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Graphene oxide nanosheets were obtained by the partial reduction of graphene oxide using a seaweed sap extracted from the red seaweed *Kappaphycus alvarezii* at 60 ^{o}C. Synergistic effect of both flavonols and transition metals present in the seaweed sap were found to be responsible for the partial reduction.

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Abstract: Large-scale and low-cost production of graphene is achieved widely by the chemical reduction of graphene oxide (GO). In this study, partially reduced GO (prGO) was prepared using a seaweed sap extracted from the red seaweed *Kappaphycus alvarezii* (**1**). The seaweed sap is a plant growth stimulant used as a foliar spray. The sap is consisted of both organic and inorganic micro and macro nutrients such as plant growth regulators (PGRs), flavonols, choline, glycine betaine and salts of potassium, magnesium, iron, zinc *etc*. In order to understand, the exact component of the sap responsible for the reduction of GO, different formulations of the sap devoid of PGRs and flavonols (**2**) as well as all organic matters (**3**) were prepared. It was observed that partial reduction of GO took place in both **1** and **3**, while the reduction was not successful in **2**. It was concluded that synergistic effect of both flavonols and transition metals present in the sap was responsible for the reduction. The recovered sap obtained after the isolation of prGO had the similar chemical composition in comparison to the original sap and hence could be used as plant foliar spray.

Introduction

Kappaphycus alvarezii (*K. alvarezii*) is a red seaweed grows in tropical waters and commercially exploited for κ-carrageenan, a phycocolloid widely used in food and beverage industry as thickener and stabilizer. [1] The fresh seaweed can be squeezed mechanically to yield a liquid (*K*-Sap), which is proven as an effective foliar spray for the enhancement of the growth/yield as well as quality of various crops. [2,3] The sap, which is being commercially produced is found to be rich of various micro and macronutrients as well as plant growth regulators (PGRs). [4] These enormous potentials of the sap as well as prolific growth of the seaweed has led to the large scale cultivation of the seaweed and is being successfully cultivated in various countries including India. [5]

Graphene, the 2D carbon nano-sheets is one of the most sought materials in the material research due to its unique chemical, physical, electrical and mechanical properties and being used in various domains of research. [6] There are number of ways to prepare graphene but reduction of graphene oxide using hydrazine is considered as one of the most convenient method for the production. [7,8] Due to certain drawbacks of this reduction method such as possible contamination of end products, toxicity *etc*., many other reducing agents are being used instead of hydrazine. Various natural compounds such as L-ascorbic acid, gallic acid, amino acids and plant extracts such as green tea, *C. esculenta* leaf, *M. ferrea Linn.* Leaf, *C. sinensis* peel and *R. damascene* (rose water) *etc*. are also being reported to reduce graphene oxide. [9-16] We have also developed a green method for the simultaneous production of hydroxymethylfurfural and rGO in ethyl lactate as well reduction of GO by bio-ionic liquids. [17] Although there is a report on exfoliation of GO sheets using concentrated salts [18] but no attempts has been made so far to use seaweeds or sap expelled out from seaweeds as a media to reduce GO.

Considering abundance of the seaweed *K. alvarezii* in both natural seawater and its successful cultivation it is worthwhile to use the sap expelled out from the seaweed as a sustainable alternative to existing systems to reduce GO. Unlike other natural extracts used for the job, the sap can be reused as plant stimulant after the recovery of prGO. Moreover, studies were made to understand the role of individual components of the sap responsible for the reduction.

Experimental Section

Materials: Graphite nano powder (average particle size: 400 nm) was purchased from SRL Chemicals, Mumbai, India. The chemicals used for the oxidation of graphite including sodium nitrate, concentrate sulphuric acid, potassium permanganate ($KMnO_4$) and hydrogen peroxide (H_2O_2, 30 wt%) were all AR grade and procured from S.D. Fine Chemicals, Mumbai, India and were used without any further purification. *Kappaphycus alvarezii* was collected from the cultivation site at Mandapam (9°15′N, 78° 58′E), Tamilnadu (India).

Synthesis of graphene oxide:

Hummer's graphene oxide was synthesized following literature procedure. [19] In a typical reaction 0.5 gm of graphite nano-powder, 0.5 gm sodium nitrate and 25 ml of concentrated sulphuric acid was added in a round bottom flask fitted in an ice bath to maintain the temperature of 0-4 ºC, into which 3 gm of potassium permanganate ($KMnO_4$) was slowly added with constant stirring. The suspension thus obtained was transfered to a 35 ± 5 °C water bath and further stirred for another 1h followed by the addition of 40 ml of milli-Q water and the temperature was raised to 90 ± 5 °C for 30 min with continuous stirring. Finally, 100 ml of milli-Q water was added into the above reaction mixture followed by the slow addition of 3.0 ml of H_2O_2 (30%) till the colour of the solution changed from dark brown to yellowish. The resulting solution was centrifuged and the residue was washed with milli-Q water for several times (residue was first washed with 5 % diluted HCl, 0.3 % diluted H_2O_2 and finally with pure milli-Q water to remove all the undesired impurities from oxidised graphite) followed by centrifugation. Finally the residue was lyophilised to obtain graphene oxide (GO).

*Extraction of sap from the red seaweed Kappaphycus alvarezii (**1**):*

Freshly harvested *K. alvarezii* was mechanically crushed to obtain pinkish coloured sap (**1**) which was stored with preservatives as literature procedure. [4a]

*Preparation of charcoal treated sap from pure sap (**2**):*

To remove colour and plant growth regulators present in the pristine sap it was treated with activated charcoal. In a typical reaction 4 g of activated charcoal was added into 100 mL of sap (1) and the mixture was stirred at room temperature for 2h. After that, the solution mixture was filtered through Whatman 41 filter paper to obtain colourless liquid (**2**).

*Preparation of sap free from organic matter (**3**):*

To prepare sap free from organic substances, 100 mL of sap (**1**) was freeze dried to obtain 4.60 g of solid pinkish powder. This powder was calcined under air at 550 ºC in a furnace for 5h. The ash contain was measured to be 3.5 g (80 % of the freeze dried powder). This ash was reconstituted with required amount of deionised water to make it 3.5% w/v (reconstituted sap devoid of organic substances) (**3**).

*Method for the reduction of graphene oxide in K-sap (**1**):*

For the reduction of GO, aqueous suspension of GO was prepared by dispersing 100 mg of GO in 20 ml of deionised water under sonication for 30 min followed by the addition of 80 ml of filtered K-sap (**1**) at room temperature. Thus obtained suspension was heated at 60 °C until the colour of the brown suspension was changed to black. Finally rGO thus obtained was isolated by centrifugation and washed repeatedly with milli-Q water.

Characterization:

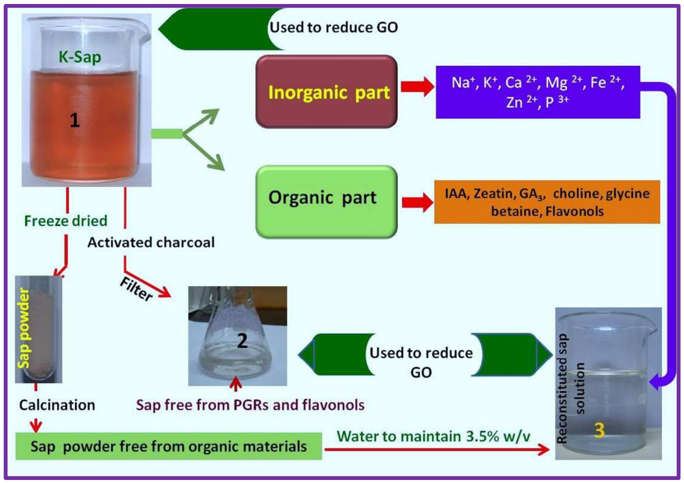
UV-Vis absorbance spectra were recorded on a CARY 500 Varian 8.01 UV-Vis spectrophotometer. Powder X-ray diffraction patterns were recorded at 298 K on a PANanalytical system using Cu anode, K_α radiation (λ = 0.15405 nm) with 2θ range from 5° to 80° at a scan speed of 0.1° sec^{-1}. Fourier transform infrared (FT-IR) was performed on a Perkin-Elmer FT-IR machine (Spectrum GX, USA) using KBr pellets in the range 4000-400 cm^{-1}. Thermo gravimetric analysis (TGA) was carried out on a NETZSCH TG 209F1 Libra TGA209F1D-0105-L machine using a temperature programmer 30-800 °C at a heating rate 5 °C min^{-1} under a nitrogen atmosphere. Transmission electron microscope (HRTEM) images were recorded on a JEOL HR-TEM (JEOL JEM 2100, Japan) instrument operated at accelerating voltage of 200 kV. Atomic force microscopy (AFM) imaging was carried out on an Ntegra Aura atomic force microscope (NT-MDT, Moscow) instrument in semi-contact mode using an NSG 01 silicon probe. Scanning electron microscopy (SEM) images were recorded on a SEM (Carl-Zeiss) model LEO 1430 VP, Germany, at an accelerating voltage of 20 kV. Electrospray ionization (ESI)-MS measurements were carried out on a Q-TOF micro mass spectrometer (USA), equipped with an electrospray ionization source, time- of- flight (TOF) analyser and micro-channel plate (MCP) detector. The mass spectrometer was run employing direct infusion technique (DIMS). Mass fragmentation patterns were recorded in ESI positive mode (ESI^+). Parameters such as desolvation temperature (150 ºC), source temperature (90 ºC), syringe rate (10 µl/min), ion energy (2.0 V) and collision energy (7.0 V) were maintained constant for all of the ESI-MS measurements whereas capillary voltage and sample cone voltage were optimized for each sample. Nitrogen gas was used as a nebulizer gas to accelerate the droplets formed during electro spray at a flow rate of 450 L/h. Mass spectra were recorded in the mass range of m/z 50-500 and data was generated using Masslynx 4.0 software (Waters corp., UK). For MS/MS studies, the parameters were once again optimized to obtain proper mass fragmentation.

Results and discussion

The brownish colour suspension of GO was turned to black after heating at 60 ^{o}C for 6 h in presence of *K*-sap (**1**) as shown in Figure 1 indicating reduction of GO and formation of graphene sheets. The UV-Vis spectra of the black suspension are depicted in Figure 2. The much lower absorbance for GO at λ_{max}= 230 nm corresponds to π-π* transition of the C–C aromatic rings. [20] After the treatment of GO with **1**, λ_{max} was shifted to 271 nm indicating reduction of GO. Further the C/O ratio measured using elemental analysis was 1.3 for GO, which was increased to 1.48 after the treatment with **1** further confirming decrease in the oxygen functionalities in GO. Since *K*-sap contains both organic matters and inorganic salts (Supporting Table S1) and hence to understand the role of individual components responsible for the reduction a strategy to prepare sap samples devoid of particular component was adopted as outlined in Scheme 1.

Figure 1. Reduction of graphene oxide in the presence of the seaweed sap extracted from *Kappaphycus alvarezii*.

At first, the sap **1** was treated with activated charcoal. The sap thus obtained after filtration was found to be free from PGRs and flavonols but metal salts were present in it (**2**) [Supporting Scheme S1]. Both the saps **1** and **2** were tested for the presence of flavonols by qualitative method [Supporting Figure S1]. Furthermore **1** was freeze dried and calcined at 550 °C resulting elimination of all organic matters, the metal salt mixture thus obtained was reconstituted to give **3** as shown in Scheme 1. UV-Vis spectra of GO suspension in **2** after heating at 60 °C for 6h did not show appearance of sharp λ_{max} with red shift. However, UV-Vis spectra of GO suspension showed sharp λ_{max} at 256 in **3** (Figure 2). In the case of **1** and **3**, the occurrence of red shift in the absorption is owing to the increase in the electron concentration due to the removal of sp^3 carbon as well as restoration of sp^2 carbon atom indication of reduction of GO. [21] Furthermore the time duration required for the complete reduction as observed by UV-Vis experiment was 6h (Supporting Figure S2).



Scheme 1. Preparation of different formulations of *Kappaphycus* sap

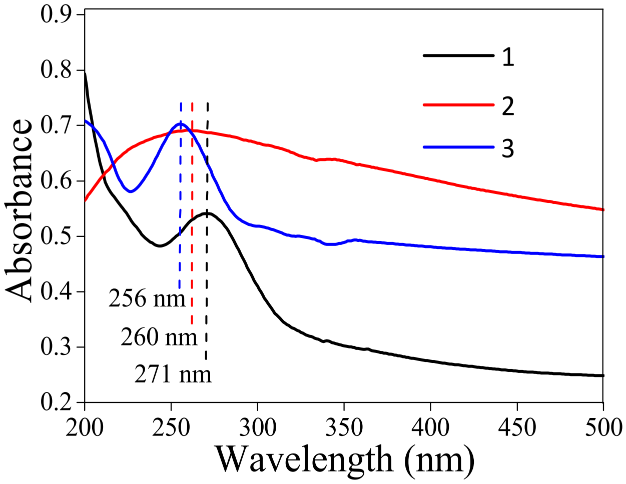


Figure 2. UV-Vis spectra of GO reduced using different formulation of *K. alvarezii* sap, pure K-sap (**1**), charcoal treated k-sap (**2**) and organic matter free *K*-sap (**3**) measured at 50 µg/ml.

The FT-IR spectral bands of synthesized GO by Hummers & Offeman's method matches well with the bands reported for the oxide. [22] Absorption bands in the range of 1205-1315 cm^{-1} is due to C-O-C asymmetric stretching vibration of the epoxides. Sp^2 hybridized C=C in plane stretching vibration are shown between 1500-1675 cm^{-1}. Absorption bands in the range 1695-1790 cm^{-1} is due to C=O stretching vibration of carbonyl of COOH and all C-OH vibration appeared at 3420 cm^{-1} and 1073 cm^{-1} is from COOH and H_2O (Figure 3a). The GO obtained after the treatment with **1** and **3** showed reduction in the intensity of the band of C=O of COOH indicating reduction of GO (Figure 3b and Supporting Figure S3). However the bands due to other oxygen functionalities are very little affected indicating partial reduction of GO after the treatments. Moreover, no such notable reduction in the intensity of C=O was observed for the GO treated with **2**.

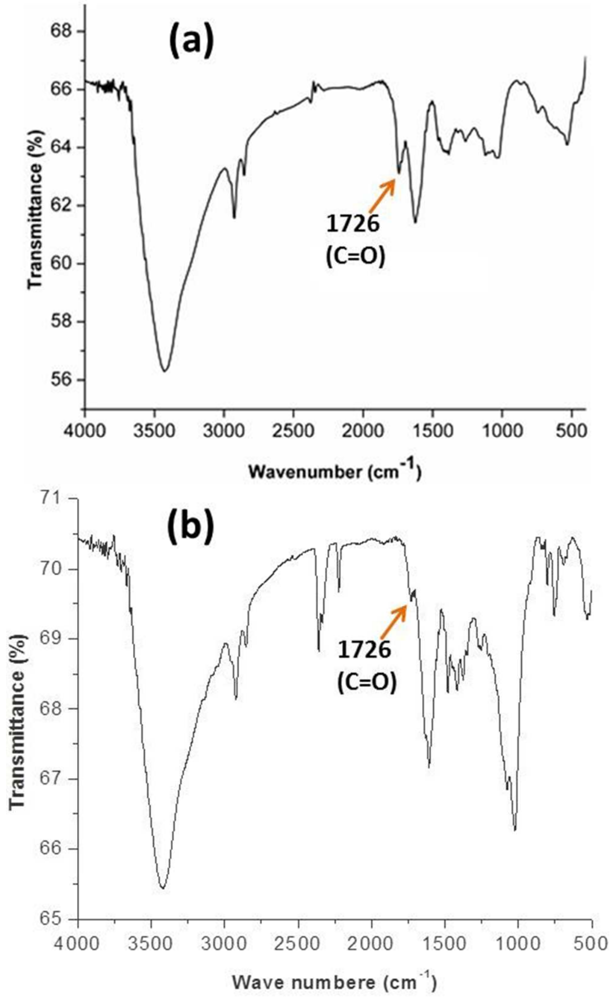


Figure 3. FT-IR spectra of (a) graphene oxide and (b) GO treated with *K*-sap (**1**).

The powder X-ray diffraction (PXRD) pattern of pure GO showed a characteristic peak of (001) at 2θ = 11.58° having interlayer spacing of ~7.64 Å, confirmed the presence of oxygen functionalities after oxidation of graphite. The peak of (002) at 2θ = 26.53° and the peak of (010) at 2θ = 42.42° are original graphite peaks. [23] The XRD pattern of GO treated with **1** and **3** exhibited a new peak broadening closer to (002) at 2θ = 20.73°, having much lower interlayer spacing of ~3.36 Å (Supporting Figure S4). However the sharpness of the peak was nbo0t very good, which perhaps due to the exfoliation of GO sheets by the metals salts present in the sap solutions. This observation confirmed partial reduction of GO by these two sap solutions.

To further confirm the reduction of GO, the micro-Raman spectra of the GO samples were recorded. Pure graphite showed D/G intensity ratio of 0.45 and pure GO showed the value of 0.89. For GO treated using **1,** the value of the ratio was increased to 0.98 (Figure 4) indicating formation of more sp^2 domains after the *K*-sap treatment and supported the reduction of GO described above. [24] The GO treated with **3** also showed D/G intensity ratio of 0.99 confirming the reduction (Supporting Figure S5). However the typical 2D peaks due to graphene were not visible in both the cases. This was perhaps due to the exfoliation of GO sheets by the metals salts present in the solutions. The observation confirmed formation of partially reduced GO sheets.

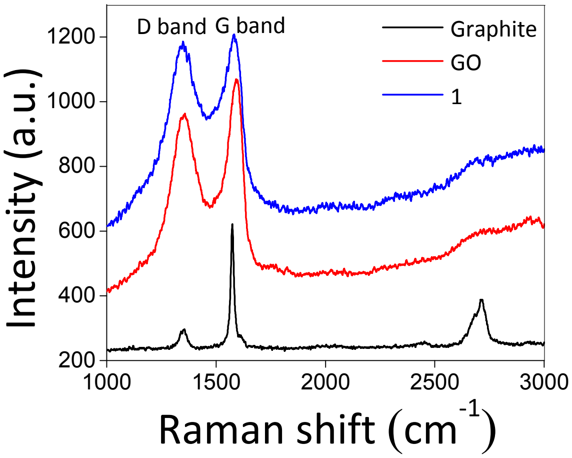


Figure 4. Raman spectra of graphite (black), GO (red) and GO treated with pure K-sap (**1**) (blue).

The scanning electron microscopic images (SEM) (Supporting Figure S6) and AFM (Supporting Figure S7) images clearly displayed the sheet like structure of the pure untreated GO (dia. 2.1µm and height 20 nm by AFM) and partially reduced GO (dia. 2.5µm and height 15 nm by AFM) using **1**. The HR-TEM images of pure untreated GO showed presence of multiple graphene layers (12 nos) with interlayer spacing of 0.37 nm while only four graphene layers with inter layer spacing of 0.34 nm were visible in the GO treated with **1** (Figure 5). This supports exfoliation of GO sheets during the reduction process. Similar observation was also made for the partially reduced GO obtained using **3** (Supporting Figure S8).

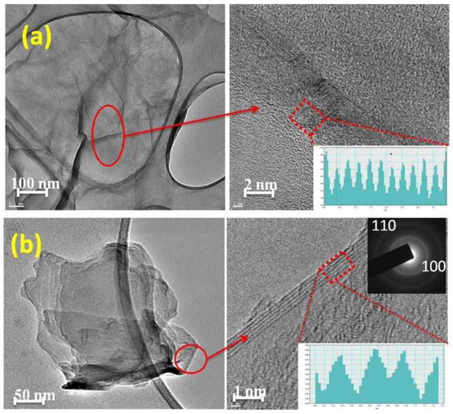


Figure 5. HR-TEM image of (a) GO and (b) partially reduced GO obtained using pure K-sap (**1**).

From above analytical measurements it is clear that, pure *Kappaphycus* sap (**1**) and the sap free from all organic constituents (**3**) were able to partially reduce GO. Since the sap free from PGRs and flavonols was not able to reduce GO (**2**) and hence it is imperative that, the PGRs or flavonols must have played important role in the reduction process. Further, it was observed that, the prGO isolated using **1** was found to have indole acetic acid and trans-zeatin. This is perhaps due to the ability of these two PGRs (presence of carboxylic and N-H group) to form hydrogen bonds with rGO, whereas due to the bulky structure and unfavourable steric orientation of carboxylic group in GA_3 it did not form the hydrogen bonds with rGO (Supporting Scheme S2). Furthermore, standard GA_3 solution was not able to reduce GO. All these observations confirmed that PGRs were not responsible for the reduction of GO. Furthermore, transition metal salts in the form of chloride or sulphates were present in **2** but the metal salts were present in oxide form in **3**, which perhaps helped the reduction. To confirm this hypothesis, a simulated mixture of NaCl, KCl, $CaCl_2$, $MgCl_2$, $ZnCl_2$ and $FeCl_3$ with a concentration similar to that present in the sap was prepared and used as such and after calcination at 550 ^{o}C for the treatment of GO. From UV-Vis spectra it was observed that, the GO did not reduce by the mixture of salts before calcination, however the calcined mixture of salts could able to reduce the oxide (Supporting Figure S9). This proves the hypothesis that the metal oxides perhaps helped the reduction.

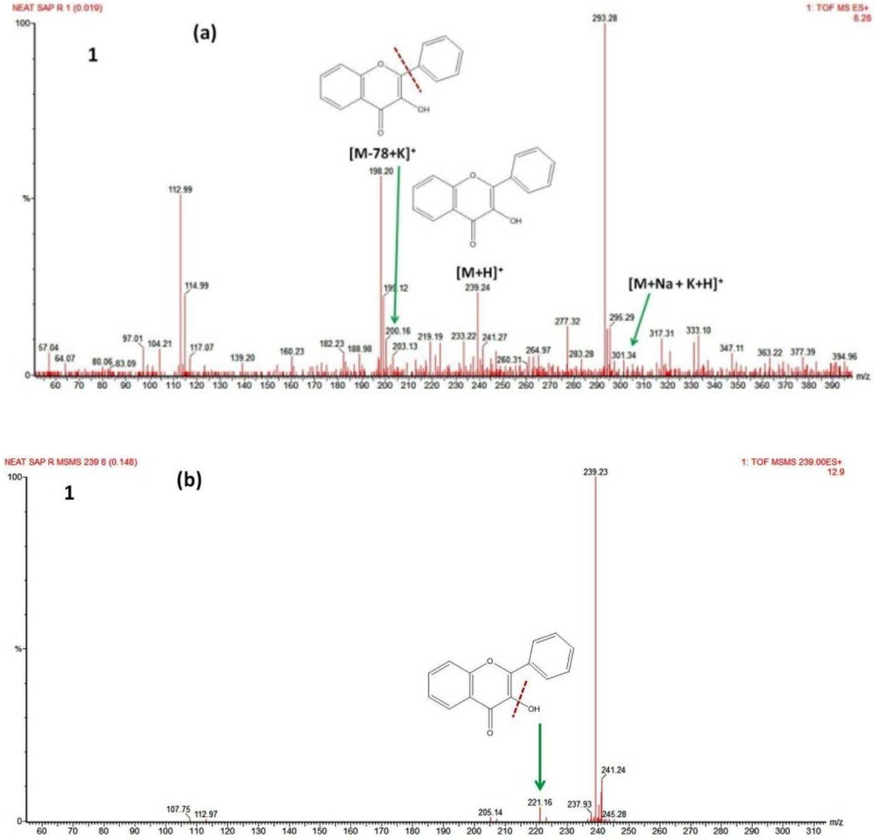


Figure 6. (a) ESI-MS analysis of pure sap (**1**) and (b) MS/MS of *m/z* of 239

The seaweed *K. alvarezii* is reported to contain flavonoids and such phenolic compounds are known to reduce GO and hence the flavonoids perhaps also responsible for the reduction of GO. Since positive indication for the presence of such compounds were observed due to the formation of fluorescence upon addition of ammonium hydroxide in **1**, while **2** did not give formation of fluorescence indicating absence of flavonoides in the sap [Supporting Figure S1]. [25] In order to confirm presence of flavonoid structures, the ESI-MS and MS/MS analysis was carried out, which confirmed presence of flavonol skeleton in **1** (Figure 6a and 6b) and presence of same skeleton was also observed the sap obtained after autoclaving (Supporting Figure S10). It was observed earlier that, autoclaving of the sap destroys the PGRs, while flavonols remained intact in the sap. This results indicates that, the flavonols were responsible for the partial reduction of GO not the PGRs.

Further, as discussed above it was observed that, the sap devoid of any organic matter (**3**) was also able to reduce GO and it is evident from Table S1 that the sap contains transition metals such as Zinc and Iron. Further, it was also observed that the transition metal oxides were perhaps responsible for the reduction. Furthermore, it was also evident from Figure 1 that the reduction ability of **3** was lower in comparison to **1** and hence it can be concluded that synergistic effect of both flavonols and transition metals present in *K*-sap was responsible for the partial reduction of GO. The composition of the *K*-sap after the isolation of prGO was found to be similar to that with pure K-sap (Table S2) and hence can be reused as foliar spray, the same way the pure sap is used (Supporting Scheme S3).

Conclusion

In summary, we have demonstrated that the sap extracted from the red seaweeds *Kappaphycus alvarezii* as a potential sustainable media for the partial reduction of graphene oxide and for the production graophene oxide nanosheets. The synergistic effect of both flavonols and transition metals present in *K*-sap was found to be responsible for the partial reduction of GO.

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