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ORIGINAL PAPER

Spectroscopic characterization of humic substances isolated from tropical mangrove sediments

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Abstract

Mangroves are tropical wetlands that are among the most productive ecosystems on Earth which cover up to 75% of the coastlines. Humic substances are the ever-present natural organic compounds, being a major component of organic carbon in the global carbon cycle. The present study focuses on the organic carbon dynamics as well as spectroscopic characterization (UV, FT- IR, and NMR) of humic substances—humic acids (HAs) and fulvic acids (FAs)—isolated from three tropical mangrove sediments representing different environmental settings belonging to Kerala, in the southwest coast of India during a premonsoon sampling conducted in April 2018. The study revealed a higher concentration of HAs than FAs in all the stations which was also complemented by a higher concentration of tannin and lignin as well as TOC. The E4/E6 ratios of FAs were also higher than that of HAs confirming its low molecular weight and less polymerized nature. The FTIR spectrum of both HAs and FAs showed peaks corresponding to the existence of carboxyl, phenol, carbonyl, and amide group. The proton NMR spectrum showed the presence of aliphatic regions slightly more controlled with long chains and/or alicyclic moieties rather than methyl groups which are also supported by the higher hydrogen content in HAs than FAs, and these peaks were absent in FAs. It also showed the presence of more or less common aromatic core involved in unsaturated structures as well as other aromatic groups like phenols and ionized carboxylic groups slightly higher in FAs than in HAs.

Keywords Mangroves · Humic acids · Fulvic acids · Spectral characterization · Aliphaticity · Aromaticity

Introduction

Mangroves or tropical tidal wetlands are among the most productive coastal habitats and dynamic zones of fishery potential areas (Kaladharan et al. 2003) and cover up to 75% of the coastlines (Pernetta 1993; Ranjan et al. 2010). The average productivity of mangroves is 2500 mg C day^{-1} (Kristensen et al. 2008) making them one of the most productive ecosystems on the earth (Rani et al. 2021; Bouillon et al. 2008; Jennerjahn and Ittekkot 2002). Due to high productivity and turnover rates, mangroves play a crucial role in the biogeochemical cycling of P, C, N, and other nutrients in coastal environments (Kristensen et al. 2008; Singh et al. 2005; Ranjan et al. 2010). Although mangroves cover only 0.1% of the continents' surface, they can account for more than 10% of the dissolved organic matter transported to the oceans (Jennerjahn and Ittekkot 2002; Dittamar et al. 2006). Organic matter of both autochthonous and allochthonous origin derived from coastal wetlands, mangrove forests, riverine input, freshwater, and marine phytoplankton accumulates in these ecosystems (Ranjan et al. 2010). Mangroves act not only as a carbon source (carbon out-welling) but also as a carbon sink for the burial of mangrove assimilated carbon in sediments (Enog 1993; Zhang et al. 2011; Rani et al. 2021). The average sediment carbon density of mangroves swamps is 0.05 ±0.004 g/cm^3 (Chmura et al. 2003; Ferreira et al. 2009; Zhang et al. 2011) contributing to a large pool of carbon which plays a major role in the global carbon biogeochemical cycle in the coastal system (Zhang et al. 2011). The studies dealing with soil organic matters in mangrove sediments are limited (Sierra et al. 2004; Ferreira et al. 2009; Ranjan et al. 2010; Zhang

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et al. 2011). It was considered by high liquid content and low solid content. The large number of holes that were occupied air, water, gas (Matsui et al. 2015) and is going to limit the biogeochemical processes involved in sedimentary organic matter in mangrove areas (Zhang et al. 2011). The elevated organic carbon production of mangrove wetlands influenced the accumulation of this aliphatic rich humic-composition. As the chief constituent of soil organic matter, humus is the central exporter of fractional carbon and nutrient pools, and the degree of humification is one of the significant features disturbing the build-up of soil organic matter in forest biomes (Kwiatkowska-Malina 2018; Yang et al. 2019).

Humic substances (HS) are the most well-known and ever-present natural non-living organic materials in aquatic and terrestrial environments, and characterize a major proportion of the organic carbon in the global carbon cycle (Allard et al. 1991; Giovanela et al. 2004; Yang et al. 2019). HS constitutes a major fraction of the soil organic matter (up to 80%) and the largest fraction (up to 60% of the dissolved organic carbon) of natural organic matter in aquatic systems (Stevenson 1994; Giovanela et al. 2004; Fernandes et al. 2010; Sarlaki et al. 2020). The properties and structure of HS may vary significantly, depending on the characteristics of the water or soil, of the compounds of origin, maturation of the HS, and specific conditions of extraction (Fong and Mohamed 2007; Garcia et al. 2016; Muscolo et al. 2013; Rigobello et al. 2017). Humic substances originate from the decomposition and further subsequent polymerization of organic deposits of plant and animal origin leading to heterogeneous supramolecular species with large molecular weights (Reddy et al. 2014; Carlose et al. 2011; Martin et al. 2014) being polydispersive molecules with different functional groups, the elemental configuration and properties of which differ rendering to the source of organic matter and the surroundings in which it is designed (Rashid 1985; Fernandes et al. 2010). Conversely, the richness of carboxylic and phenolic substituents is associated with terrestrial contributions, demonstrating that lignins and, perhaps, tannins play a significant role in the construction of humus in continental settings (Stevenson 1994; Fernandes et al. 2010). Generally, the degradation of humic substances give rise to their decolorization, and the constitutions of humic substances with a lighter color change gradually (Ikeya et al. 2015;Yangi and shindo 2016;Yang et al. 2019). Based on their solubility, HS are classified into three main fractions, humic acids (HA), which are soluble in alkali, but insoluble in acid; fulvic acids (FA), soluble in alkali and acid; and humins, insoluble in both (Rigobello et al. 2017). These fractions vary significantly in molecular size and functional group content, the main functional groups being carboxylic and phenolic along with minor amounts of other groups like amino acids (Cezikova et al. 2001; Anuradha et al. 2011), and HAs are generally larger than FA (Samios et al. 2007; Fong and Mohamed 2007; Rigobello et al. 2017).

No single analytical tool, however, can provide definitive structural or functional information about the natural organic matter because of its heterogeneous, ill-defined nature (Chen et al. 2002). Spectroscopic techniques such as nuclear magnetic resonance (NMR), Fourier-transform infrared (FTIR), electron paramagnetic resonance (EPR), ultraviolet-visible (UV/Vis), and fluorescence have been applied for both quantitative and qualitative characterization of NOM (Stevenson and Goh 1971; Schnitzer and LeVesque 1979; Schnitzer and Preston 1986; Leenheer et al. 1987; Sensi et al. 1989; Sensi et al. 1991; Gu et al. 1994; Chen et al. 2002; Anuradha et al. 2011; Prashob Peter 2014; Rigobello et al. 2017; Jennees et al. 2019). The objective of this study is the isolation and characterization of humic substances such as humic acid and fulvic acid from three different mangrove ecosystems belonging to the state of Kerala situated along the southwest coast of India. This study makes use of sp\ectroscopic techniques as well as the elemental composition of isolated humic materials from these diverse mangrove environments situated in a tropical region like Kerala to get an idea about the structural characteristics of isolated materials concerning environmental settings.

Materials and methods

Study area and sampling

Mangroves in India account for about 5% of the world's mangrove vegetation which expands over an area of about 4500 km^2 beside the coastal States/UTs of the country (Mini et al. 2012). Mangroves are highly localized in Kerala, but the species diversity and their associates are comparatively rich and are confined to estuaries, lagoons, backwaters, and creeks. In Kerala, mangrove forests that once occupied about 700 km^2 have now dwindled to 17 km^2; the vegetation has diminished in its extent drastically and has acquired a "threatened" status in Kerala (Basha 1991, 1992). In Kerala, the maximum extent of mangroves is reported from Kannur district, belonging to Northern Kerala (1100 ha.), followed by Ernakulam (600 ha.), and the minimum extent is reported in three districts, Malappuram (26 ha.), Thiruvanthapuram (belonging to southern Kerala as well as the state capital (28 ha.), and Thrissur (30 ha.) (Vidyasagaran and Madhusoodann 2014). Figure 1 represents the location map of the study area.

In the present study, mangrove sediments were collected from three locations by geographically dividing it as the northern, central, and southern part of Kerala and also based on the mangrove area covered by the respective stations, northern side maximum, followed by central and the southern side with minimum mangrove cover. Station 1 (Dhrmadam) is located in Kannur district belonging to Northern Kerala between 11° 47′ 24.27795″ N latitude and 75° 27′ 51.43521″ E longitude.

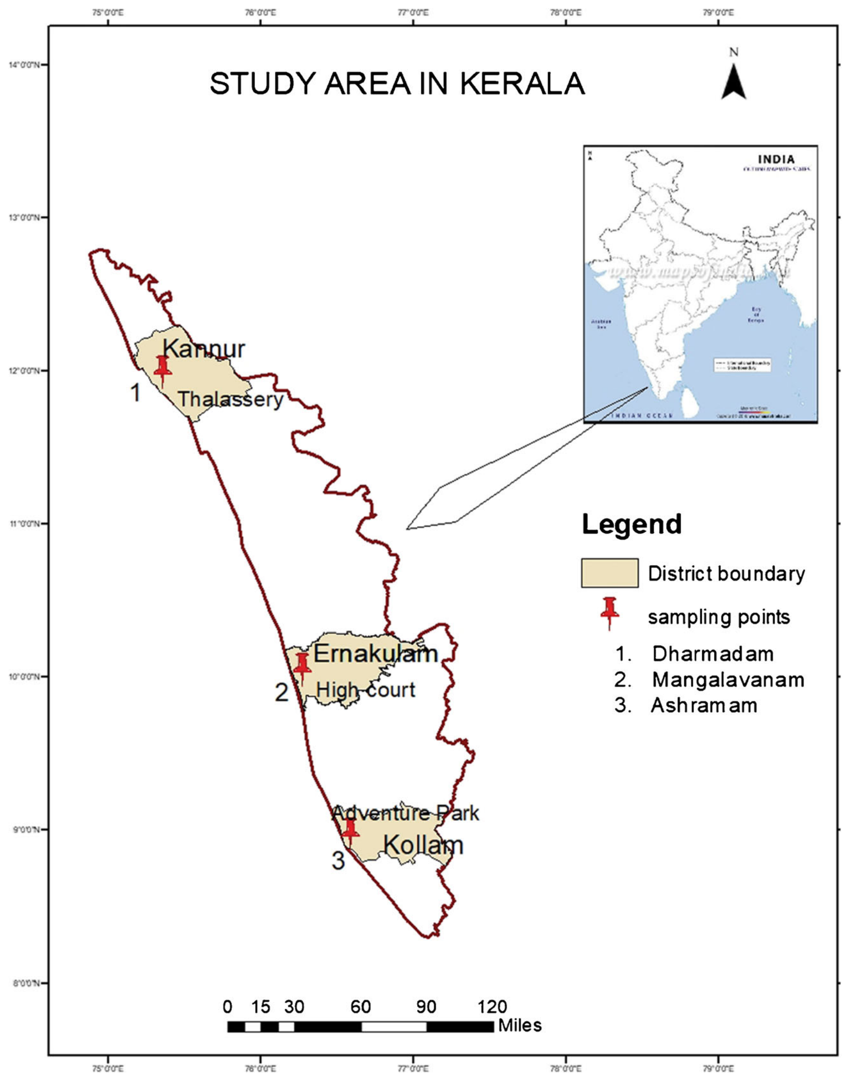

Fig. 1 Map showing of sampling area

Dharmadam is on the estuary of the Anjarakkandy River, which surrounds the island on three sides (the Laccadives Sea is on the fourth). Station 2 is Mangalavanam located in Ernakulum district between 9° 59′ 133″ N latitude and 76° 16′ 26″ E longitude, and in this study, it is taken as a representative of the central region of Kerala. This mangrove is an ecologically sensitive area situated at the center of the city of Kochi and covers about 2.74 hectares. This acts as a nesting ground for a large variety of migratory birds and also supporting a variety of mangroves and is often regarded as the “green lung of Kochi” since it plays a pivotal role in controlling the air pollution of Kochi city.

Station 3 is Asramam Adventure Park situated in Kollam district belonging to southern Kerala, near the backwaters of Ashtamudi Lake (between 8° 53′ 7560″ N Latitude and 76° 35′ 1151″ E longitude). In addition to being a major spawning ground for several edible marine species, the Asramam mangroves are also home to otters as well as several migratory birds.

In this study, mangrove sediment samples were collected during the pre-monsoon season (April 2018) from the three geographically distinct regions of Kerala. The surface sediment samples were collected from each station using a pre-cleaned plastic scoop. Core samples were collected from these sites using PVC pipes with 75-mm diameter and 1.5-m length. The core samples were divided into two parts such as the surface sediments and the core sediments representing 1-m depth on each sampling site. The samples were carried to the laboratory in an icebox and kept in a deep freezer at −200 °C until analysis. The samples were freeze-dried for further analysis.

Analytical methods

Extraction of humic substances

The humic substances were isolated using the procedure IHSS 2010. In this method, 200 g freeze-dried sediment was washed with 1 l of 0.1 M HCl to discard weakly bound molecules like hydroxides, carbonates, and sulfates. The sediment/HCl mixture was continuously stirred and allowed to settle. The supernatant was then separated. One liter of 0.1 M NaOH was added to the sediment mixture. For the next 24 h, it was kept on a rotatory shaker. The dark-colored extract was separated from sediment by centrifugation at 10,000 rpm for 30 min. 6 M HCl was then added to it and the pH was adjusted between 1 and 2. The precipitate thus obtained was maintained at −4 °C for 16 h. The precipitate was then separated again by centrifugation for 10 min at 10,000 rpm. The obtained precipitate of humic acid (HA) was freeze-dried for further analysis while the supernatant was used for the analysis of fulvic acids (FAs).

The above acid-soluble supernatant containing FA solution was allowed to pass through a XAD-8 resin previously cleaned and then desalted by passing Milli-Q water and ensured it was chloride-free by testing with 0.1 M $AgNO_3$. To desorb the absorbed FA, 0.1 M NaOH was passed through the column and then the eluate was passed through a previously cleaned cation-exchange resin (Collins et al. 1971). The FA solution was freeze-dried for further studies.

Characterization of isolated HAs and FAs

The characterization of isolated HAs and FAs by the above methods was done by using UV-visible, FTIR, ^{1}H NMR spectroscopic methods, CHNS elemental analysis, and TOC.

The total organic carbon (TOC) in the samples was analyzed by direct analysis done with the aid of the PRIMACSMCS TOC analyzer of SKALAR. The UV-visible spectroscopic analysis was done with the Thermo Fischer Model No 117. For this, 2 mg humic substance was dissolved in 10 ml, 0.05 N sodium bicarbonate of pH 8, and observed the optical densities at 465 and 665 nm and hence the E4/E6 ratio was determined (Chen et al. 1977). The E2/E3 ratio (optical densities at 250 and 365 nm (Chen et al. 1977)) was calculated by dissolving 2 mg humic substance in 10 ml of 0.05 N sodium hydroxide followed by determining optical densities at 270 and 407 nm.

C, H, N, and S compositions were determined by direct analysis on an ash-free basis with the aid of the CHNS Elemental analyzer of Elementar Vario ELIII. The oxygen level was calculated using the formula 100 − (sum of the percentages of CHNS).

FTIR spectra of HAs and FAs are documented using Avatar 370 (FTIR spectrometer) with a high resolution of 4 cm^{-1} obtained from the mid-infrared region (4000–400 cm^{-1}). The samples were prepared to eliminate excess moisture; aliquots of humic substance (HAs and FAs) were placed over P2O5 at 45 °C overnight. Four hundred milligrams of spectroscopic grade KBr was mixed with 30 mg dried humic substance samples (HA and FA), and then the combination was squeezed to form pellets. The spectra were picked up with high resolution.

^{1}H NMR spectroscopic characterization was done using TMS, and the signals were determined with Bruker Advance 400 MHz NMR spectrometer fitted with QNP 1H probe.

The tannin and lignin concentrations were estimated by using the standard procedure adopted by the sodium tungstate phosphomolybdic acid method (APHA 1995). The textural characteristics of the sediments were analyzed by using a particle analyzer, LISST-PORTABLE | XRD (SEQUOIA).

Results and discussions

Quantification of humic substances

In this study, the quantities of humic acids and fulvic acids isolated were higher in the surface compared to the bottom sediments (Table 1). The surface samples of station 1, taken as a representative of central Kerala mangroves, showed (12.53 g) higher concentration of HAs compared to the other stations as well as depth. This was almost double than that of isolated from the surface samples stations 1 (5.62 g) and 3 (5.32 g). Station 3, representing southern Kerala, showed slightly lower quantities of HAs for both surface (5.32 g) as well as bottom (1.02 g) sediments. The amount of fulvic acids isolated from all the three stations was much lower than that of the HAs. Though not much different, the quantity of isolated FAs followed the order station 2 (central) > station 1 (northern) > station 3 (southern).

Station 2 was characterized by the higher amount of HAs compared with other stations maybe because of the rich organic content provided by the migratory birds and plant foliage that are plenty in this bird sanctuary. This mangrove forest is currently a site that is being highly protected for preservation. The large quantities of humic substances are isolated from areas characterized by high decomposition from plant foliage, microbial action, and soil condition, for example, pH and temperature (Swift et al. 1979). This station was noticed by a higher percentage of TOC, i.e., 4.9399% for surface and 3.1768% for bottom sediments (Table 2)

Table 1 Quantification of isolated humic substances

Stations	Humic acid (g)	Fulvic acid (g)
Station 1–surface	5.62	0.56
Station 1–bottom	2.36	0.45
Station 2–surface	12.53	0.58
Station 2–bottom	6.53	0.49
Station 3–surface	5.32	0.52
Station 3–bottom	1.02	0.40

Table 2 TOC, texture, and tannin and lignin content of the sediments

Stations	Sand%	Clay%	Silt%	TOC% of sediments	Tannin lignin (mg/g)
Station 1–surface	23.36	50.37	26.27	4.6069	19.46
Station 1–bottom	35.43	35.17	29.4	2.6462	10.73
Station 2–surface	24.5	25.2	50.3	4.9399	25.03
Station 2–bottom	30.7	17.6	51.7	3.1768	18.69
Station 3–surface	82.3	16.2	1.5	3.03	21.27
Station 3–bottom	95.4	3.62	0.98	0.002	8.99

compared to the other two stations which may be due to extraordinary primary production , the elevated contribution of autochthonous sedimentary organic matter, and also high sedimentation rate (Marchand 2017; Rani et al. 2021).

Station 2 was also noted for its higher percentage of silt (more than 50% for the surface as well as the bottom) and a significantly higher proportion of tannin and lignin compared to the other stations (25.03 mg/g for surface and 18.69 mg/g for bottom) (Table 2). In station 3, the quantity of humic substances isolated was less and it is also supported by low TOC (3.03% in surface and 0.002% in the bottom) as well as predominantly sandy sediment texture (> 80% was sandy for the surface as well as the bottom). This is due to the improvement of mud in the mangroves area over a long period in dissimilar stages under tidal effects and deposition of wind instinctive material. Maybe at the previous stage, there was high involvement of sand deposits (from sea and wind) and then the coarser material transformed to finer material through physical weathering or the settling of fine particles at the surface through sedimentation in suspension (Alsumaiti and Shahid 2018; Suthhof et al. 2000). The association of organic matter with fine-grained sediment is well established (Nair et al. 1993; Sarala Devi et al. 1995; Hedges and Keli 1995).

The surface samples of all three stations are noted for the higher TOC values (Table 2) which may be due to the decomposition of dead organisms and mangrove detritus, domestic sewage, and anthropogenic inputs. The high levels of organic carbon in mangrove sediments are due to decomposition of dead organisms, decomposition of mangrove detritus, and anthropogenic inputs, particularly oil spills, sewage, etc. which are transported by tides and strand on low energy ecosystems such as mangrove forests (Rini 2002). The TOC content demonstrated a sharp decrease from top to bottom which recommended forceful mineralization (Bouillon et al. 2004; Manju et al. 2020). In station 3, tidal flushing might have removed the organic matter from the surface sediment and sandy nature allows the decomposition to takes place more rapidly allowing the microbes and oxygen to penetrate the sediment, hence resulting in a comparatively low percentage of TOC (Table 2).

Tannin and lignin, familiar aromatic polycyclic phenolic compounds biosynthesized by complex plants (Hernes and Hedges 2000), have been introduced into an aquatic environment through terrestrial runoff (Kalesh et al. 2001). The tannin content of mangrove leaves is higher and more polymerized than that found for 40 other dicotyledonous plant species (Hernes et al. 2001). Lignins have been implicated as major source material for terrestrial humic substances (Flaig et al. 1975; Kalesh et al. 2001). Mangrove species exhibit a typical vascular-plant lignin signature, with great variations between leaves and wood, the latter being richer in lignin oxidation product (Kristensen et al. 2008). They act as a major fraction of refractory organic matter, and its quantitative resolution offers valuable information on the input of terrestrially derived organic detritus into the sediments (Manju et al. 2016). In this study, the tannin and lignin concentrations (mg/g) varied from 8.99 to 25.03 which showed a higher concentration in the surface than the bottom sediments (Table 2). The majority of the species of mangrove leaves contained a greater amount of tannin and lignin during pre-monsoon (Katherisan and Veera 1990). Since these leaves are the main source, its concentration was maximum in the system during this season (Geetha 2002). The maximum amount of tannin and lignin was noted during pre-monsoon in selected mangrove ecosystems of Kerala, especially from Mangalavanam (station 2 in this study) (Geetha et al. 2008). *Rhizophora mucronata* is profusely distributed in Kannur and Ernakulam districts whereas it was not seen in Kottayam and Trivandrum districts (Vidyasagaran and Madhusoodann 2014). Mangroves, especially those belonging to the Rhizophoraceae family, contain a large amount of tannins (Bask et al. 1999; Hernes and Hedges 2004; Maie et al. 2008).

Elemental analysis

Tables 3 and 4 display the elemental composition (CHNS %) as well as the elemental ratios of humic acids and fulvic acids respectively of the isolated samples under study.

The content of C, H, O, N, and S provides essential information on the origin of samples. The elemental ratios like H/C, O/C, and C/N indicate the structure and molecular shapes of isolated humic substances. Atomic ratios (H/C and N/C) have been preferentially employed to establish the organic matter source, degree of condensation, diagenetic transformation, and environmental conditions under which the

Table 3 Elemental composition (CHNS %) and elemental ratios in humic acids

Stations	C%	H%	N%	S%	O%	H/C	C/N	O/H	O/C	N/C
Station 1 surface	50.5	12.3	2.5	1.9	32.8	0.2435	20.2	2.66	0.6495	0.0495
Station 1 bottom	35.26	8.3	1.01	1.4	54.03	0.2353	34.91	6.50	1.5323	0.0286
Station 2 surface	54.03	9.76	4.6	2.6	29.01	0.1806	11.74	2.97	0.5369	0.0851
Station 2 bottom	52.34	10.08	1.32	4.1	32.16	0.1925	39.65	3.19	0.6144	0.0252
Station 3 surface	47.3	18.03	2.5	2.8	29.37	0.3811	18.92	1.62	0.6209	0.0528
Station 3 bottom	17.06	19.6	1.7	3.8	57.84	1.1488	10.03	2.95	3.39	0.0996

humic substances are generated (Stuemer et al. 1978; Rice and Mac Carthy 1991; Meyers and Ishiwatari 1993). Marine sediments are normally less aerated than superficial sediments and have an aliphatic and nitrogen-rich source of organic matter, and produce HS with high H/C and N/C ratios (Rashid 1985; Rice and Mac Carthy 1991). Due to the large inputs of terrestrial plants, terrestrial samples are rich in lignin moieties presenting, consequently, low H/C and N/C ratios (Stuemer et al. 1978).

While observing the elemental composition of isolated HAs, it is seen that in most of the stations, the percentage of carbon was higher than that of oxygen. In the bottom soils of stations 1 and 3, the percentage of oxygen was higher than that of carbon. Next to carbon and oxygen, the percentage of hydrogen was higher in all the samples of HAs. The percentage of sulfur was higher than that of nitrogen especially in the samples of station 3 (both surface and bottom) and also in the bottom sediments of stations 1 and 2 (Table 3). While discussing the percentagewise composition of elements in fulvic acids, it is seen that the highest percentage was for oxygen, followed by carbon, hydrogen, sulfur, and nitrogen (Table 4). The values obtained are similar to those attained by previous researchers (Rice and Mac Carthy 1991; Giovanela et al. 2004; Anuradha et al. 2011; Prashob Peter 2014).

When the sample is aliphatic than aromatic, it is generally observed that the hydrogen content will be higher (Traina et al. 1990). In the samples collected, the humic acids showed higher hydrogen content, indicating the aliphaticity of the isolated humic acids (Bravard and Richi 1991; Rigobello et al. 2017). An increase in hydrogen content indicates a greater number of aliphatic carbons (CH 2) than the aromatic carbons (C=C) (Traina et al. 1990). The carbon content was also observed to be higher in humic acids compared to fulvic acids. On the other hand, the oxygen content was greater in fulvic acid when compared to humic acid (Tables 3 and 4). HAs contained more carbon than nitrogen; the formation of FA is accompanied by the loss of carbon and nitrogen and the gain of oxygen (Schnitzer 1999). The percentage of nitrogen was higher in HAs than FAs (1.01–4.6% in HAs and 0.65–1.92% in FAs) which can be attributed to the fact that unlike FA they are not completely separated from the more hydrophilic and possibly nitrogenous compounds for example proteins and amino acids, by the adsorption step onto XAD-8 resins (Giovanela et al. 2004).

While discussing the elemental ratios, the H/C ratio of the fulvic acid was found to be lower (0.14–0.49%) than that of humic acid (0.14–1.14%), indicating that humic acids had more aromatic fractions and contained a large fraction of unsaturated structures (zhang et al. 2011; Rigobello et al. 2017; Abakumov et al. 2018). Prashob Peter (2014) reported an H/C ratio of 1.1 in Kochi mangrove sediments. Low H/C values support lignin mineralization rather than humification, whereas, increased O/H values support accelerated humification (Anuradha et al. 2011). In this study, the reported range of O/C was 0.5369–3.39 in HAs and 1.15–7.80 in FAs (Tables 3 and 4), and Prashob Peter (2014) reported O/C ratio of 0.566 in HAs of Kochi mangroves that is almost similar to our values reported from station 2 which is a Kochi mangrove (Mangalavanam). The O/C ratios are low in humic acids when compared to that of fulvic acids, which indicates the higher humification of the complex (Bravard and Richi 1991; Giovanela et al. 2004; Sierra et al. 2004). The O/C atomic

Table 4 Elemental composition (CHNS %) and elemental ratios in fulvic acids

Stations	C%	H%	N%	S%	O%	H/C	C/N	O/H	O/C	N/C
Station 1 surface	41.56	6.04	1.23	2.22	48.17	0.14	33.78	7.97	1.15	0.04
Station 1 bottom	28.68	6.25	0.68	2.32	60.77	0.21	42.17	9.72	2.11	0.069
Station 2 surface	40.73	8.25	1.86	1.98	47.18	0.20	21.89	5.71	1.16	0.045
Station 2 bottom	31.25	6.05	0.88	1.73	58.59	0.19	35.51	9.68	1.86	0.076
Station 3 surface	38.36	8.75	1.92	2.95	46.72	0.22	19.97	5.33	1.21	0.083
Station 3 bottom	10.32	5.06	0.65	2.08	80.56	0.49	15.87	15.92	7.80	0.189

ratio is related to the carbohydrate and carboxylic acid content (both aromatic and aliphatic) and degree of oxidation of HS. The lower values of this ratio indicate a higher degree of humification due to the reduction in the carbohydrate content in the O-bearing structures (polysaccharides) (Prashob Peter 2014). The N/C and H/C ratio in samples from all stations in HAs and FAs was found to be low, indicating the geographical location as "terrestrial" due to high lignin mineralization (Stuemer et al. 1978; Giovanela et al. 2004; Rigobello et al. 2017).

The high C/N ratios have a large contribution to humification resulting from the decomposition of vascular plants (Rocha et al. 2007). Humification is prominent in static mangrove sediments where organic molecules are plenty in the form of leaf litter and organic wastes like bird excreta such as in the case of station 2 in this study. Mangalavanam is a tiny forest and a shelter of several exotic and more varieties of migratory birds of different extremes (Anuradha et al. 2011). The C/N ratios of HAs and FAs reported are comparatively higher (10.03–39.65 for HAs and 15.87–42.17 in FAs). In stations 1 and 2, C/N ratios are higher for the bottom samples than the surface in both HAs and FAs (Tables 3 and 4). Low C/N ratio indicates that N is preferentially mineralized relative to carbon (Resmi 2004). Organic compounds rich in nitrogen (low C:N ratio) favor net mineralization whereas those poorer in N (high C:N) favor net immobilization (Resmi 2004). Nonvascular aquatic plants contained a low C/N ratio, normally among 2.0 and 10, while vascular land plants, which enclose cellulose, lignin, and tannins, have ratios of 20 and upper (Giovanela et al. 2010). The C/N ratios in this study varied from 10.03 to 39.65 in HAs (Table 3) and 15.87 to 42.17 (Table 4) in FAs. These values are comparatively high, which designates that terrestrial vascular plants are the main sponsors of these samples.

The contribution of lignin moieties to the HAs can be inferred from the N/C ratio (Sainto and Hayano 1981). HAs with lower N/C ratios are generally rich in lignin moieties (Stuemer et al. 1978; Sierra et al. 2004). The N/C ratios reported in this study are in the range 0.0252–0.0996 for HAs (Table 3) and 0.04–0.189 for FAs (Table 4), and Prashob Peter (2014) reported N/C ratio of HAs as 0.074 for Kochi mangrove sediments and our study reported N/C ratio of 0.0851 in station 2, which is a mangrove from Kochi (Mangalavanam). A higher N/C ratio in terrestrial humic acids suggests the presence of vanillyl and syringyl acids, aldehydes, and ketones, along with cinnamic compounds (p- coumaric and ferulic acids) (Ertel and Hedges 1984; Lallier et al. 2008). The most likely occurrence of varying degrees of vascular lignin moieties and non-vascular lignin moieties is confirmed by higher C/N ratios (Prashob Peter 2014).

UV-visible spectroscopic methods

The ratio of the absorbance of dilute aqueous humic substance solutions at 465 and 665 nm is extensively used for the determination of the characterization and to evaluate the degree of humification and investigate the aliphatic or aromatic nature of the humic substance (Prashob Peter 2014). This ratio, which is discussed as the E4/E6 ratio, is originated to be independent of the concentration of humic materials, but it varies for humic materials, extracted from diverse soil types (Kononova 1966; Schintzer and Khan 1972). E4/E6 ratio (condensation degrees) designed from the investigational data is used to clarify the rigidity of the condensed aromatic rings in HS (i.e., degree of aromaticity), particle size, molecular weight, and acidity (Chen et al. 1977; Uyguner and Bekbolet 2004). Many of these complexes are held to be joint structural subunits in humic matter and E2/E3 as absorbance ratios measured at 250 and 365 nm resemble the correlation of molecular size and aromaticity (Peuravuori and Pihlaja 1997).

The E4/E6 ratios and E2/E3 ratios of isolated HAs and FAs are displayed in Table 5. The E4/E6 ratio, i.e., a decrease in absorptivity with an increase in wavelength, characterizes the changing content of aliphatic and aromatic fragments existing in the HAs. The E4/E6 ratio of HAs was in the range varying between 3.54 and 11.5 and that of FAs was in the range varying between 5.97 and 18.61. These values lie in the upper side of the known range (3–5.9) (Kononova 1966; Chen et al. 1977) and recommended the occurrence of inflexible aromatic core in the isolated humic substances, and are more in FAs than HAs. The E4/E6 ratios of fulvic acid are usually higher than the HA, to associate the fact that the previous ones are with low molecular weights and are less polymerized. Comparatively extensive ratios of FAs than those of HAs reproduce a low degree of aromatization and the presence of a relatively large proportion of aliphatic structures in FAs (Stevenson 1994). Fulvic acid had a higher E4/E6 ratio in comparison to humic acid. It could be due to the higher degree of aromaticity in carbon atoms of humic acid (Eshwar et al. 2017) supported by Tahiri et al. 2016), Srilatha et al. (2013) and Satisha and Devarajan (2011). The low H/C values (Table 3 and Table 4) indicate greater aliphaticity and lesser content of aromatic structures in humic substances (Rigobello

Table 5 UV-visible spectroscopic characterization

Stations	Humic acids (nm)			Fulvic acids (nm)		
	E_4/E_6	E_2/E_3	Aromaticity	E_4/E_6	E_2/E_3	Aromaticity
S1–surface	10.2	0.95	42.95	18.61	0.66	29.84
S1–bottom	7.52	1.96	88.63	16.02	0.43	19.44
S2–surface	11.5	0.60	27.13	16.82	0.52	25.77
S2–bottom	6.95	0.73	33.01	13.03	0.36	16.27
S3–surface	8.33	0.53	23.96	13.52	0.46	20.80
S3–bottom	3.54	0.96	43.41	5.97	0.36	16.27

et al. 2017, Amir et al. 2004), and therefore, its decrease infers an increase in the degree of humification. Stevenson (1994) displayed that absorbance at wavelength 465 nm is the same as light absorption of components related to the first phases of the humification process (young humic substances). Light absorption at 665 nm is related to healthy humified components. Small E4/E6 ratio values (< 4) show high (HS) quality. In contrast, a high E4/E6 ratio of pure humic acid shows that the molecule is smaller in size and has low molecular weight and C content, rich in O content, COOH group and total acidity and assumes the occurrence of comparatively large proportions of aliphatic structures (Stevenson 1994; Prashob Peter 2014). This is also supported by the fact that HAs in this study presented lower O/C values than the FAs. But, for all samples, the HA fraction offered values greater than 0.5, suggesting the presence of a larger amount of oxygenated functional groups such as carboxylic and carbohydrates (Tan 2014).

The surface sample gives a higher E4/E6 ratio than the core samples (Table 5) in both HAs and FAs. This is because the surface samples were of young origin, whereas the bottom regions are more ancient due to higher microbial activity and decomposition (Stevenson 1994; Zeck et al. 1997). While comparing among the stations, E4/E6 ratio in station 3 was slightly less than that of stations 1 and 2. Since E4/E6 is an index of the maturity of the HAs, the low E4/E6 ratio indicates a lesser degree of humification and maturity (Yavmetdinov et al. 2003). The reduced free acidic groups in the isolated compounds can also lead to increased ratios (Prashob Peter 2014). A high E_{465}/E_{665} ratio (or a rapid decrease in absorptivity with an increase in wavelength) may result primarily from the absorption by ketonic C=O functional, and on the other hand, a low E_{465}/E_{665} ratio may be largely attributed to the absorption by aromatic C=C functional groups (Prashob Peter 2014). Additionally, a high degree of condensation of the aromatic rings and the large molecular weight of SHA are believed to contribute to its relatively high absorption in the visible range (Schintzer and Khan 1972). Typically E4/E6 is larger for non-humified material by the presence of proteins and carbohydrates, which increase the absorptivity at the UV region of the spectrum (Faustino et al. 2009). These results are in line with those of Haddad et al. (2015), Petrus et al. (2009), and Banik and Sanyal (2006). Higher E2/E3 ratios are normally connected with lower molecular weight and a lesser degree of aromaticity (Vergnoux et al. 2011). The E_2/E_3 ratios varied from 0.53 to 1.96 in HAs and 0.36 to 0.66 in FAs, besides, to associate the fact that FAs have more aromatic nature than HAs which is also confirmed by E4/E6 ratios. To determine the aromaticity of the molecule, an equation proposed by Peuravuori and Pihlaja (1997) was used, in which aromaticity = 52.5 − 6.78 E2/E3.

FTIR spectroscopic studies

Fourier-transform infrared spectroscopy has been commonly used for the description of hydroxyls, aromatic and aliphatic carboxyls and carbonyls, aliphatic C-H and amides that are typical to HAs (Stevenson 1994; Amir et al. 2004; Sierra et al. 2004), which can offer considerable evidence about the structural as well as functional group properties of these compounds. Irrespective of their origin, all FAs and HAs isolated in this study offered nearly similar spectra maybe because of the presence of polar chemical functionalities absorbing infrared radiation such as phenolic and alcoholic hydroxyls, aromatic and aliphatic carboxyls and carbonyls, aliphatic C-H and amides, in all the HAs and FAs, irrespective of their origin (Giovanela et al. 2004). Such explanation is in line with the conceptions attributed to humus mixtures, which around that it consists of associations of molecules, with a “universal average formula unit,” held together via supramolecular interactions (Schintzer and Khan 1972; Piccolo et al. 1996; Sein Jr et al. 1999; Cozzolino et al. 2001; Giovanela et al. 2004).

The FTIR spectral characteristics of the isolated humic acids and fulvic acids are given in Fig. 2 (for HAs) and Fig. 3 (for FAs). A broad, high-intensity band at 3400 cm^{-1} displays the occurrence of H-bonded –OH groups of alcohols and phenols as well as hydrogen-bonded N - H groups (Amir et al. 2004; Sierra et al. 2004; Stevenson 1994). These peaks are evident in the FTIR spectrum of isolated humic acids and fulvic acids of this study. In the 1600–1720 cm^{-1} region, two peaks were detected: a fewer intensive absorption band around 1720 cm^{-1} which has been recognized to carbonyl groups in aldehydes, ketones, and carbonic acids, and a sharp high-intensity absorption band about 1635 cm^{-1} which has numerous assignments with aromatic –C=C– vibrations, –C=O stretching of amide groups (amide I band), strongly H-bonded –C=O of quinones, and/or H-bonded and conjugated carbonyl –C=O (Stevenson 1994; Smid et al. 2015). The absorbance at these wavelengths recommends the existence of extremely conjugated delocalized π electron through condensed double bond character (–C=O) of quinones (Silverstein et al. 1991). The peak around 1635 cm^{-1} is existing in all the samples in both isolated HAs and FAs.

Absorption bands of nitrogen-containing functional groups in HAs were perceived in the FTIR absorption region between 1570 and 1510 cm−1 (Orlov 1985). The occurrence of protein-like fractions was characterized by two peaks, one at 1660–1630 cm−1 (C=O stretching of amide groups) and additional at 1560–1510 cm−1 (N - H deformation and C ≡N stretching of amide groups) (Zhang et al. 2011). Peaks nearby 1590 cm−1 are realized in the isolated FAs only in station 1 (surface and bottom), station 2 (bottom), and station 3 (surface). In the 1400–1500 cm−1 range, peaks characterize OH deformation and CO stretching of phenolic OH, C-H deformation of CH 3 and CH2 groups and C-O – O- (Sierra et al.

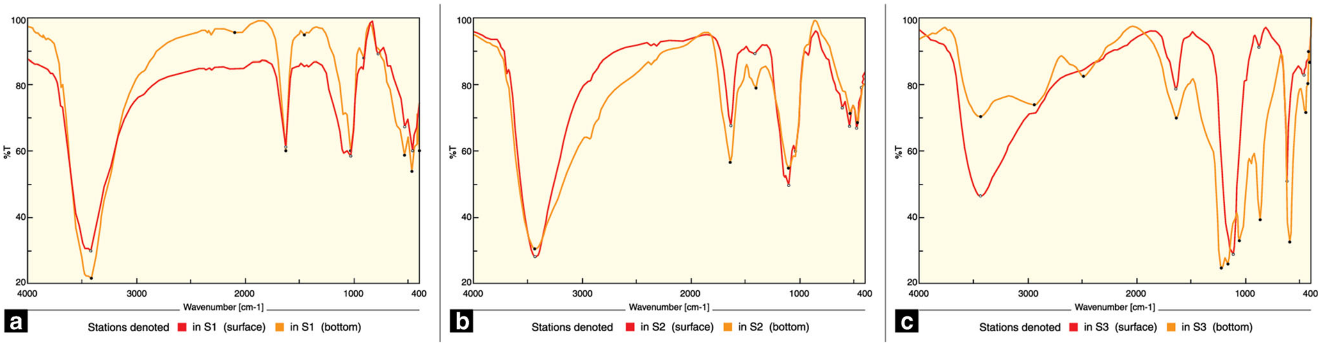

Fig. 2 FTIR spectrum of HAs. **a** S1. **b** S2. **c** S3

2004; Stevenson 1994). These peaks are similarly joint in both isolated FAs and HAs in this study, but in station 1 bottom, station 3 (surface and bottom) for HAs and station 2 (surface and bottom) for FAs. The peak at 1400–1390 cm−1 (COO -1 antisymmetric stretching) and the peak around 1220 cm−1 (C-O stretching and OH deformation of COOH) designated the occurrence of a huge quantity of carboxylic acids in both the FA and HA (Zhang et al. 2011). Peaks conforming to this were further shared in FAs in this study than the HAs. This was also reinforced by the higher percentage of oxygen in FAs than HAs (Tables 3 and 4).

Peaks in the range 1100–1225 cm^{-1} are distinguished from the second overtone C-H stretching, NH3+ of lysine rocking, and occurrence of cellulose (Sierra et al. 2004; Adani et al. 2006). In the isolated FAs, sharp peak at 1120 cm^{-1} was perceived in all the samples from the three stations. In the isolated HAs, sharp peaks in this range were realized only in station 2 (surface along with bottom) and station 3 (surface in addition to bottom). Numerous earlier research work on humic substances has attained comparable results (Jennees et al. 2019, Prashob Peter 2014, Moreda–Pineiro et al. 2004, and Giovanela et al. 2004). The FTIR study clearly showed the existence of hydroxyl, methyl, methylene, carbonyl, carboxyl, phenol, alcohol, and amide groups in the humic substances isolated from sediment samples collected from different environmental settings.

^{1}H NMR spectroscopic studies

The ^{1}H NMR spectra of isolated humic acids and fulvic acids are given in Fig. 4 (for HAs) and Fig. 5 (For FAs). Resonances in the range 0.65–1.75 ppm arise from protons on terminal methyl groups of methylene chains and methyl groups of extremely branched alkyl groups and alicyclic compounds (Francioso et al. 2001; Graham et al. 2002). In this study, the ^{1}H NMR spectrum of isolated HAs exhibited resonance peaks in this range for the surface as well as bottom sediment samples. In the surface sample of station 1, resonance peaks are detected at 0.842, 1.147, 1.227, and 1.470 whereas in the bottom sediments two resonance peaks were observed at 0.732 and 0.838. In station 2, in surface peaks are observed at 0.827, 1.149, 1.229, and 1.480. In this station, the peaks were obtained at 0.832, 0.870, 1.155, 1.224, and 1.646 in the bottom sediment sample. In station 3, surface sediment showed peaks at 0.791, 0.823, 1.144, and 1.222, whereas, in bottom sediment sample peaks were displayed at 0.842, 1.147, 1.227, and 1.470. Peaks in the range 1.10–1.40 are suggestive of methyl groups of highly branched aliphatic structures, and those in the region 1.4–1.65 designate the occurrence of methylene protons of alicyclic compounds. Amount of peaks further than 1.40 in station 1 (surface), station 2 (surface), station 2 (bottom), and station 3 (bottom) displays the occurrence of aliphatic regions slightly more controlled with long chains and/or alicyclic moieties rather than

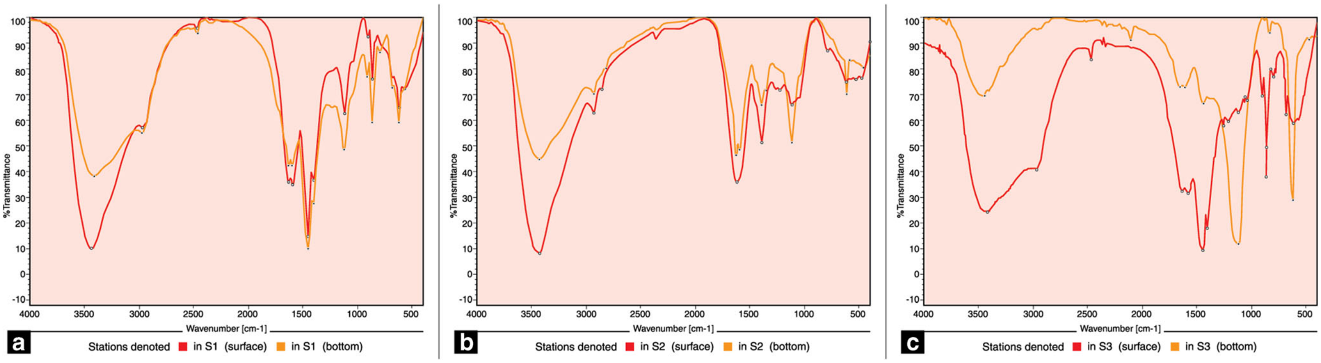

Fig. 3 FTIR spectrum of FAs. **a** S1. **b** S2. **c** S3

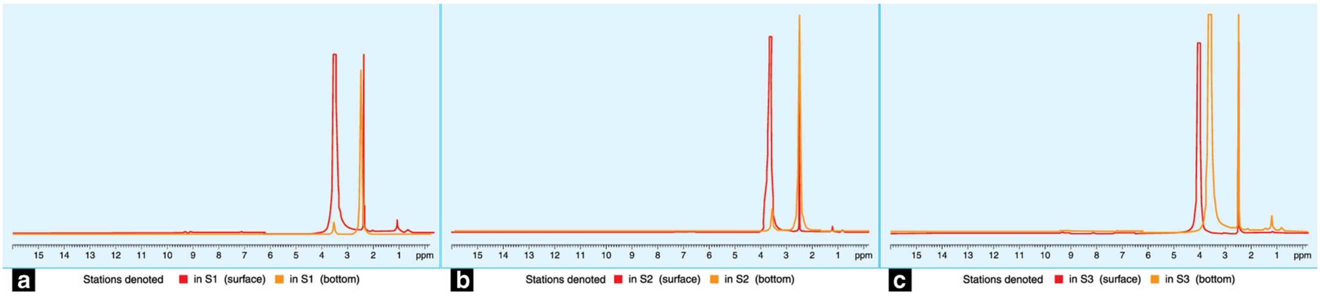

Fig. 4 ^{1}H NMR spectrum of HA. **a** S1. **b** S2. **c** S3

methyl groups (Hatcher et al. 1981; Graham et al. 2002). This is also supported by the increased hydrogen content in the isolated HAs than FAs which shows the occurrence of more number of aliphatic carbons (CH_2) than aromatic carbons ($C = C$) (Traina et al. 1990; Prashob Peter 2014).

Aliphatic protons adjacent to carbon atoms together with highly electronegative atoms (O or N), unsaturated groups or aromatic groups, are usually seen in the range 2.00–3.00 (Sainto and Hayano 1981; Francioso et al. 2001; Graham et al. 2002; Qi et al. 2004). Peaks corresponding to this are observed at 2.175 and 2.5 (station 1 surface), at 2.5 (station 1 bottom), at 2.18 and 2.5 (station 2 surface), at 2.5 (station 2 bottom), at 2.5 (station 3 surface), and at 2.175 and 2.5 (station 3 bottom). The occurrence of sharp resonance peaks detected in this region may be due to the protons near to benzylic carbons (Hatcher et al. 1980; Majid and Ripmeester 1990). Poorly established proton resonance in this range could also be due to the decreased presence of aliphatic protons nearby unsaturated or aromatic groups (Graham et al. 2002; Qi et al. 2004). In this study, all the samples reported a sharp peak at 2.5 ppm.

The resonance in the range 3.10–4.75 ppm is mostly due to the protons on the carbons bonded with amines, alcoholic hydroxyls (polysaccharides), proteins, and polyether or methoxyl linkages of HAs (Francioso et al. 2001; Adani et al. 2006). These peaks are noticed at 3.6 (station 1 surface, station 2 surface as well as the bottom), at 3.5 (station 1 bottom), and 3.4 (station 3 bottom). Peaks in the spectral range 3.3–3.8 exhibit the influence of proton maximum which takes place from –CHOH and -CH_2 OH functional groups, which point out the major contribution of polysaccharides and/or lignin and lignin like moieties (Yasuda et al. 1999; Kingery et al. 2000; Francioso et al. 2001). A resonance peak was observed at 4.5 ppm in station 3 surface sample. The number of peaks in this region was relatively less for the isolated HAs. The occurrence of sugar like moieties in HAs decreased because of acidification process ($pH < 2$) carried out during the isolation of HAs from sediments that damaged a major amount of polysaccharides (Schnitzer and Preston 1983; Gonzales-Villa et al. 1999; Montoneri et al. 2003; Adani et al. 2006).

Resonances in the range 6.40–8.45 ppm originate from aromatic protons phenols and quinones (Hatcher et al. 1980; Wilson 1981; Wersaw 1985; Jokica et al. 2004). Sterically hindered aromatic protons and nitrogen heteroaromatics display peaks in this region. In this study, the bottom sediments of stations 1 and 2 did not show peaks in this region which concludes the absence of aromatic structures in the isolated HAs from these stations. In the surface sample of station1, four peaks were detected at 7.085, 7.212, 7.236, and 7.341. In this sample, two more peaks were detected at 9.153 and 9.183, and another peak was noticed at 9.399. In station 2 surface, peaks were displayed as follows: one at 6.669, two at 7.105 and 7.136, another two at 7.236 and 7.261, and two more at 7.362 and 7.387. Here one more peak was observed at 8.244. In station 3 surface, peaks were observed as follows, two at 7.059 and 7.084, two at 7.184 and 7.210, and two more at 7.315 and 7.339. In this sample, one peak was seen at 9.130, two more at 9.316 and 9.332, and one more at 9.417. In station 3, bottom sediment sample peaks were observed at 7.085, two at 7.212 and 7.236, one at 7.341 and two more at 9.153 and 9.183 and finally at 9.339. These results correspondingly recognized the occurrence of deshielded protons and sterically

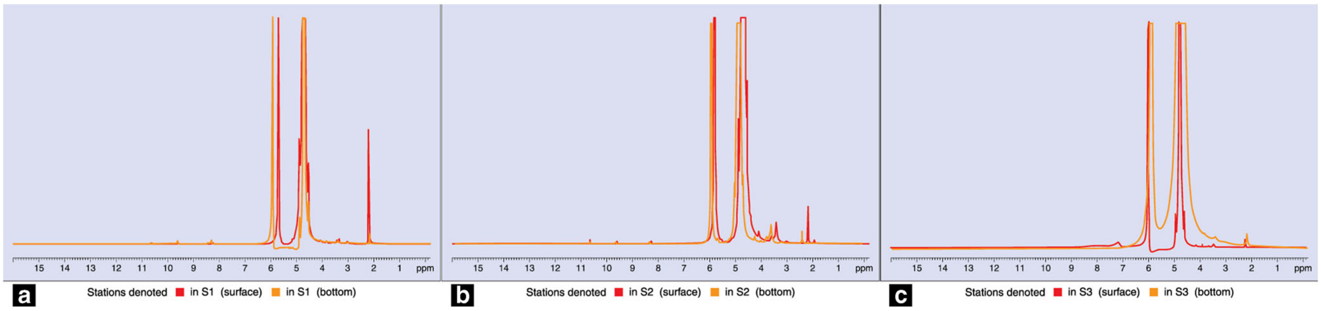

Fig. 5 ^{1}H NMR spectrum of FA. **a** S1. **b** S2. **c** S3

hindered aromatic protons (Grasso et al. 1990; Qi et al. 2004). This also concludes that all are having more or less common aromatic core closed to unsaturated carbons ($>CH = CR_2$) and also the presence of aromatic groups such as phenols, reduced quinines through ionized carboxylic groups which contributed to electron shuttling and redox properties related with HAs (Bradley et al. 1998; Hernandez and Newman 2001).

Peaks demonstrating terminal methyl groups of alkyl chains, methyl groups of highly branched aliphatic structures, and methylene protons of alicyclic compounds are absent in the ^{1}H NMR spectrum of isolated FA samples which fully ruled out the probability of the presence of aliphatic side chains in FAs. Peaks in the range 2.00–2.30 display protons attached to α- methyl and methylene groups on aromatic rings. Single resonance peak is detected in this range in all the stations at 2.3 ppm (station 1 surface), 2.1 ppm (station 1 bottom), 2.2 ppm (station 2 surface and bottom), 2.1 ppm (station 3 surface) and 2.2. ppm (station 3 bottom). Peaks in the range 3.10–3.25 show the presence of aromatic amines. Very few peaks are observed corresponding to this as observed at 3.04 ppm (station 1 surface), 3.03 ppm (station 1 bottom), 3.028 ppm (station 3 surface), and 3.112 ppm (station 3 bottom).

Peaks in the range 3.65–4.00 showed the existence of protons attached to α- carbons to oxygen groups like methyl aryl ether and carbohydrate HCHO. The appearance of peaks is due to protons adjacent to ester. The main dissimilarity between the (Abakumov et al. 2018) H NMR spectra of HAs and FAs is that the occurrences of aliphatic and alicyclic groups are completely absent in FAs and they show the presence of aromatic rings with α- methyl and methylene groups, aromatic amines, oxygen groups like methyl aryl ether and carbohydrate HCHO (FAs contain more oxygen-containing functional groups than HAs). Peaks in the range 3.65–4.00 are seen in all the samples. This is detected at 3.844 ppm (station 1 surface), 3.852 ppm (station 1 bottom), 3.610 (station 2 surface), 3.843 (station 2 bottom), 3.848 (station 3 surface), and 3.871 (station 3 bottom). Peaks in the range 4.00–4.75 are also seen in the ^{1}H NMR spectra of isolated FAs. In the surface sediment of station 1, peaks were observed at 4.079 ppm and 4.6 ppm, while in the bottom sediment sample peaks were displayed at 4.109 and 4.6. Also in station 2, the surface sediment showed peaks at 4.09 ppm and 4.7 ppm, while peaks were displayed at 4.078 ppm and 4.6 ppm in the bottom sediment sample of this station. In station 3, the surface sample showed peaks at 4.104 ppm and 4.6 ppm, but in the bottom sediment, resonance peak was seen at 4.6 ppm.

As said previously, resonances in the range 6.40–8.45 ppm arise from aromatic protons with phenols and quinones, sterically hindered aromatic protons and also of nitrogen heteroaromatics (Hatcher et al. 1980; Wilson 1981; Wersaw 1985; Jokica et al. 2004; Prashob Peter 2014). These peaks were generally in the case of isolated FAs in this study. They are observed at 8.244, 9.617, and 10.667 ppm in station 1 surface sample and at 8.309, 9.626, and 10.618 ppm in the bottom sediment of the same station. In station 2 also more or less similar peaks were observed at 8.281, 9.626, and 10.637 ppm in surface and 8.293, 9.608, and 10.647 ppm in the bottom sediment sample. In station 3, the surface sample exhibited peaks at 7.152 and 8.120 ppm, while in the bottom sediment it was seen at 10.628 ppm. As seen in the case of HAs, at this point also peaks are recognized at values > 9 ppm which displays the occurrence of deshielded protons and sterically hindered aromatic protons (Grasso et al. 1990; Qi et al. 2004). This confirms the probability of having more or less common aromatic core involved to unsaturated carbons ($>CH = CR_2$) and aromatic groups such as phenols, decreased quinines through ionized carboxylic groups which may be slightly higher in FA than in HA (Bradley et al. 1998; Hernandez and Newman 2001; Prashob Peter 2014). The presence of signals in the low field region 7.1–10.7 ppm indicates the presence of more plentiful OH groups with the phenolic OH groups(Schnitzer and Skinner 1974), and that in the region 9.3–13.2 ppm labeled the existence of carboxylic OH groups (Chamberlain 1974).

Conclusion

The study revealed differences in the amount of isolated humic substances—humic and fulvic acids—with respect to differences in environmental settings. The existences of humic substances were found to be positively correlated with the organic carbon content of the sediments, tannin and lignin content, and fine texture of the sediment (silt), thus attesting the fact that environmental settings control the humification status of mangrove sediments. The results obtained from elemental analysis, UV-visible spectroscopic studies, FTIR, and NMR of the samples and their respective HS showed that almost all of the signals indicated the presence of the functional group properties and the higher degree of humification in the samples isolated from the study area.

Sediment samples collected from different mangrove wetlands contained higher level of carbon, nitrogen, hydrogen, and sulfur and also higher amount of humic substances which indicate higher rates of decomposition and humification happening in these environments. The presence of higher hydrogen content indicated the aliphaticity of the isolated humic substances from the Kerala mangroves. The N/C and H/C ratio in HAs and FAs in all the samples from the selected stations was found to be low, indicating the geographical location as “terrestrial” due to high lignin mineralization. The most likely occurrence of varying degrees of vascular lignin moieties and non-vascular lignin moieties is confirmed by higher C/N ratios from the Kerala mangroves. The spectral characteristics also supported the elemental ratios of the isolated humic substances. The E4/E6 ratios of fulvic acids were found to be higher than those of humic acids supporting the fact that the fulvic acids are with low molecular weights and are less polymerized. The surface samples from Kerala mangroves showed a higher E4/E6 ratio than the bottom sediment samples in both HAs and FAs which indicates that the surface samples were of young origin, whereas the bottom regions are more ancient due to higher microbial activity and decomposition. These ratios also help us to differentiate the study area based on humification degree and maturity; the low E4/E6 ratio indicates a lesser degree of humification and maturity which was observed towards the mangroves collected from the southern part of Kerala. The presence of hydroxyl, methyl, methylene, carbonyl, carboxyl, phenol, and amide in the humic substances was confirmed with FTIR spectrum of the isolated humic substances. ^{1}H NMR spectral characteristics were also complementary to the FTIR results. The occurrence of active functional groups, diverse elements, and favorable structural properties observed in this study indicate the richness of isolated humic materials which enhances microbial activity, soil productivity, and the potential to form the varied complexes in the study area. The structural characterization helps us to understand the mechanism of interaction of these materials with contaminant metals and other toxic chemicals in the environment, which definitely has the scope of a spatial comparison with respect to mangroves from different environmental settings in this study.

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Declarations

Conflict of interest The author(s) declare that they have no conflict of interest.

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