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Characterization of Antibacterial Compounds from Plants by FTIR and GC-MS Analyses

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Biofouling is a critical concern in several environmental conditions and hence significant economic resources have been dedicated to the development of effective biofouling monitoring and preventive strategies. The present study focused on isolation, characterization and evaluation of compounds from various medicinal and common plants and seaweed which are found to be inhibiting biofilm formation, using FTIR and GC-MS. The solvent extracts were scanned by using the KBr pellet method and the characteristic peaks were detected. For GC-MS analysis, ethanol, chloroform, ethyl acetate, water and methanol extracts were used. The compound detection employed the NIST Ver. 2.0 library.The FTIR spectrum confirmed the presence of functional groups such as carbonyls, amines, alcohol, alkene and aromatic compounds in different plant and seaweed extracts. The results of the GC-MS analysis provided different peaks determining the presence of several compounds with different therapeutic activities. Thus this study creates a platform to screen many bioactive components to act as a biofouling agent.

Keywords: Environmental biofilms, Inhibition, GC-MS, FTIR, Cluster analysis.

Bacteria live as free floating cells in aquatic environment and nearly majority of them depend on other microorganisms for energy, carbon and other nutrients and live in microecosystems filled with hundreds of other microorganisms. It is estimated that in the natural world more than 99% of all bacteria exist as biofilms (Costerton *et al*, 1987). When bacteria form biofilms, they become more resistant to many environmental perturbations, such as fluctuation of nutrients and oxygen, alteration of pH, and antibiotics effects. One of the reasons for the chronic nature of some infections caused by these pathogens is the ability of the pathogens to form biofilms in which the bacteria are protected from host defences and killing by antibiotics (Whiteley *et al*, 2001). While biofilms of bacteria are more resistant to antimicrobial agents the medicinal plants have been used as alternate source to obtain anti-biofilm forming agents.

Herbal molecules are safer and overcome the resistance produced by the pathogens as they exists in a combined form or in a pooled form of more than one molecule in the protoplasm of the plant cell (Lai *et al*, 2004 and Tapsell *et al*, 2006). The effectiveness of the herbal plants depend on the use of proper plant part and its biological potency which in turn depends upon the presence of required quantity and nature of primary and secondary metabolites in a raw drugs (Vinoth *et al*, 2011). An acquaintance of the chemical constituents of plants is enviable for the discovery of anti-biofilm agents and for discovering the actual significance of folkloric remedies. Therefore a meticulous validation of the herbal drugs has emerged as a new branch of science emphasizing

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and prioritizing the standardization of the natural drugs and products because several of the phytochemicals have complementary and overlapping mechanism of action. As bacterial resistance of biofilm forming communities is becoming a larger problem for environmental health, some potential new materials-based approaches for preventing biofilm formation would have to be investigated.

In the past decade a number of remarkable advancement has been made in analytical techniques including HPLC, UV, FTIR, NMR and GC-MS that were powerful tools for separation, identification and structure determination of phytochemicals (Roberts and Xia, 1995). Though, simple, cost-effective and rapid tests for detecting phytocomponents are necessary, spectroscopic (UV-Vis, FTIR) methods together or separate can be used in this sense as well as conventional methods (Aysal *et al*, 2007).The Fourier Transform Infrared (FTIR) spectroscopy allows the analysis of a significant amount of compositional and structural information in plants. Furthermore, FTIR spectroscopy is a well-known time saving method to characterize and identify functional groups (Grube *et al*, 2008). Mass spectrometry, together with chromatographic separations such as gas chromatography (GC-MS) is generally used for direct analysis of components existing in traditional medicines and medicinal plants. In recent years GC-MS studies have been progressively useful for the analysis of medicinal plants (Khare *et al*, 2007).

Hence the main aim of this work was to resolve the bioactive compounds present in the 17 common and medicinal plants *Croton bonplandianus, Cassia fistula, Cadaba fruticosa, Cassia alata, Gelidiella aerosa, Justicia gendarussa, Acalypha indica, Trigonella foenum-graecum, Azadirachta indica, Ocimum sanctum, Anethum graveolens, Phyllanthus emblica, Cuminum cyminum, Murraya koenigii, Mentha longifolia, Curcuma longa, Terminalia chebula* and one sea weed *Gracilaria edulis,* with the application of GC-MS and FTIR techniques, which may provide an insight in its use of traditional medicine. The study was also extended to identify the key compounds from these medicinal plants, their functional groups that are accountable for the anti-biofilm activity of the compounds.

MATERIALS AND METHODS

Collection of plant material

The 17 medicinal and common plants were collected in Chennai, Tamil Nadu and were identified.The leaf, seed or fruit sample was taken for extraction using ethanol, methanol, chloroform, ethyl acetate and water as listed in Table 1.

GC-MS analysis

The powdered plants and seaweed sample were soaked with 30 ml ethanol overnight and filtered through ash less filter paper with sodium sulphate. The extracts were concentrated to 1 ml by bubbling nitrogen into the solution. The extract contained both polar and non-polar phyto components. 2µl of the ethanolic extracts of the plants and seaweed samples were employed for GC-MS analysis (Merlin *et al*, 2009). The Clarus 680 GC used in the analysis employed a fused silica column packed with Elite-5MS [30.0m, 0.25mmID, 250µm df] and the components were separated using Helium as carrier gas at a constant flow of 1 ml/min. The 2µl sample extracts injected into the instrument were detected by the Turbo gold mass detector (Perkin Elmer) with the aid of the Turbo mass 5.4.2 software. During the 36thminute GC extraction process, the oven was maintained at a temperature of 110°C with 2 minutes holding. The injector temperature was set at 250°C (mass analyser). The different parameters involved in the operation of the Clarus 600 Mass Spectrometer, were also standardized (Inlet line temperature: 240°C; Source temperature: 240°C). Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 50 to 600 Da. The MS detection was completed in 36 minutes.

FTIR analysis

FTIR analysis was performed using KBr pellet method, which was used to detect the characteristic peaks and their functional groups. 97% of KBr and 3% of the samples were added and mixed vigorously using mortar and pistol. After mixing, the samples were loaded in a KBr die set. The KBr die set was pressed under KBr to convert the powder form to pellet form. The pellet was analysed with FTIR spectrophotometer. The peak values of the FTIR were recorded. Each and every analysis was repeated twice for the spectrum confirmation. The functional group data as obtained from the FTIR analysis were tabulated

and subjected to clustering analysis using the SPSS v.16 software in order to identify the distribution of the various functional groups in the compounds. The cluster mapping was performed for the data obtained from the FTIR to classify the extracts based on the predominant occurrence of the various functional groups occurring in the extracts.

Identification of components

The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The detection of the compounds employed the NIST (National Institute of Standards and Technology) (NIST08s). The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name and molecular weight of the components of the test materials were ascertained.

RESULTS AND DISCUSSION

GC-MS Analysis

The methanolic, ethyl acetate and ethanolic extracts of seaweed and plant samples were subjected to GC-MS analysis. Interpretation on mass spectrum GC-MS were conducted using the database of National Institute of Standards and Technology (NIST) which has more than 62,000 patterns. The name, molecular weight and structure of the components of the test materials were ascertained. The presence of diverse bioactive compounds justifies the use of the seaweed and plant extracts in antifouling mechanisms. However isolation of individual phytochemical constituents and subjecting it to biological activity will definitely give successful results. The GC-MS profile showed the existence of several compounds such as adipic acid, alpha amyrin, ar-tumerone, asarone, beta amyrin, beta sitostirol, betulin, campesterol, curlone, diosgenin, gamma-sitosterol, gamma-tocopherol, hexadecanoic acid, hexanedioic acid, inositol, limonene, lupeol, n-hexadecanoic acid, phenol, phytol, stigmasterol, taraxasterol and vitamin E. Most of the compounds listed have been proved to be antibacterial, anticancer, antioxidant and antifungal agents. The effectiveness of these compounds needs to be studied further to prevent the biofilm formation.

Functional groups identification

The FTIR spectra of the different solvent extracts of selected plants with high inhibitory activity were presented in Fig. 1. The FTIR spectrum was used to identify the functional groups of the active components present in extract based on the peaks values in the region of IR

Table 1. List of plants with the solvents used for the extraction. The extract number was given in parenthesis.*

Ethyl acetate	Ethanol	Methanol
Croton bonplandianus (1)	*Cassia alata* (8)	*Croton bonplandianus* (2)
Cassia fistula (3)	*Trigonella foenum-graecum* (12)	*Cassia fistula* (4)
Cadaba fruticosa (5)	*Gelidiella aerosa* (14)	*Cadaba fruticosa* (6)
Trigonella foenum-graecum (11)	*Gracilaria edulis* (16)	*Terminalia chebula* (10)
Gelidiella aerosa (13)	*Azadirachta indica* (18)	
Gracilaria edulis (15)	*Ocimum sanctum* (20)	
Azadirachta indica (17)	*Justicia gendarussa* (22)	
Ocimum sanctum (19)	*Acalypha indica* (24)	
Justicia gendarussa (21)	*Anethum graveolens* (26)	
Acalypha indica (23)	*Phyllanthus emblica* (28)	
Anethum graveolens (25)	*Cuminum cyminum* (30)	
Phyllanthus emblica (27)	*Murraya koenigii* (32)	
Cuminum cyminum (29)	*Mentha longifolia* (34)	
Murraya koenigii (31)	*Curcuma longa* (36)	
Mentha longifolia (33)		
Curcuma longa (35)		

***Cassia alata* (7) and *Terminalia chebula* (9) were also extracted with chloroform and water respectively.

radiation. When the extract was passed into the FTIR, the functional groups of the components were separated based on its peaks ratio. The results of FTIR analysis confirmed the presence of carbonyls (C=O), amines (C-N), (N-H), alcohol (O-H), ether (C-O), Alkene (=C-H), = - CH_3), alkane (C-H) and aromatic (C=C) (Table 2). Hence the extracts subjected to FTIR analysis were used for the identification of chemical constituents present in the plant extracts. In addition, FTIR spectroscopy is proved to be a reliable and sensitive method for detection of biomolecular composition.

Table 2. FTIR peak values and their characteristic functional groups.

Functional groups	Characteristics Absorptions (cm^{-1})
Carbonyls (C=O)	1670-1820
Amines(C-N)	1080-1360
Amines(N-H)	1600
Ether (C-O)	1000-1300 (1070-1150)
Alcohol (O-H)	3200-3600
Alkene (=C-H)	3010-3100
Alkene(= -CH_3)	2850-3000
Alkane (C-H)	1350-1480
Aromatic(C=C)	1400-1600

Cluster analysis was performed using SPSS v.16.0 to generate a cluster map of the functional profiles of the plants. The dendrogram plotted using the Ward's method indicated the classification of the plants based on the functional groups distributed in their respective solvent extracts (Fig. 2.). According to the cluster map, the plants were classified based on their functional groups into five clusters and the members of each cluster were shown in Table 3. These results will

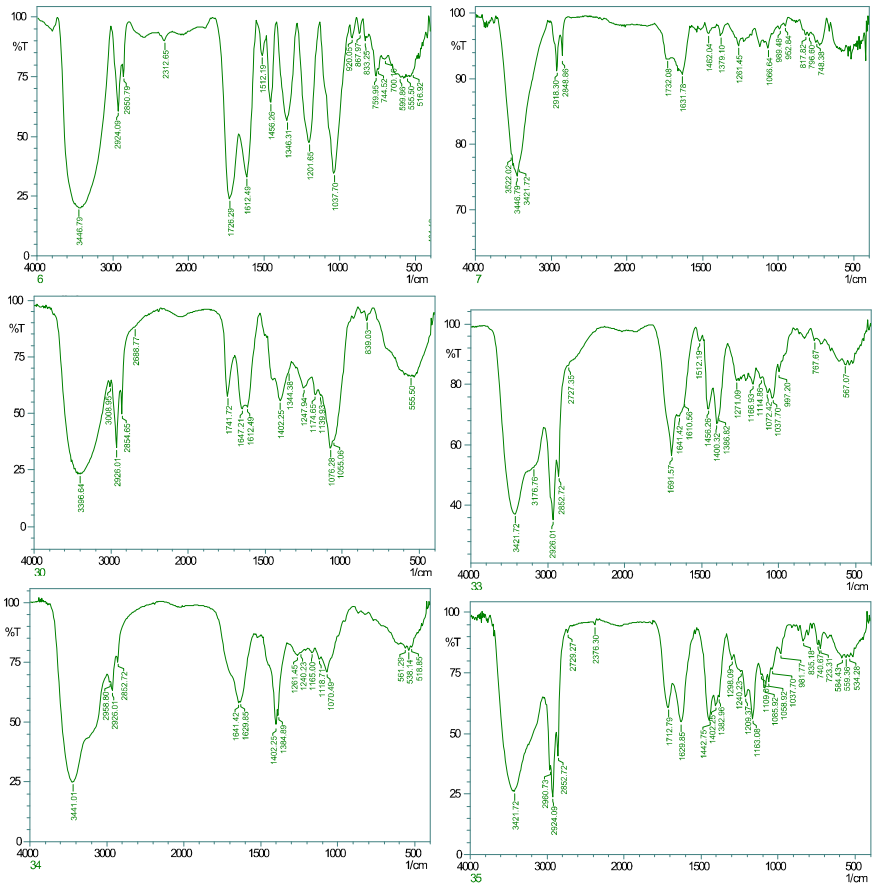

Fig. 1. FTIR Spectra of selected extracts which showed higher inhibitory activity (Extract number has been mentioned at the left bottom of the spectrum)

help in understanding the presence of active compounds with specific functional groups.

The solvent extracts of these plants were tested against eight bacterial strains which are known to have biofilm forming ability. No solvent extract was capable of inhibiting all the bacterial strains tested in this study. Based on the cluster formation the level of inhibitions showed marked variations. The plant extracts in cluster 3 and 5 were found to be less inhibitive when compared to the other plant extracts grouped in clusters 1, 2 and 4. The variation observed in the level of inhibition might be attributed to the relative concentration of the bioactive compounds in the extracts. Another plausible explanation might be due to the resistance developed by the biofilm forming bacteria from the environment to the chemicals.

The results of the analysis of variance tests suggested the predominant roles of various functional groups among the clusters and they were presented in Table 4. The cluster analysis

Table 3. Effect of solvent extracts from plants on biofilm forming bacteria

Cluster	Sample No.	Plant	Solvent	% of bacterial strains inhibited*
1	14	*Gelidiella aerosa*	Ethanol	37.5
	2	*Croton bonplandianus*	Methanol	12.5
	18	*Azadirachta indica*	Ethanol	50
	12	*Trigonella foenum-graecum*	Ethanol	0
	23	*Acalypha indica*	Ethyl acetate	50
	7	*Cassia alata*	Chloroform	75
2	24	*Acalypha indica*	Ethanol	50
	4	*Cassia fistula*	Methanol	50
	1	*Croton bonplandianus*	Ethyl acetate	0
	13	*Gelidiella aerosa*	Ethyl acetate	0
	15	*Gracilaria edulis*	Ethyl acetate	0
	11	*Trigonella foenum-graecum*	Ethyl acetate	12.5
3	26	*Anethum graveolens*	Ethanol	0
	28	*Phyllanthus emblica*	Ethanol	25
	21	*Justicia gendarussa*	Ethyl acetate	0
	8	*Cassia alata*	Ethanol	37.5
	16	*Gracilaria edulis*	Ethanol	0
	6	*Cadaba fruticosa*	Methanol	75
4	20	*Ocimum sanctum*	Ethanol	0
	36	*Curcuma longa*	Ethanol	25
	27	*Phyllanthus emblica*	Ethyl acetate	37.5
	3	*Cassia fistula*	Ethyl acetate	37.5
	17	*Azadirachta indica*	Ethyl acetate	50
	33	*Mentha longifolia*	Ethyl acetate	62.5
	34	*Mentha longifolia*	Ethanol	62.5
5	31	*Murraya koenigii*	Ethyl acetate	12.5
	32	*Murraya koenigii*	Ethanol	0
	30	*Cuminum cyminum*	Ethanol	62.5
	19	*Ocimum sanctum*	Ethyl acetate	0
	29	*Cuminum cyminum*	Ethyl acetate	37.5
	35	*Curcuma longa*	Ethyl acetate	62.5

* Total number of strains tested in this study n = 8.

Table 4. Cluster mapping of the functional groups

Cluster	1	2	3	4	5
1	—	N-H, = - CH_3	N-H, C-H	N-H	-C-O-, N-H, =C-H, C-H
2	N-H, = - CH_3	—	O-H, C-H	O-H, N-H	-C-O-, O-H, = C-H, C-H
3	N-H, C-H	O-H, C-H	—	N-H	-C-O-
4	N-H	O-H, N-H	N-H	—	-C-O-, N-H
5	-C-O-, N-H, =C-H, C-H	-C-O-, O-H, =C-H, C-H	-C-O-	-C-O-, N-H	—

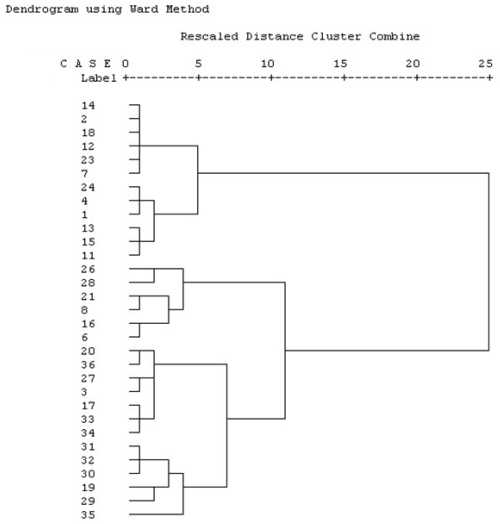

Fig. 2. Cluster analysis of the plant extracts based on the functional groups. Case label indicates the extract number.

indicated that the functional group amines were occurring predominantly in the compounds followed by the alcohol groups. The cluster map also revealed the presence of the functional group amine to be predominantly present in the active compounds of the plant extracts. It is therefore necessary to study in detail about the characteristic impact of the presence of amine group in the compounds would have an efficient role in forming an anti-biofilm agent.

CONCLUSION

Surface-associated communities of bacteria, called biofilms, spread through natural environments. Mature biofilms are resistant to a wide range of antimicrobial treatments and therefore pose persistent pathogenic threats. The use of surface chemistry to inhibit biofilm growth has been found to only transiently affect initial attachment. Hence an attempt has been made to study the active plant compounds as a source of anti-biofilm agents. The study was made amongst various medicinal plants with the application of GC-MS and FTIR techniques. These mass spectra are fingerprint of the compound which can be identified from the NIST data library. These metabolites can be further exploited for the use of antifouling through eco-friendly manner.

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