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FTIR ANALYSIS AND REVERSE PHASE HIGH PERFORMANCE CHROMATOGRAPHIC DETERMINATION OF PHENOLIC ACIDS OF *HYPNEA MUSCIFORMIS* (WULFEN) J.V.LAMOUROUX.

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ABSTRACT

Sea weeds have been one of the promising resources of biologically active metabolites and their extraction has significantly expanded in the last few decades. *Hypnea,* a proven candidate among the sea weeds for its rich mucopolysaccharides. In this study, *Hypnea musciformis* (Wulfen) J.V.Lamouroux. was screened for phenolic acid profile and further subjected to FTIR analysis of the different solvent extracts to know the functional groups.Reverse phase high-performance liquid chromatographic (RP-HPLC) method with gradient elution was adapted to fractionate and quantify free phenolic acids (gallic, vanillic, chlorogenic, sinapic, p-coumaric, ferulic, hydroxybenzoic, phloroglucinol, catechol and cinnamic acid). Chromatographic separation was performed with C18 column and detection was conducted at the wavelengths 254, 278 and 300 nm according to the absorption maxima of the analyzed compounds. Validation procedures were conducted and the method was proven to be precise, accurate and sensitive. The experimental results revealed high concentrations of sinapate, coumarate, phloroglucinol and ferulate, whereas,other phenolic acids were in low concentrations. Sinapate was established as the dominating phenolic acid. Diverse FT-IR peaks for each solvent extract has been determined and identified their specific functional groups. Remarkable correlation was found between the phenolics and FTIR peaks. 1743 cm^{-1} may be considered as optimal indicator of phenolics concentration. FTIR spectroscopy is recommended as rapid and reliable tools to predict the phytochemical composition of herbals. Further studies are warranted to isolate, purify the lead molecule and to evaluate its biological potentialities.

Key words: Sea weeds, RP-HPLC, FT-IR, phenolic acids, functional peaks.

INTRODUCTION

Seaweeds possess a wide array of phytochemicals which were significant in pharmaceutical and nutraceutical industries. Seaweeds are also marine algae resource of renewable energy and mostly dominated along the shallow waters. In India seaweeds are found abundantly along the south eastern and north eastern parts of the coast. There were approximately 681 known species of seaweeds reported in India,out of which 60 are commercially important.The people of Japan, New Zealand, Canada, Ireland, Norway, Iceland and Scotland have been consuming seaweeds since from ancient times.The seaweeds were used as animal and human food, soil manure, salt extractions (soda, iodine, etc.), colloid production (agar, alginates, carrageenan, furcellaran, etc.), cosmetics and pharmaceuticals. Thus represent an important economical resource in the south East Asia countries where they are not only largely harvested but also intensively and largely employed in the human nutrition[1].There were

reports that the *Hypnea musciformis* was an important carrageenan resource in the industry of phycocolloid gelling agent[2].There were numerous reports of macro algae derived compounds that have broad range of biological activities, such as antibiotic, antiviral, anti-neoplastic, antifouling, anti-inflammatory, cytotoxic and antimitotic. For centuries, many of the seaweed secondary metabolites have been used in traditional medicines due to their therapeutic potentials[3]. Marine algae were continuously exposed to many biotic and abiotic pressures which influence their physiology, which in turn leads to the production of multifunctional natural secondary metabolites. Phenolic compounds are commonly found in edible brown, green and red seaweeds, whose antioxidant properties have been correlated with their phenolic contents[4]. Seaweeds extracts were also considered to be rich source of flavanoids. The other major compounds were terpenes, fatty acids derivatives and other nitrogenous compounds. There were several reports on antimicrobial and pharmacological activities of different solvent extracts from marine algae[5][6]. But only scattered reports were available on intensive phytochemical studies using HPLC and FTIR studies on the marine seaweeds. In the past few years, Fourier transform infrared (FTIR) spectroscopy has developed to become versatile, flexible technique for the differentiation and identification of phytochemicals. The spatial resolution achievable with infrared micro spectroscopy allows for the analysis of macromolecular pools in individual cells that allow species-specific measurements within heterogeneous microscopic communities[7][8]. In this juncture, the present study was aimed to study the FTIR analysis of petroleum ether, ethyl acetate and ethanolic extracts of the red seaweed *Hypnea musciformis* and also RP-HPLC analysis of phenolic acids of methanolic extract.

MATERIALS AND METHODS

Sample preparation: The marine algae *Hypnea musciformis* was collected during April 2015, from the Mandapam coast (latitude 9˚ 17’ N, longitude79˚ 22’ E), Gulf of Mannar. The thalli of *Hypnea musciformis* were cut into pieces, sun dried and powdered in a grinder to 40-mesh size powder. The ground samples were then kept in air-tight container and stored for further analysis. Various extracts were prepared according to the methodology of Indian Pharmacopoeia [9]. 20 g of dried algal powder were taken in a Soxhlet apparatus and extracted successively with 250 ml each of solvents such as petroleum ether, ethyl acetate and ethanol. The extraction was repeated 2 to 3 times. The extracts were filtered through Whatsman filter paper No.1. It was then kept at room temperature for evaporation. The extracts were subjected for further analysis.

Determination of Total Phenolic Content: The amount of total phenolics in the seaweed was determined with the Folin-Ciocalteau reagent. Gallic acid was used as standard and the total phenolics were expressed as mg/g gallic acid equivalents (GAE). This blue colour was measured spectrophotometrically at 765nm and expressed as mg GAE/g of the sample.

HPLC Analysis: HPLC method was performed on Shimadzu LC-10 AT VP HPLC system, equipped with LC-10AT pump, UV–VIS detector an auto injector SIL-10AT. A Hypersil -BDS C-18 column with C-18 guard column was used.An isocratic HPLC witht wo LC-10 AT VP pumps (Shimadzu), variable wave length programmable photo diode array detector SPD-M10A VP, CTO-10AS VP column oven (Shimadzu), SCL-10A VP system controller (Shimadzu) and reverse phase Luna5-C18 (2) column 250 mm x 4.6 mm was used.The mobile phase components Potassium hydrogen phosphate and acetonitrile in a ratio of 75:25 were filtered through 0.2 l membrane filter before use and were pumped from the solvent reservoir at a flow rate of 1 ml/min which yielded a column backup pressure of 260–270 kgf/cm^2.The column temperature was maintained at 27 ºC. 20 ml of the respective sample was injected by using Rheodyne syringe. The elution was carried out with gradient solvent systems with a flow rate of1 ml /min at an ambient temperature (25–28 ºC). The mobile phase was prepared daily, filtered and sonicated before use. Total running time was 15 min. The sample injection volume was 20 µl whilst the wavelength of the UV–VIS detector was set at 254 nm. [10]

FT-IR analysis: The FT-IR studies have been followed as per the method described by Jagmohan[11]. Dried powder of different solvent extracts of algae was used for FTIR analysis. 10 mg of the dried extract powder was encapsulated in 100 mg of KBr pellet, in order to prepare translucent sample discs and subjected to pressure of about 5x106 Pa in an evacuated die to produce a clear transparent disc of diameter 13 mm and thickness 1mm. IR spectra region 4000-400 cm^{-1} were recorded at room temperature on a perkin Elmer fourier transform spectrometer equipped an air cooled DTGs (deuterated triglycine sulfate) detector. For each spectrum, 100 scans were CO added at a spectral resolution of 4cm^{-1}. The frequencies for all sharp bands were accurate to 0.01 cm^{-1}.

RESULTS AND DISCUSSION

Many live style diseases and pathological situations were connected with reactive oxygen species (ROS) or free radicals. ROS produced *in vivo* include superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). H_2O_2 and $O_2^{\bullet}$ can react in the presence of transition metal ions to form highly-reactive oxidizing species, the hydroxyl radical ($^{\bullet}OH$).Phytochemicals like polyphenolic groups such as flavonoids and phenolic acids were growing interest because of their potent antioxidant potentiality. The existence of any substances function as an antioxidant or free radical scavenger may protect the cells from the consequences of oxidative stress. Thus, antioxidants safe guard the cells against oxidative burst caused by reactive species [12] [13].Similarly, low molecular mass antioxidants like ascorbate and E, carotenoids, tannins also scavenge the free radicals from the cellular system. Preliminary phytochemical screening in the seaweed showed the presence of phenols in substantial amount i.e., 0.335 ± 0.03 mg gallic acid equivalent / g dry seaweed.

Subsequently, the HPLC analysis was attempted to fractionate polyphenolics. In this study C18 column was used to quantify the polyphenolic contents. Ten standard phenolic acids were used to compare with the chromatograms produced by the methanolic extract. From Figure 1, it can be observed that a good separation can be achieved within ashort period. Symmetrical, sharp and well-resolved peaks were observed for the 10polyphenolic standards. The elution order and the quantity of gallic, vanillic, chlorogenic, sinapic, p-coumaric, ferulic, hydroxybenzoic, phloroglucinol, catechol and cinnamic were 100.94, 109.12, 107.7, 5524.9, 764.22, 337.14, 10.4, 442.06, 129.58, and 107.34 µg/g respectively. Phenolic compounds were important secondary metabolites involved in scavenging ROS due to their OH groups. Out of the ten phenolic compounds sinapate was found in the significant level compared to others amounting 5524µg/g whereas coumarate was found at 764µg/g(Table 1). Phenolic acids show beneficial effects in terms of antioxidant, anti-ageing and protect cardiovascular issues. Further, their attributes reduce oxidative stress, lipid peroxidation, free radical generation and low density lipoprotein (LDL) cholesterol-oxidation. Moreover,other phenolic acids noticed were gallate, hydroxyl benzoate, chlorogenate, phloroglucinol, vanillate, cinnamte, and ferulate. These also possess positive biological effects on human health and ameliorate oxidative stresses. Phenolic compounds are found remarkably in medicinal plant species, including seaweeds, and have been reported to have wide range of biological activities including antioxidant [14] [15][16][17].HPLC evaluations of polyphenolics in *Gardenia jasminoides* revealed diverse phenolics, flavonoids and their involvement in free radical scavenging potential[18]. Zavoi et al.,compared the yield of phenolics with hepatoprotective potential in medicinal plants[19]. The HPLC analysis of the algae *Amphiroa anceps* revealed that the polyphenolic compounds present in them were an effective source of antioxidants. Therefore the seaweed extracts could have potential applications in food industries [20].Nagai and Yukimoto also reported that the phenolic compounds are one of the most effective antioxidant [21]. The HPLC analysis of *Gracilaria corticata* and *Spirulina platens* was correlated with the bioactive compounds having antioxidant activity [22].

FT-IR technique was used for evaluation the type of organic and inorganic complexes in plants. The analyze were carried out on drying and low aching temperature material of different parts of plants. The FT-IR spectra (4000-900 cm^{-1}) of ethanolic, petroleum ether and ethyl acetate extracts of sea weeds were analyzed and the specific wave numbers, intensities were compared. Fig. 2 a, b, c presents the FT-IR spectra of the sea weeds and Table 2, 3, 4 includes the corresponding absorption peak areas for specific regions. The functional groups discrimination was based on the FTIR peaks featured to stretching and bending vibrations. Region 1 includes < 1000 cm^{-1} corresponds to C-H bending vibrations from isoprenoids, region 2 (997-1130 cm-1) to stretching vibrations C-O of mono-, oligo- and carbohydrates, with signals at 1030, 1054, 1104, and 1130 cm^{-1}, while zone 3 show 1150-1270 cm^{-1} corresponds to stretching vibrations of carbonyl C-O or O-H bandings. Zone 4 forms 1300-1450 cm^{-1}correspond to stretching vibrations C-O (amide) and C-C stretchings from phenyl groups. Similarly, zone 5 includes1500-1600 cm^{-1} aromatic domain and N-H bending vibrations. Zone 6 the complex one 1600-1760 cm^{-1} corresponding to bending vibrations N-H amino acids, C=O stretchings of aldehydes and cetones, esters as well to free fatty acids (1710 cm^{-1}) and glycerides (1740 cm^{-1}). Zone 7 (2800-2900 cm^{-1}) possess C-H stretching vibrations specific to CH3 and CH2 from lipids, metoxy derivatives, C-H (aldehydes), including cis double bonds. Zone 8 (3350-3600 cm^{-1}) corresponds to stretching vibrations of OH groups (from water, alcohols, phenols, carbohydrates, peroxides) as well from amides (3650cm^{1}). In petroleum ether extracts revealed absorption peaks in the domain 3458.37,3429.43, 3136.25, 1460.11,723.31,2924.09, 2852.72, 931.62, 1417.68,1261.45,869.90, 800.46, 1373.32, 678.94,651.94,682.80, 3136.25,2924.09,2852.72,

2733.13,2675.27,1712.79, 1101.35,1026.13, 1712.79 cm-1. Meanwhile, ethyl acetate contain absorption peaks in domain 3294.42, 3267.41, 2922.16, 854.65, 1369.46, 3078.39, 1456.26, 678.94,651.94, 1718.582922.16,2854.65,1456.26,1718.58,3078.39, 1369.46, 1234.44, 678.94,653.87,1718.58, 1456.26cm-1 and ethanolic extracts shows 3537.45,3512.37,3456.44, 3431.36,3408.22,3379.29 ,3346.50,3325.28,3304.06,3284.77,3265.49,1554.63, 1521.84 ,1554.63,1521.84, 2920.23,2850.79,1365.60, 1712.79, 1712.79,1629.85, 1647.21, 650.01, 1112.93,1049.28, 1280.73, 1259.52,1224.80, 831.32,719.45,609.51,580.57, 557.43,536.21,514.99 cm-1.The FTIR analysis confirmed the presence of alcohols/phenols, alkanes , primary and secondary amines, alkyl halides, carboxylic acids, nitro groups, alkenes, alkynes(Table 2,3,4). Marimuthu et al ., also reported the presence of several functional groups such as amides, alcohols,phenols and phosphorous compounds in the FTIR analysis of crude powder of *Sargassum wightii*, which can be corroborated by the present findings[23]. Li et al., reported FTIR fingerprint spectrograms of traditional Chinese herbal *Marsdeniatena cissima*[24]. The species was used massively for treating many disorders in humans. Most FT-IR studies on algal seaweeds and their extracts revealed the toxic interaction sites of carboxyl, amino acid and hydroxyl groups on the algal surface. Biological samples such as algae show complex vibrational spectra that include overtones and combination of all bands. But metal-legend stretching frequencies and properties of functional groups coordinated to toxic centers offer useful information. C–O stretching, NH rocking C-O and CH2 stretching bands were metal sensitive and were shifted as the metal was changed, but NH2 vibrations were sensitive to the effect of intermolecular interactions (e.g., hydrogen bonding) which makes it difficult to discuss the strength of the metal-nitrogen bond from the frequency shift[25].

Similar FTIR studies on the fresh water algae *Spirulina* powder also confirmed the presence of several functional groups which were responsible for improving the health status and normalized in atrazine toxicity[26].Fourier transform infrared (FTIR) spectroscopy was also used in studies to identify and determine spectral features of *Chlorella vulgaris* and *Scenedesmus obliquus* (Turpin) by band assignments. As individual bands may have contributions from number of molecular groups representing different macromolecular components, the high level of correlation between certain bands has been used in aiding band assignment[27] .Marine habitats form the source of functional materials, including polyunsaturated fatty acids, polysaccharides, essential minerals, vitamins, antioxidants, enzymes and bioactive peptides[28]. FT-IR is a valuable tool for measuring many chemical constituents in plants[29]and seaweeds and it is also used to reveal some qualitative aspects regarding the organic compounds.

CONCLUSION

This is the first report on the phenolic profile of the sea weed *Hypnea musciformis*. The present results clearly indicate that *Hypnea musciformis* extract possess significant phenolics and therefore capable of antioxidant activities like ascorbate. This study indirectly showed that the phenolic compounds were pivotal for its free radical scavenging activity. Similarly, these antioxidant substances may be responsible for other therapeutic values like anti-inflammatory and chemoprotective properties.FT-IR spectroscopy for each of the solvent extract reveals diverse specific functional groups. FTIR analysis validates it as ideal tool to investigate the fingerprint and to predict the composition of therapeutic plants.Future perspectives include isolation, purification of lead molecule and to justify its biological potentialities.

Table 1.RP-HPLCprofileof phenolic acids in *Hypnea musciformis*

Compound	Concentration (µg/g)
Chlorogenic acid	107.7
Sinapicacid	5524.9
Hydroxylbenzoic acid	10.4
Gallic acid	100.94
Phloroglucinol	442.06
Vanillic acid	109.12
Cinnamic acid	107.34
Catechol	129.58
Ferulic acid	337.14
Coumaric acid	764.22

Table 2.FT-IR profile of functional groups (cm^{-1}) in petroleum ether extract of *Hypnea musciformis*

Compound	Bond	Petroleum ether extract
Alcohols/phenols	OH Stretch, H -bonded	3458.37,3429.43,3136.25
Alkanes	C-H Bend, C-H-rock	1460.11,723.31,2924.09, 2852.72
Alkenes	OH-Bend	931.62
Aromatics& aromatic amines	C-C Stretch, CH-"OOP"	1417.68,1261.45,869.90, 800.46,
Nitro groups	N=O bend	1373.32
Alkyl halides	C- Br Stretch, C-H wag(-CH_2X)	678.94,651.94,682.80
Carboxylic acids	OH Stretch	3136.25,2924.09,2852.72, 2733.13,2675.27,1712.79
Aliphatic amines	C-N Stretch	1101.35,1026.13
Carbonyls	C=O Stretch	1712.79

Table3. FT-IR profile of functional groups (cm^{-1})in Ethyl acetate extract of *Hypnea musciformis*

Compound	Bond	Ethyl acetate extract
Primary, Secondary Amines	NH stretch, OH stretch	3294.42,3267.41
Alkanes	H-C-H Asymmetric and symmetric Stretch, C-H rock	2922.16,2854.65,1369.46
Alkenes	=C-H stretch	3078.39
Amines-Secondary	N-H Bend	1456.26
Alkyl halides	C- Br Stretch	678.94,651.94
Ketones, Saturated aliphatic	C=O stretch	1718.58
Carboxylic acids	OH Stretch	2922.16,2854.65,1456.26, 1718.58
Aromatic ring	C=C-H Asymmetri Stretch	3078.39,1369.46
Aliphatic amines	C-N Stretch	1234.44
Alkyl halides	C-Br Stretch	678.94,653.87
Alkynes	-C (triple bond)C-H:C-H bend	678.94,653.87
Carbonyls	C=O Stretch	1718.58,1456.26

Table 4. FT-IR profile of functional groups(cm^{-1})in Ethanolic extract of *Hypnea musciformis*

Compound	Bond	Ethanolic extract
Alcohols/phenols	OH Stretch, H -bonded	3537.45,3512.37,3456.44, 3431.36,3408.22
Amines- Secondary, Amides,	N-H Stretch, N-H bend	3379.29,3346.50,3325.28, 3304.06,3284.77,3265.49, 1554.63,1521.84
Nitro groups	N=O stretch	1554.63,1521.84
Alkanes	C-H Stretch, CH rock	2920.23,2850.79,1365.60
Carboxylic acids	C=O Stretch	1712.79
Ketones,Saturatedaliphatic	C=O Stretch	1712.79,1629.85

Alkenes	C-C=C Symmetric stretch	1647.21
Alkynes	-C (triple bond)C-H:C-H bend	650.01
Aliphatic amines	C-N Stretch	1112.93,1049.28
Alkyl halides	C-H wag ($-CH_2X$), C-Cl stretch, C-Br Stretch	1280.73,1259.52,1224.80, 831.32,719.45,609.51,580.57, 557.43,536.21,514.99

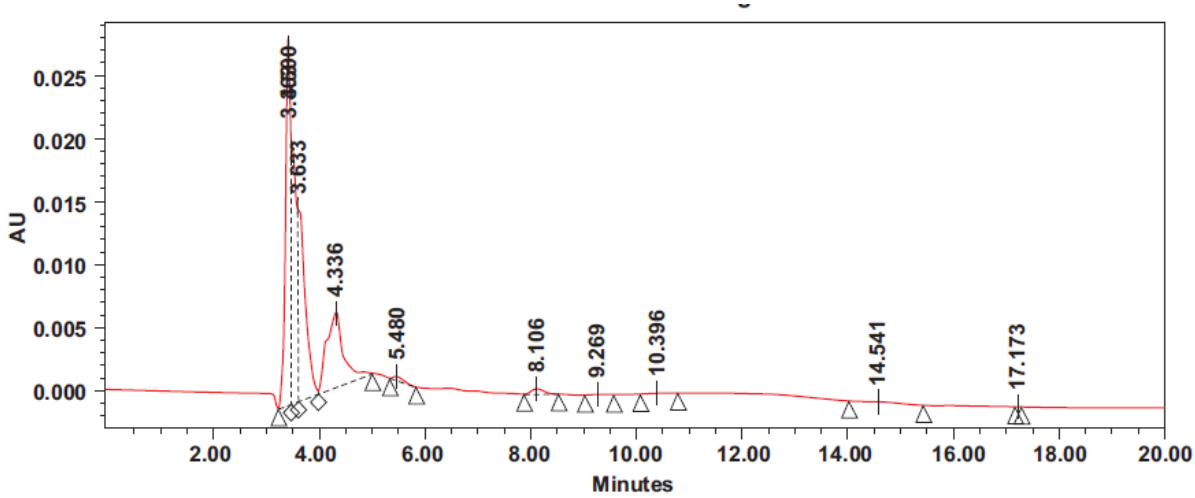

Figure 1. HPLC chromatogram of methanolic extract of *Hypnea musciformis*

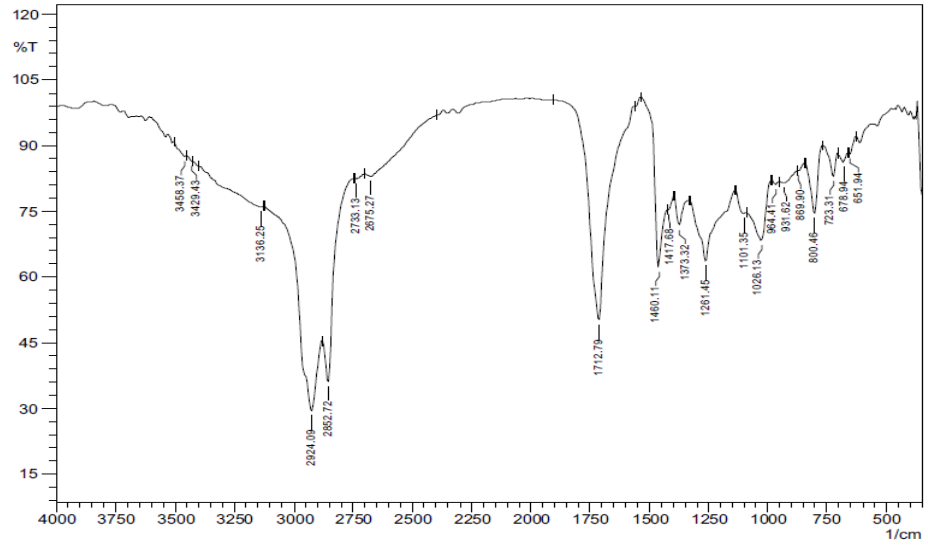

Figure 2. FTIR spectrum of petroleum ether extract of *Hypnea musciformis*

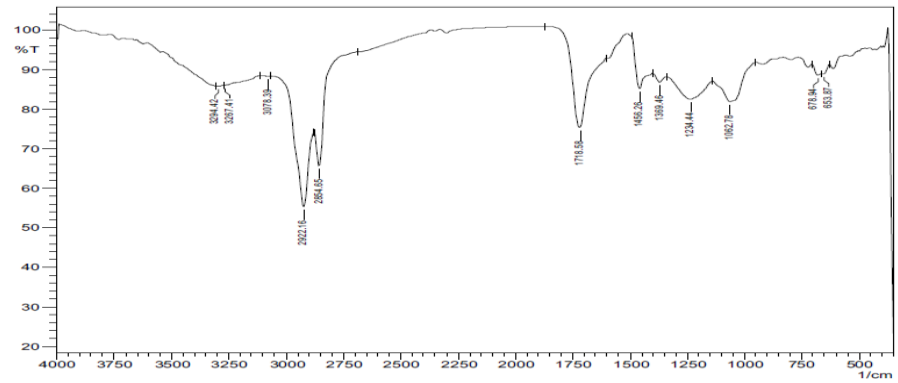

Figure 3. FTIR spectrum of ethyl acetate extract of *Hypnea musciformis*

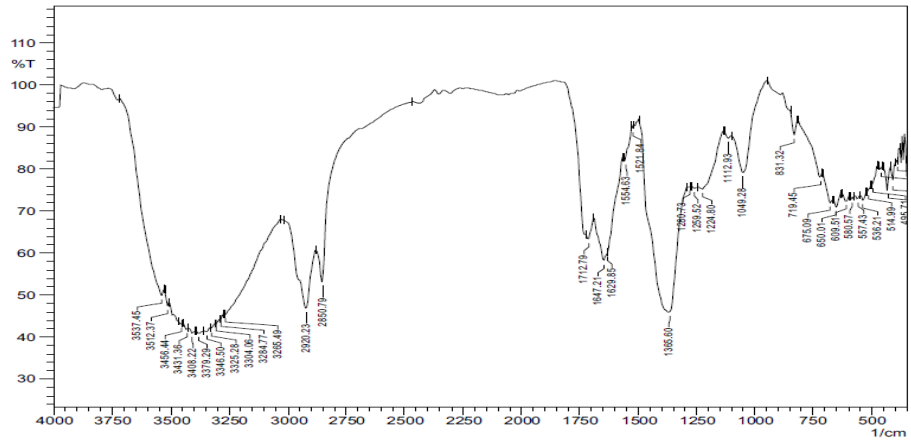

Figure 4. FTIR spectrum of ethanol extract of *Hypnea musciformis*

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