min using a binary pump. The chromatographic system was connected to a triple quadruple mass spectrometer (SHIMADZU). MS analysis was carried out in ESI positive ionization mode with the following parameters: drying gas flow of 17 L/min, nebulizing gas flow of 3 L/min and a total flow rate of 0.7 µL/min. Compounds were identified by comparing the mass spectra with the Plant Metabolite Database (PmDB).

Elemental analysis of raw alga and its extracts

A known quantity of raw RMA and its extracts were digested using 3 different acid mixtures: triple acid (9:2:1 ratio of concentrated nitric, sulfuric and perchloric acid), N -diacid (5:2 ratio of concentrated sulfuric and perchloric acid) and S-diacid (1:2 ratio of concentrated nitric and perchloric acid). The clear solution from the N-diacid digestion was diluted with double distilled water (DDw) for N estimation, while the S-diacid and triple acid digests were diluted with 1 % nitric acid respectively for S and other elements analysis. The diluted contents were filtered through Whatman No. 42 filter paper and volume made up to 100 mL using corresponding diluents. Nitrogen was determined by the Kjeldahl method, P and K was determined with UV-Visible spectrophotometer at 420 nm and a flame photometer respectively. The secondary and micronutrients as well as heavy metals were determined using an inductively coupled plasma optical emission spectrometer (ICP-OES, Thermo fisher, 7000 Series).

In-vitro study

A laboratory *in vitro* assay was carried out using maize (CO6 hybrid of TNAU) as test species. Ten maize seeds were placed on germination paper in petri plates. Various concentrations (2.5, 5.0, 7.5 and 10 %) of aqueous and cow urine extracts of RMA were applied to the petri plates along with water only treatment as control. The experiment was performed in duplicates, adopting a completely randomized block design (CRD). Seed germination was monitored and counts were recorded on 7^{th} and 10^{th} days after imposing treatments. The plates were incubated at 27 ± 1 °C with a 16 h light/8 h dark cycle. Germination was considered successful when the radical protruded more than 2 mm. Using the collected data, the parameters viz., germination % (GP), germination Index (GI), mean germination time (MGT), seedling Vigour Index (SVI) and germination energy (GE) were calculated as detailed below.

a. GP = no of germinated seeds/total number of seeds × 100,

b. GI is calculated as (Gt/Tt), where Gt is the number of seeds germinated on day t and Tt is the number of days (12)

c. MGT is calculated as $\Sigma (D \times n)/\Sigma n$, where n is the number of seeds germinated on day D and D is the number of days since the test started (13).

d. SVI = seedling length (cm) x germination percentage (14).

e. GE % is calculated as number of germinating seeds/ total seeds per treatment after germination for d days × 100, seedling length and biomass.

After 10 days of sowing, the maize seedlings were removed from each plate and growth parameters viz., seedlings length, biomass and roots characters were determined. Root parameters including root length (cm), surface area (cm^2), average diameter (mm), number of tips and root volume (cm^3) were recorded using a root image analysis system with WinRHIZOPRO software.

Statistical analysis

a.The Analysis of Variance for Completely Randomized Block Design was worked out to examine the collected data from the study. The treatments with significant variations were subjected to F-test (p = 0.05) using OPSTAT (15) for comparison. Correlation and regression analysis were performed with MS-Excel (MS office 2007) package.

Results

Elemental composition of RMA, cow urine and extracts

The physicochemical properties and elemental composition of RMA, cow urine and their extracts are shown in Table 1. The pH and EC of 1 % RMA solution were recorded as 6.14 and 12.15 dS m^{-1} respectively. For cow urine alone, aqueous extract (AQE) and cow urine extract (CUE), the pH values were 8.12, 5.91 and 8.41 with the EC values of 3.25, 5.35 and 53.2 dS m^{-1} respectively. Organic carbon analyzed was found to be 63.9, 1.59, 1.01 and 1.30 % in RMA, cow urine, AQE and CUE respectively. The primary nutrients, N, P and K ranged from 0.017-0.78, 0.003-0.047 and 0.49-3.80 % respectively, irrespective of RMA, cow urine or their extracts. The secondary elements, Ca, Mg and S were present in the range of 0.003-0.069, 0.001-0.17 and 0.32-4.80 % respectively. The concentration of micronutrients elements and heavy metals were also analyzed and the results are presented in Table 1.

The elements extraction efficiency (EEE) by the water and cow urine from RMA was calculated by comparing it with raw alga composition. The results are presented in Table 2. The elemental composition present in cow urine was not taken into account while calculating EEE by the cow urine. There was a significant variation in nutrients element composition between cow urine and aqueous RMA extracts. The EEE was noticed to be higher for cow urine extract compared to water, ranging from 23.23-44.74, 1.83-30.43 and 2.24-10.10 % in CUE and 0.38-7.01, 0.24-24.32 and 1.48-32.43 % in AQE, for macronutrients (N, P, K, Ca, Mg, S), micronutrients (Fe, Zn, Cu, Mn, B) and heavy metals (Pb, Cr, Ni, Cd) respectively.

Phytochemicals and metabolites of red marine alga in GC-MS and LC-MS

The raw marine algae powder was extracted using methanol and subjected to GC-MS and LC-MS analysis to identify phytochemicals, metabolites and plant growth substances (Fig. 1-3). The mass spectra of the RMA and the intensity of compounds detected in GC-MS are presented in Fig. 1 and 2 and summarized in Table 3. About 30

Table 1. Physico-chemical characteristics and elemental composition of RMA, cow urine and their extracts.

Parameters	Raw seaweed	Cow urine	AQE*	CUE*
pH (1 % solution)	6.14	8.12	5.91	8.41
Electrical conductivity (dS m^{-1})	12.15	3.25	5.35	53.20
Organic carbon (%)	63.90	1.59	1.01	1.30
Nitrogen (N) (%)	29	0.61	0.017	0.78
Phosphorous (P) (%)	0.047	0.005	0.003	0.018
Potash (K) (%)	3.80	0.49	0.124	1.83
Calcium (Ca) (%)	0.069	0.027	0.003	0.011
Magnesium (Mg) (%)	0.17	0.066	0.001	0.096
Sulfur (S) (%)	4.80	3.60	0.32	1.30
Iron (Fe) (mg kg^{-1})	170.45	58.40	5.50	97.5
Copper (Cu) (mg kg^{-1})	7.10	1.00	0.10	0.20
Manganese (Mn) (mg kg^{-1})	14.8	2.10	3.60	1.10
Zinc (Zn) (mg kg^{-1})	85.00	7.30	0.20	6.20
Boron (B) (mg kg^{-1})	106.15	14.25	0.50	2.20
Nickel (Ni) (mg kg^{-1})	3.70	6.20	1.20	1.00
Chromium (Cr) (mg kg^{-1})	6.75	10.20	0.10	0.70
Lead (Pb) (mg kg^{-1})	12.50	0.90	0.30	0.30
Cadmium (Cd) (mg kg^{-1})	2.10	0.20	ND	ND

*AQE- aqueous extract; CUE- Cow urine extract; ND- not detected

Table 2. Elements extraction efficiency by cow urine and water from red marine alga.

Parameters	AQE (%)	CUE (%)
Nitrogen (N)	0.57	86.67
Phosphorous (P)	6.38	34.62
Potash (K)	3.26	44.74
Calcium (Ca)	8.90	23.23
Magnesium (Mg)	0.93	41.25
Sulfur (S)	7.01	28.55
Iron (Fe)	3.23	30.43
Copper (Cu)	1.41	2.47
Manganese (Mn)	24.32	6.51
Zinc (Zn)	0.24	6.72
Boron (B)	0.47	1.83
Nickel (Ni)	32.43	10.10
Chromium (Cr)	1.48	4.13
Lead (Pb)	2.40	2.24
Cadmium (Cd)	0.001	-

*AQE- aqueous extract; CUE- Cow urine extract

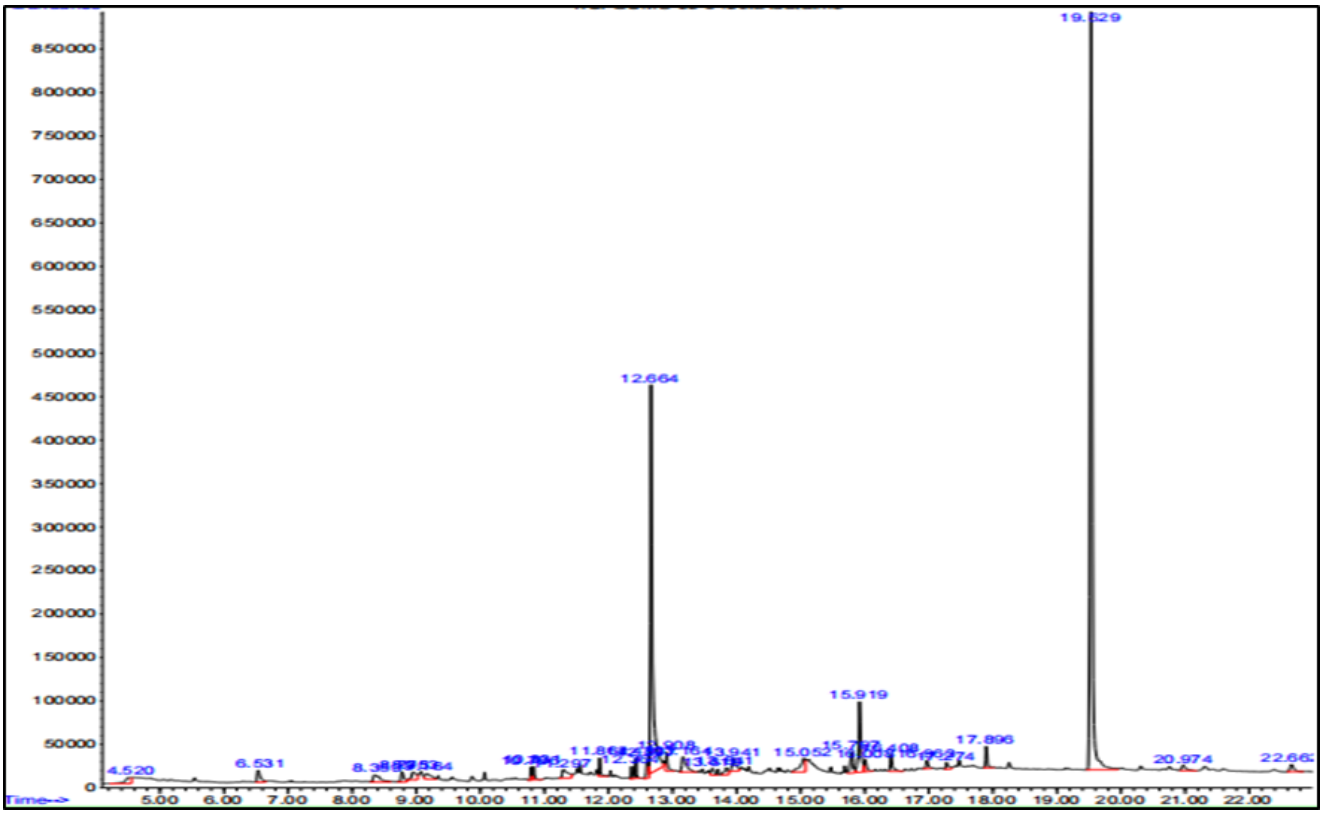

Fig. 1. GC-MS chromatogram of red marine alga showing the presence of bioactive compounds.

compounds were detected in RMA by GC-MS which belong to categories such as fatty acids, alcohols, butyric acid derivatives etc. The dominant (based on peak area intensity) compounds detected in raw RMA are cholesterol (46.64 %), n-Hexadecanoic acid (21.84 %), Hexadecanoic acid (4.56 %) (Fig. 2). The phytochemicals identified with the help of NIST library analysis of the GC-MS spectra (Table 3) were classified into different phytochemical classes and their proportional content is presented in Fig. 4a.

The mass spectra of the RMA methanolic extract, analyzed by LC-MS, and the m/z ratio intensities of the detected compounds are shown in Fig. 3 and Table 4. Compound identification was facilitated using the PmDB open-source database, based on their m/z ratios. Although the TIC spectrum revealed over 100 compounds and about 25 compounds were identified using open source and were categorized into different metabolomics groups (Fig 4b). Major essential amino acids such as proline and isoleucine were detected at higher intensities in the raw RMA. Growth hormones including indole acetic acid and indole-3-carboxylic acid were also identified, along with flavonoids like 3-hydroxydaidzein and dalbergin. Additionally, 7-hydroxycoumarin, p-coumaric acid and sinapic acid were detected at higher levels in the RMA.

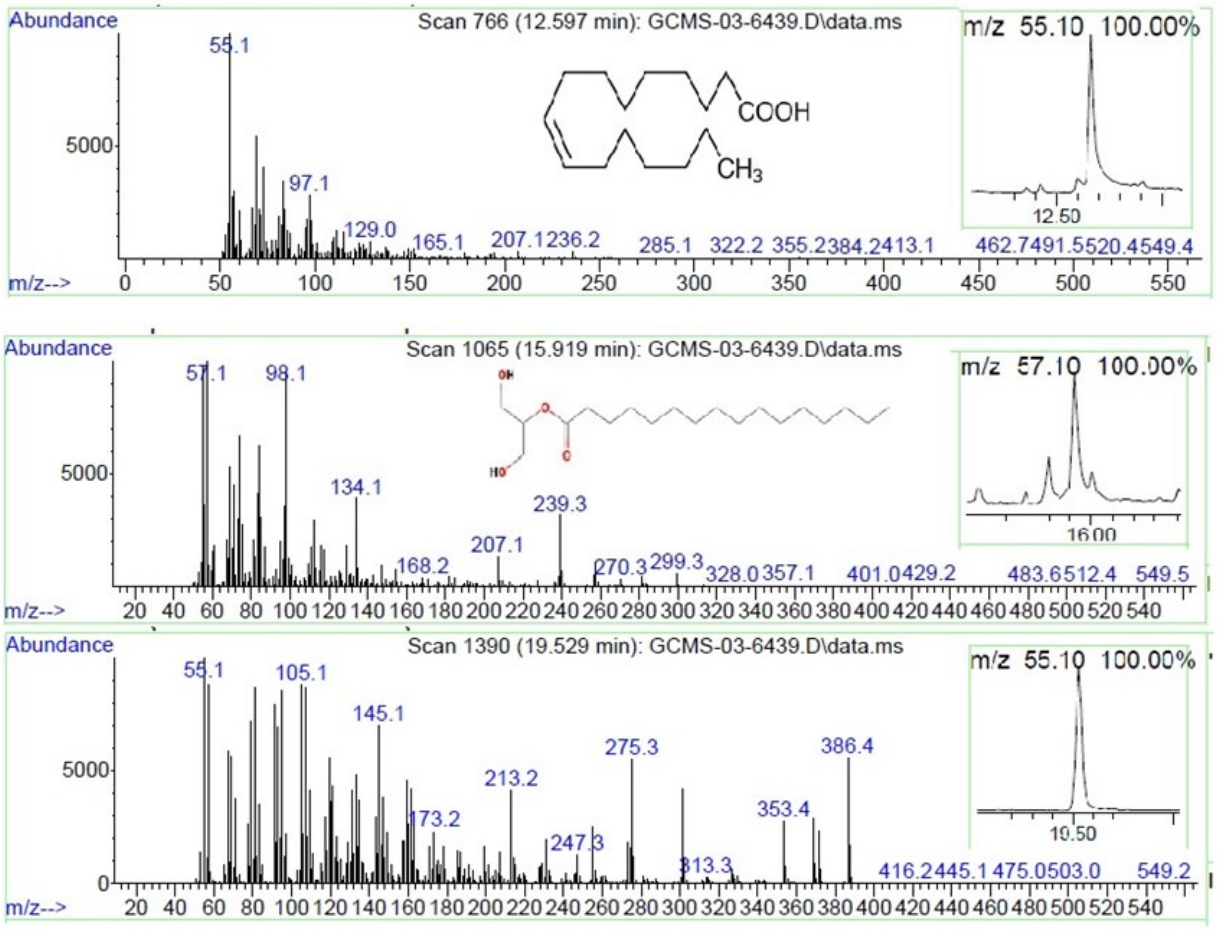

Fig. 2. Mass spectra and qualifier mass chromatogram (from GC-MS) of major phytochemicals identified with the help of NIST library.

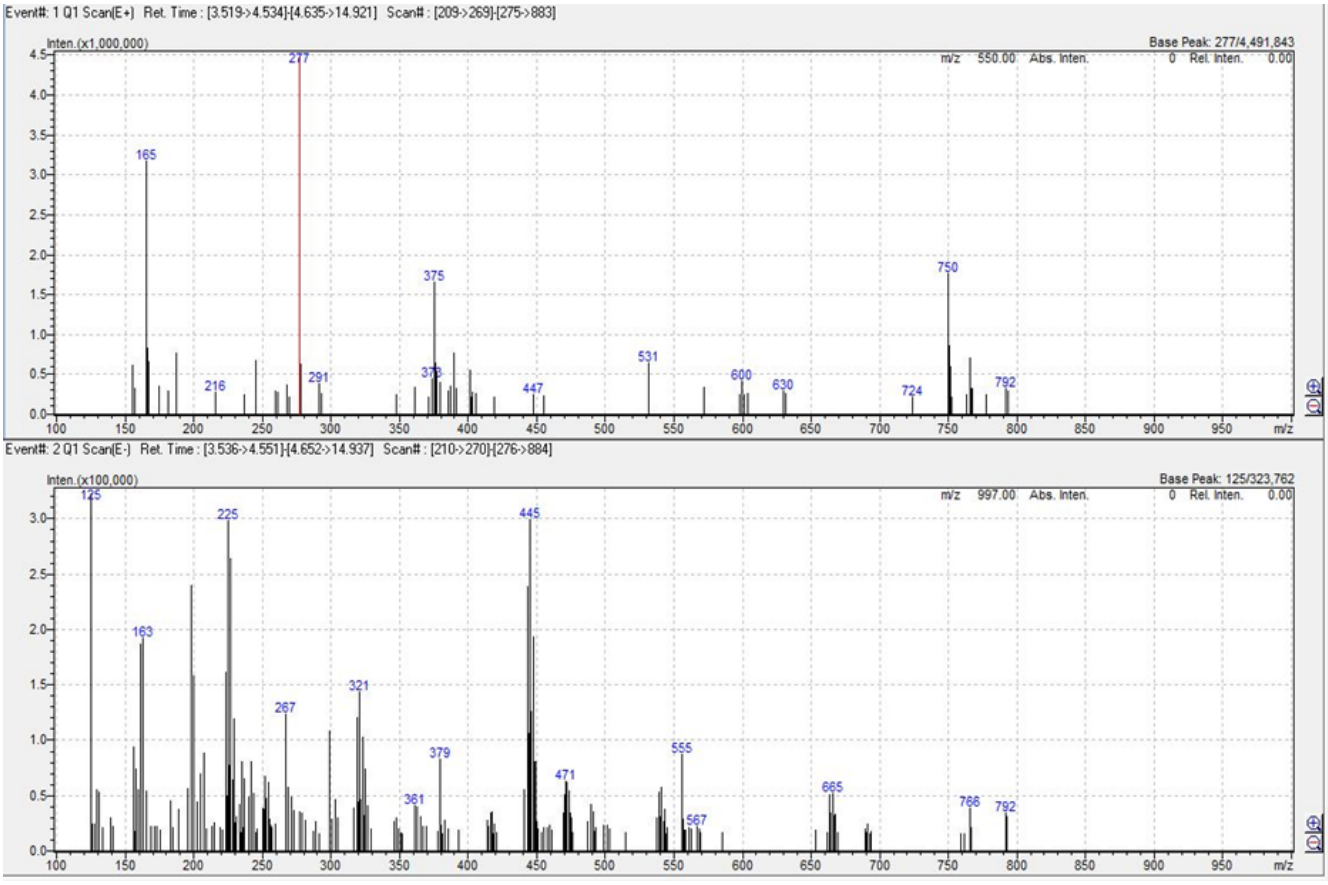

Fig. 3. The total ion count (TIC) chromatogram of raw *Kappaphycus alvarezii* analysed by LC-MS equipped with ESI detector in both positive (upper) and negative (lower) modes.

Table 3. Major phytochemicals profile of *Kappaphycus alvarezii* analyzed by GC-MS.

S No	Compound identified by NIST library in GC-MS	m/z	RT (min)	Intensity (%)	Class
1.	Diglycerol	61.10	4.520	1.17	Fatty acid
2.	2,6-Nonadienal, (E,Z)-	69.10	6.531	1.08	Plant metabolite
3.	4b-Methyl-6,8-dioxa-3-thia-bicyclo (3,2,1)octane	60.10	8.353	1.42	-
4.	N,N'-(1,2-Propylene)-thiourea	116.10	8.775	0.84	Amino group
5.	Ethyl .alpha.-d-glucopyranoside	60.00	8.953	1.12	Glucose derivatives
6.	2-(2-Butoxyethoxy)ethoxy-trimethyl silane	116.10	9.164	0.96	Organo silicon
7.	Dodecylbis(trifluoromethyl)phosphine sulfide	55.10	10.786	0.53	organo-thio phosphorus
8.	Tritetracontane	57.10	10.831	0.59	hydrocarbon
9.	Octadecanoic acid	73.00	11.297	1.47	fatty acid and plant metabolite
10.	2-Pentadecanone, 6,10,14-trimethyl	58.10	11.864	0.72	Fatty acid
11.	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	57.10	12.364	0.56	Oxaspiro compound
12.	Pentadecanoic acid, 14-methyl-, methyl ester	74.00	12.430	0.84	Fatty acid
13.	cis-9-Hexadecenoic acid	55.10	12.597	1.25	Fatty acid
14.	n-Hexadecanoic acid	73.10	12.664	21.84	Fatty acid and plant metabolite
15.	Ethanol, 2-(dodecyloxy)-	57.10	12.908	0.96	Ethanol
16.	Pentadecanoic acid	55.10	13.164	1.99	Fatty acid and plant metabolite
17.	Oleyl alcohol, heptafluorobutyrate	71.10	13.619	0.53	Butyric acid derivatives
18.	cis-Vaccenic acid	55.10	13.841	0.72	Fatty acid and plant metabolite
19.	Oleic Acid	55.10	13.941	1.43	Fatty acid, antioxidant and plant metabolite
20.	Polygalitol	99.10	15.052	2.01	Sugar alcohol
21.	Carbonic acid, hexadecyl 2,2,2-trichloroethyl ester	55.10	15.797	1.48	Carbonic acid
22.	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	57.10	15.919	4.56	Fatty acid and plant metabolite
23.	1,19-Eicosadiene	57.10	16.008	0.70	-
24.	Oleyl alcohol, heptafluorobutyrate	55.10	16.408	1.07	Butyric acid derivatives
25.	N,N-Dimethyl-4-nitroso-3-(trimethylsilyl)aniline	207.10	16.963	0.57	-
26.	Cyclotrisiloxane, hexamethyl-	207.10	17.274	0.53	organo silicon
27.	Cyclotrisiloxane, hexamethyl-	207.10	17.896	0.95	organo silicon
28.	Sterol (26-Nor-5-cholesten-3.beta.-ol-25…)	55.10	19.529	46.64	Algal and human metabolite
29.	1,2-Bis(trimethylsilyl)benzene	207.10	20.974	0.66	Silicon derivatives
30.	Cyclotrisiloxane, hexamethyl-	207.10	22.662	0.80	organo silicon

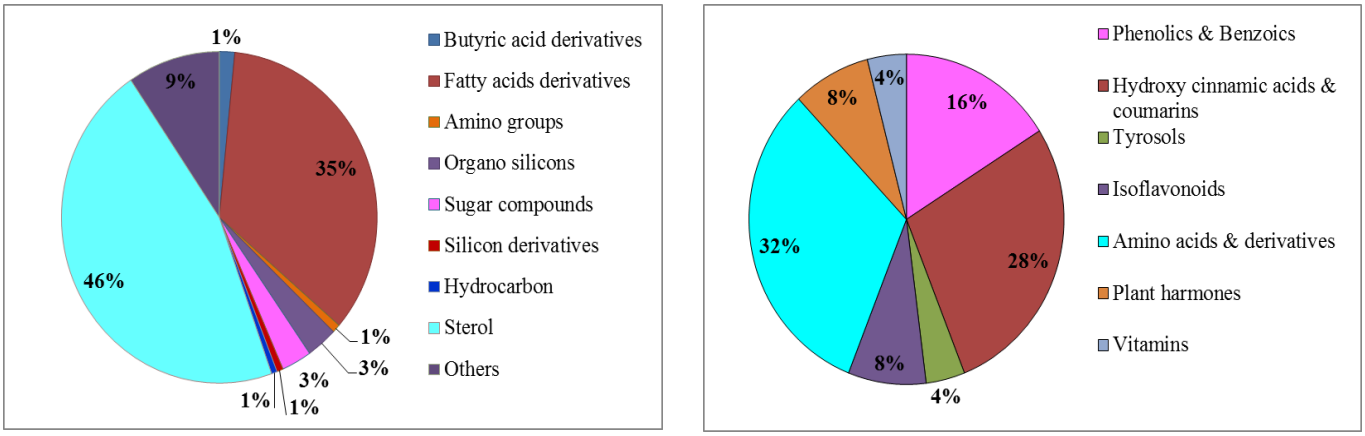

Fig. 4. Pie charts illustrating the percentage of commonly identified bioactive compounds classes in red marine alga. (a). GC-MS data; (b). LC-MS data.

Table 4. Major metabolites identified in raw *Kappaphycus alvarezii* by LC-MS.

S.No	Compounds	Ionization (ESI^+/ESI^-)	Molecular weight	Theoretical (m/z)	Observed (m/z)	Intensity (%)
	Phenolic and Benzoic compounds					
1.	Difluorophenol	[M - H]-	130.10	129.09	129.10	10.14
2.	Vanillic acid 4-sulfate	[M - H]-	247.00	245.99	246.00	5.29
3.	Benzoic acid, 2,4-dimethoxy-	[M - H]-	182.17	181.16	181.10	6.65
4.	Pentafluorobenzoic acid, pent-2-en-4-ynyl ester	[M + H]+	276.15	277.16	277.15	48.96
	Hydroxy cinnamic acids and coumarins					
5.	Caffeic acid 4-sulfate	[M - H]-	258.95	257.94	257.95	5.16
6.	Sinapic acid	[M - H]-	223.06	222.05	222.06	22.23
7.	Coumarin-3-carboxylic acid	[M - H]-	190.15	189.14	189.20	6.99
8.	7-Hydroxycoumarin	[M - H]-	162.14	161.13	161.00	38.71
9.	Furo(4',5',6,7)coumarin	[M + H]+	186.15	187.16	187.15	8.96
10.	p-Coumaric acid, trans	[M + H]+	164.16	165.17	165.10	50.57
11.	Urolithin A	[M - H]-	228.04	227.03	226.95	36.71
	Tyrosols					
12.	3,4-DHPEA-EDA	[M + H]-	319.12	318.11	318.10	16.31
	Isoflavonoids					
13.	3-Hydroxydaidzein	[M + H]-	268.06	267.05	267.2	19.08
14	Dalbergin	[M + H]-	270.05	269.04	268.05	9.13
15	3-O-Methylequol	[M + H]-	272.1	271.09	270.08	8.11
	Amino acids and its derivatives					
16.	L-Proline, 1-acetyl-	[M + H]+	156.16	157.17	157.10	72.07
17.	Isoleucine, ethyl ester, L-	[M + H]+	159.26	160.27	160.20	100.00
18.	Phenylalanine	[M + H]+	166.10	167.11	167.10	11.43
19.	DL-Valine, N-acetyl-, methyl ester	[M + H]+	173.20	174.21	174.20	5.74
20.	L-Isoleucine and Leucine	[M - H]-	132.10	131.09	131.10	7.74
21.	DL-Alanine, N-DL-alanyl-	[M + H]+	160.15	161.16	160.15	14.94
22	L-Phenylalanine	[M + H]+	166.00	167.01	167.10	11.43
	Plant growth hormones					
23	Indole acetic acid	[M - H]-	174.00	172.99	173.00	5.80
24	Indole-3-carboxylic acid	[M + H]+	161.05	162.06	162.05	5.56
	Vitamin					
25	Riboflavin	[M + H]+	376.30	377.31	377.30	5.71

Maize germination parameters

The effect of AQE and CUE on maize germination and seedling growth assessed following 3rd and 10th day after sowing and treatments application, are presented in Fig. 5. Irrespective of concentration applied, AQE resulted in lower germination ranging from 45-65 % compared to CUE, which exhibited GP values between 70 to 100 %. Notably, the application of AQE and CUE at a concentration of 2.5 % led to significantly higher germination (p=0.05), achieving 100 and 65 % respectively, compared to control. However the concentration of extracts increased, a decline in germination percent was observed (Fig. 5a). Similar trend was observed for germination index (GI), with higher values for CUE (1.51-3.05) compared to AQE (1.46-2.25). Higher and lower index was observed with 2.5% concentration and control, respectively (Fig. 5b). To assess the speed and uniformity in the emergence of seeds, germination energy (GE) was calculated using the data recorded on 3rd day. The CUE consistently recorded significantly (p=0.05) higher GE (25-75 %) compared to AQE (20-45 %). Irrespective of extracts, 2.5 % solution recorded higher GE and lower by the control. The mean germination time (MGT) was calculated for both RMA extracts at all concentrations, ranging from 3 -4 days for CUE and 3-5 days for AQE, depending on the applied concentrations. Regardless of the extracts type, lower and higher MGT was associated with control and 5 % solution concentration respectively.

Maize growth and root morphological parameters

The length and fresh weight of maize seedlings was assessed by carefully removing the whole seedlings from petri-plates after 10 days of sowing. Comparatively, the seedling treated with CUE exhibited significantly (p=0.05) higher length and weight than those with AQE and an increase concentration resulted in enhanced growth parameters of maize seedlings (Fig. 6). Specifically, 7.5 % concentration recorded significantly higher seedling length of 75.46 and 54.43 cm with CUE and AQE respectively. Conversely, the 5 % solution has significantly higher seedling weight of 15.96 and 7.56 g with CUE and AQE solutions respectively, followed by 7.5 and 10 % solutions.

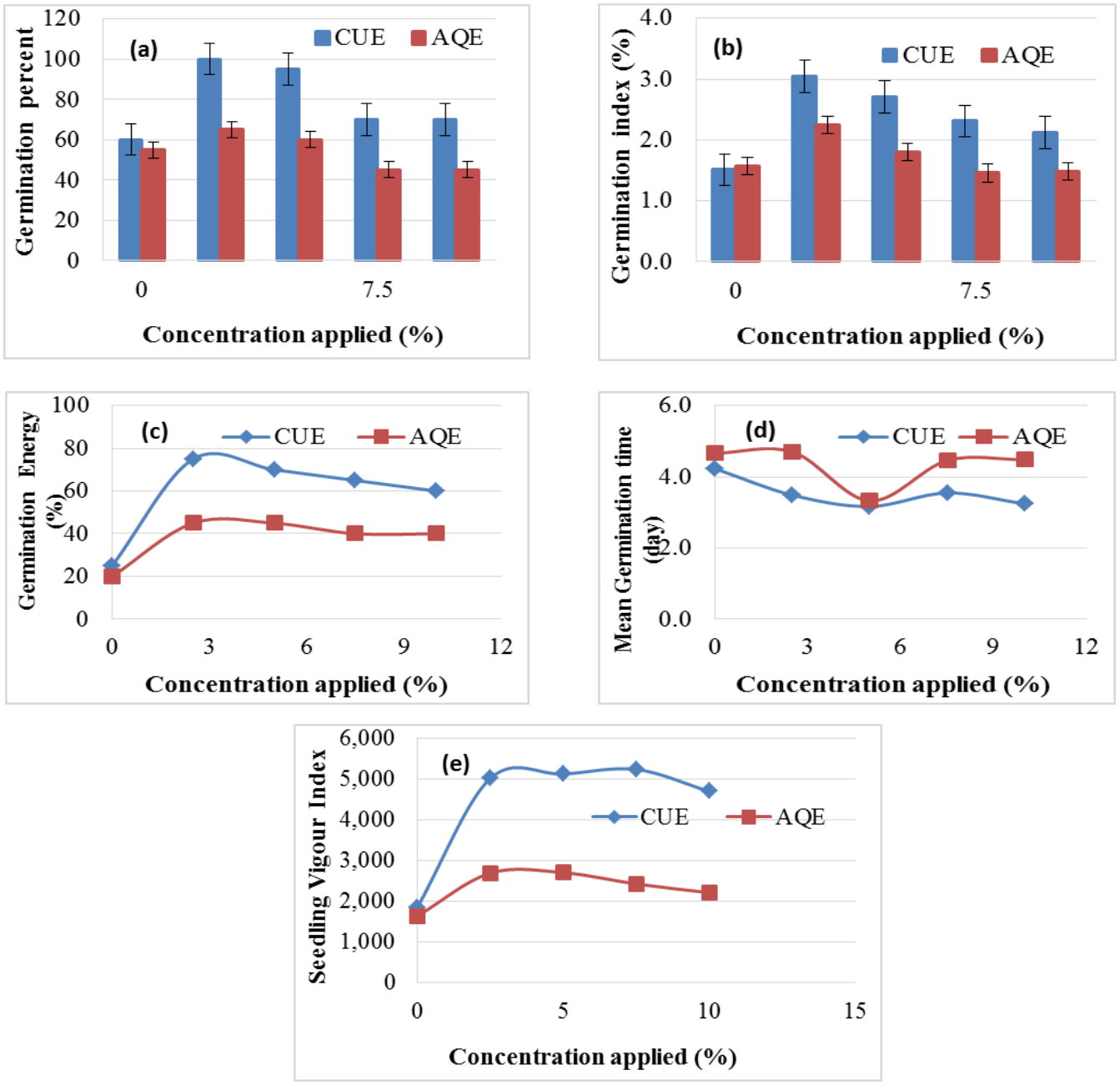

Fig. 5. Influence of various concentrations of aqueous and cow urine extracts on (a) germination percentage (%), (b) germination index, (c) germination energy (%), (d) mean germination time (days) and (e). seedling vigour index.

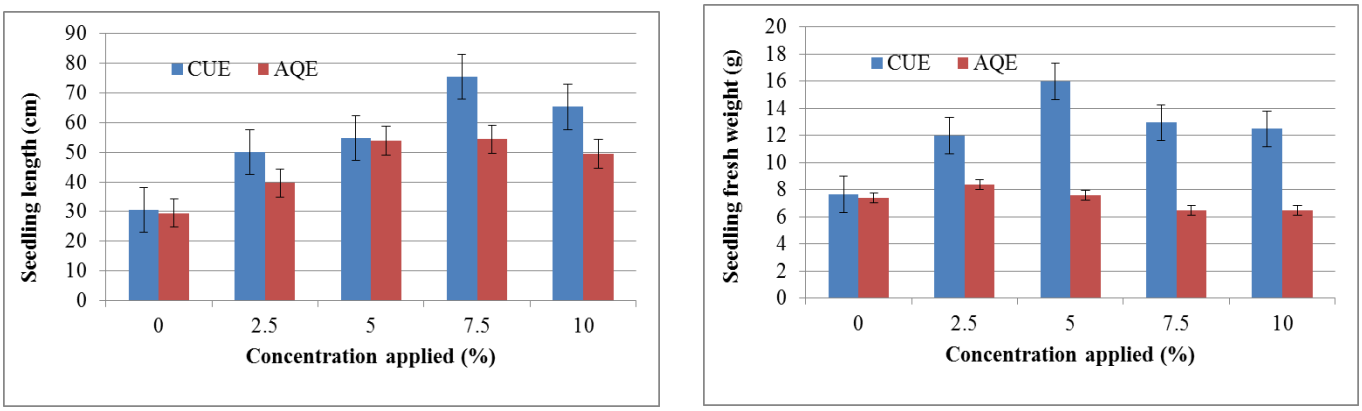

Fig. 6. Representation showing the influence of aqueous and cow urine extracts of RMA and varying concentrations on maize growth (a) seedling length, (b) seedling fresh weight.

Significant variations (p=0.05) were observed in maize root parameters, including root length (RL), root surface area (RSA), average diameter (AvD), root hairs (RH) and root volume (RV), based on the type of extracts and their respective concentrations. Notably, an increase (p=0.05) in all parameters was observed with the application of the 7.5 % solution, regardless of whether CUE or AQE was used, with the exception of the number of tips. Specifically, the number of tips was significantly higher with the 10 % solution compared to the 7.5 % solution. Conversely, the control group exhibited the lowest root parameters. Across different levels of CUE and AQE, RL, RSA, AvD, RH and RV ranged from 169.73 to 399.67 cm, 32.66 to 93.78 cm^2, 0.59 to 0.81 mm, 962 to 1899 cm^3 and 0.50 to 1.72 respectively. The number of root tips ranged from 1025 to 1899 for CUE and from 962 to 11286 for AQE respectively (Table 5).

Table 5. Effect of aqueous and cow urine extracts of seaweed on maize root morphological parameters.

Treatments	RL (cm)	RSA (cm^2)	AvD (mm)	RTs	RV (cm^3)
			Cow urine extract		
0	170.22	32.66	0.60	1025.00	0.50
2.5	201.51	45.84	0.75	1163.00	0.83
5.0	274.18	65.77	0.81	1238.00	1.26
7.5	399.67	93.78	0.70	1815.00	1.75
10.0	241.58	44.47	0.60	1899.00	0.65
Mean	**257.43**	**56.51**	**0.69**	**1428**	**0.99**
			Aqueous extract		
0	169.73	33.91	0.61	962.00	0.51
2.5	278.26	64.63	0.72	999.00	1.31
5.0	294.86	69.66	0.76	1052.00	1.42
7.5	294.43	70.49	0.75	1243.00	1.13
10.0	261.63	49.51	0.59	1286.00	0.75
Mean	**259.78**	**57.64**	**0.687**	**1108.40**	**1.02**

	SE(d)	CD (p=0.05)	SE(d)	CD (p=0.05)	SE(d)	CD (p=0.05)	SE(d)	CD (p=0.05)	SE(d)	CD (p=0.05)
S	2.64	NS	0.43	0.91	0.007	NS	10.89	22.87	0.01	0.02
L	4.17	8.76	0.68	1.43	0.011	0.024	17.21	36.15	0.02	0.04
S x L	5.90	12.39	0.97	2.03	0.016	0.034	24.34	51.13	0.03	0.05

Discussion

Seaweeds, the macroscopic algae, are utilized in agriculture as a rich source of nutrients and substances of growth promotion. Among the various species of marine algae, the red marine alga, *Kappaphycus alvarezii* is gaining attention across various industries due to its carrageenan property and the presence of numerous phyto-chemicals. However, there has been a lack of research on the composition, metabolites and effectiveness in promoting plant growth of *K. alvarezii* sourced from the Indian marine environment, particularly in South India. Thus, the current study aims to characterize *K. alvarezii* and its extracts, tailored to meet the needs of organic agriculture and to investigate their impact on the emergence and growth of maize plants.

Characterization of *K. alvarezii* and its extracts

Analysis of the raw RMA showed presence of all essential mineral elements and trace amounts of heavy metals. The pH of RMA was slightly acidic and high in EC and OC. Among the major nutrients, N, K and S were present in notable quantities, while Fe, Zn and B were found to be predominant among the micro-nutrients. Additionally, seaweeds were reported to contain significant amounts of essential nutrients, growth regulators, vitamins and amino acids (4, 5, 16, 17). Likewise, the raw cow urine utilized for RMA extraction exhibited basic nature and has lesser EC than seaweed and very low OC. Notably, the major nutrients; N and S were present in higher quantity with Fe concentration surpassing other micro-nutrients. Importantly, all heavy metals detected in both raw RMA and cow urine were found within the permissible limits. The presence of essential salts, metals, minerals, vitamins, enzymes and other substances in small amounts in cow urine has also been well documented (18).

GC-MS analyses revealed the presence of fatty acid, metabolites and other growth promoting substances in the RMA. The bioactive compounds identified from RMA were grouped into different classes and their occurrence percent is represented in pie chart (Fig. 6A). Sterols emerged as the dominant class, constituting over 46 % of the total compounds, followed by fatty acid derivatives at more than 34 %. Additionally, organo-silicon derivatives and sugar compounds were also identified, comprising 3.24 % and 3.13 % of the total compounds respectively. Among the growth promoting and protecting compounds detected in RMA, the n-hexadecanoic acid, pentadecanoic acid, octadecanoic acid, sterols, organo silicon were present at higher concentration (based on intensity). The presence of cyclic and monoenoic fatty acids viz., hexa, penta and octadecanoic acids in *K. alvarezii* and sterols and sulfur containing metabolites in seaweeds were previously documented by the researchers (16, 17).

LC-MS analysis of raw RMA revealed the presence of various plant growth-stimulating and promoting compounds (Fig. 6B). The compounds were categorized into several classes: phenolics and benzoic derivatives (16 %), hydroxy cinnamic acids and coumarins (28 %), amino acids and derivatives (38 %), hormones (8 %), isoflavonoids (8 %), tyrosols (4 %) and vitamins (4 %). Among the seven identified amino acids and derivatives, L-proline and L-isoleucine were detected at higher intensities, alongside phenylalanine, valine, alanine and leucine. Additionally, 7 hydroxy cinnamic acids and coumarin compounds, along with 2 flavonoids, hormones and vitamins, were found in the RMA. These findings suggest that RMA could be an effective bioeffector for promoting plant growth. The stimulant effect of *Kappaphycus alvarezii* extract on plants has also been reported in the literature. The presence of amino acids, vitamins, hormones, flavonoids, phenolics, benzoic derivatives, cinnamic acids and coumarins in various seaweed species has been documented previously as well (4, 5).

Nutrient elements extraction efficiency by cow urine and water

The mineralogical composition of CUE and AQE of red marine algae were analyzed and compared to the raw RMA composition to assess the extraction potential of cow urine and water. The cow urine has extracted significantly higher amount of mineral elements from the RMA compared to AQE. The average extraction efficiencies of primary, secondary, micro nutrients and heavy metals were 34, 31, 10 and 5 % for CUE and 3, 6, 6 and 12 % for AQE respectively (Fig. 7). Both cow urine and water extracted significantly higher quantities of nickel (10.10 and 32.43 % respectively) compared to other heavy elements. These showed that the extraction efficiency of cow urine was quiet superior than de-ionized water. As pioneers in this type of organic extraction of seaweed, there are no previous publications to compare with these findings. The best extraction efficiency traits were identified by comparing the concentration of nutrient elements extracted into solution, viscosity and odor of the extracts. Due to the carrageenan in seaweed, which is a naturally occurring jell like material, the extracts becomes viscous and has a foul fish odour. The viscosity of seaweed carrageenan extracts varies depending on temperature and the type of extracting solution, ranging from 3.02 to 45.91 cp (19, 20). Cow urine was able to extract significantly higher quantity of the nutrient elements from the RMA, likely due to the reduced viscosity and increased solubility of the minerals promoted by the organic compounds in cow urine. The reduced viscosity could be ascribed to partial decomposition of carrageenan by the alkali composition of the cow urine. The decreased or change in viscosity of carrageenan isolated from *K. alvarezii* by the alkali materials and heat or temperature has been previously reported (19).

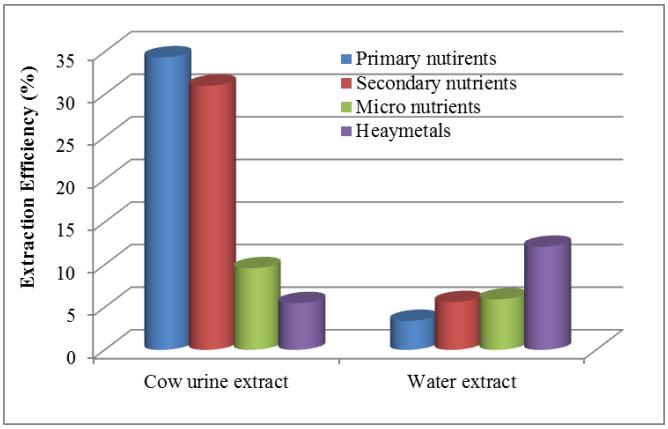

Fig. 7. Graph showing the class wise nutrient extraction efficiency by cow urine and water from red marine alga (AQE- aqueous extract; CUE- Cow urine extract).

Stimulant potential of organic RMA extracts on maize

The RMA extracted with cow urine and de-ionized water was tested to evaluate the germination and seedling growth of maize at various concentrations (0, 2.5, 5.0, 7.5 and 10 %) using a petri-dish assay. The stimulant potential was assessed by deriving various parameters viz., germination percentage, germination index, germination energy, mean germination time and seedling vigour index. All germination parameters were highest with 2.5 % concentration and decreased with higher concentrations. Similar effects of various seaweed species extract on germination and seedling vigor of wide range of crops have been documented (5). Among the CUE and AQE, the nutrients content in CUE was significantly ($p=0.05$) higher and effective in enhancing maize germination, while AQE showed negative effects at higher concentrations of 7.5 and 10 %. CUE has resulted in 30-35 higher GP and 20-30 % higher GE than AQE respectively than AQE, regardless of concentration applied. The lowest MGT was observed in the control group, with highest MGT at a 5 % solution concentration for both the extracts. The seedling vigor index was 2.65-4.40 % higher with CUE compared to AQE across all concentrations. Unlike the GP, GI and GE, the SVI was significantly higher with 7.5 % concentration of CUE (182 % higher than control) and 5 % concentration of AQE (65 % higher than control) treated assay, likely due to the second application after 1 week of germination. Previous studies also showed improved maize germination with a 5 % extract of *K. alvarezii* and decreased germination at 10-15 % concentrations (21, 22). However CUE of seaweed known to influence significantly the germination assays of the maize irrespective of its essential nutrients content and plant growth hormones.

In addition to germination parameters, seedling length, biomass and root traits were measured to assess the potential of RMA extracts on maize establishment and growth. Seedling length increased with increased extract concentration, peaking at 7.5 %, while seedling biomass was highest at 5.0 % for CUE and 2.5 % for AQE. The CUE enhanced the seedling length and biomass by 21 % (at 7.5 % solution) and 8 % (at 5 % solution) compared to AQE. This could be attributed to the higher amount of essential mineral elements in CUE, in addition to amino acids and plant growth-promoting bioactive compounds, as detected by ICP and LC-MS in their composition (4, 16, 23).

The mixture of these beneficial attributes is directly involved in metabolism and promotes cell division and elongation, irrespective of the seaweed extract concentrations documented in our study. Similar results and mechanisms of seaweed extracts have been documented in previous studies (4, 5). In the present study, GC-MS analysis also showed the presence of (N,N'-(1,2-Propylene)-thiourea and Dodecyl-bis (trifluoromethyl) phosphine sulfide) in raw RMA, which may have been extracted more by cow urine than by water, significantly influencing seedling length and biomass. In addition, the detected amino acids such as phenylalanine and alanine (by LC-MS) may serve as sources of carbon and nitrogen, which are critical to early seedling growth.

The measured root traits showed significant variation based on the sources and concentration of extracts. Regardless of concentration applied, both CUE and AQE were on most root parameters except root tips, which were significantly higher for CUE than AQE, with the mean values of 1428 and 1108 numbers respectively. This could be attributed to the stimulatory impact of phytochemicals like Indole acetic acid (the predominant auxin in higher plants) and kinetin in RMA extracts beside the increased extraction of various macro-and microelements in CUE from RMA. Similar findings were reported previously for the effects of seaweeds and cow urine on sorghum plants growth (22, 23). Roots are more sensitive to auxin and other bioactive compounds in algal extracts, which influence lateral root and hair formation, promote root growth, modify root architecture and improve root water-use efficiency (24-26). Mechanisms such as apical dominance, tissue differentiation, plastid division and elongation are directly regulated by indole acetic acid (auxin). Hence, the organic RMA extracts prove that a mixture of substances exists and their stimulant potential on the seed-to-seedling transition of maize has a positive impact.

Conclusion

The present study reveals that the red marine alga, *Kappaphycus alvarezii*, contains a wide range of essential minerals and bioactive compounds beneficial for plant growth. Cow urine extracted 23-44 % of major nutrients and 2-30 % of micronutrients from the raw alga, compared to 0.9-8.9 % and 0.2-24.2 % respectively, by water. Due to its high mineral content, CUE, regardless of the concentration tested, enhanced maize germination, seedling vigor index, seedling length, biomass and root traits (volume, length, diameter, number of root tips etc.). Bioassay results indicated that 5.0 % and 7.5 % concentrations of CUE are optimal for maize germination and seedling growth. In conclusion, the cow urine-based extract of *alvarezii* shows its potential as a suitable and environmentally safe biostimulant for organic farming, aiding in crop establishment. Further validation is necessary to fully assess its impact on maize growth and production under field conditions.

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Authors' contributions

VV carried out the investigation and wrote the original draft. PJ conceptualized the work and wrote review and edited draft manuscript. TC coordinated the study. EP involved in designing methodology and supervised the experiments. MS participated in designing and coordination. RK involved in designing study. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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