Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(3): 3874-3879
Received: 23-03-2019
Accepted: 28-04-2019

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Bio-assay guided isolation of lead bioactive molecules from *Spirogyra rhizopus* C-C. Jao

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Abstract
Scientific and technological advancement which propelled the scientists in drug discovery research to design as well as synthesize novel drug to treat multidrug resistant microorganisms. *Spirogyra* alga is the source of novel bioactive phytochemicals and contains carbohydrates, proteins and fats. Among the different crude extracts prepared from *Spirogyra rhizopus* C.-C. Jao, Chloroform crude extract showed promising activity against four bacterial strains *Salmonella typhimurium, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*. The present study aimed to investigate the bioassay guided isolation of lead bioactive molecules from active chloroform crude extract and evaluate their antibacterial potential against *S. typhimurium, E. coli, S. aureus* and *P. aeruginosa* by disc diffusion method, column chromatography isolation of crude extract leads to SP-1, SP-2, SP-3 and SP-4 bioactive molecules and structural elucidation was done with the help of ^{1}H-NMR, ^{13}C-NMR, IR and DEPT spectral studies.

Keywords: Bioassay guided isolation, antibacterial acitvity, NMR, IR, DEPT

Introduction
Algae are represented by 30,000 species worldwide supplying oxygen to the biosphere, food for fish and man, phytochemicals, medicine and fertilizers as well as source of structurally unique natural products [1, 2]. Terpenoids were predominantly isolated from marine algae in the 1970s to 1980s, further it led to the isolation of many classes including nitrogen and oxygen heterocycles, sterols, amino acids and guanidine derivatives [3], different types of algae have been intensively assessed for their antioxidant, antibacterial and antifungal activities [4, 5].
Spirogyra is a filamentous fresh water macro algae, long, thin strands which contribute to the familiar green, slimy 'blanket weed' in ponds [6]. It is most common genus of Zygnemataceae, it exhibits the diversity of 12 to 13 genera and more than 400 species are presently recognized based on morphological characteristics [7, 8]. However, Spirogyra genus is mostly found in its vegetative stage, at pH values between 6.2 and 9.1 [9], It has been commonly used in several Thai food and contains high amount of nutritional compositions, including carbohydrate, fat, proteins and mineral substances [10, 11]. *In vivo* studies indicated that *Spirogyra* sp. has several beneficial effects, including antigastric ulcer and anti-inflammatory effect [12]. *Spirogyra* sp. extract has been also shown antioxidant activity *in vitro* [13] and its phytochemical compositions recently revealed the presence of phenolics, tannins, glycosides, sterols, terpenes, variety of monosaccharides and saponins [14-16]. Kang *et al.* recently isolated gallic acid from spirogyra sp. cures heart disease through vasorelaxant antihypertensive effect [17]. The use of alternative fuels for the mitigation of ecological impacts by use of diesel has been focus of intensive research and *Spirogyra* sp. [18] shows maximum biodiesel conversions from oil to alcohol ratio (1: 8). Lee *et al.* evaluated the biosorption capacity of heavy metal ions lead (PbII) and copper (CuII) from aqueous solutions and found that *Spirogyra* species are effective in the removal of heavy metal ions [19]. Champagne *et al.* reported *Spirogyra* sp. was efficient to recover nutrients from municipal waste water sources and simultaneous production of biomass that contains value added bio chemical components for bioenergy and biofuels applications [20]. Among the different crude extracts we found chloroform extract displayed relatively superior antibacterial activity, we encouraged from the previous result hence the present study focus on column chromatographic isolation of lead bioactive molecules from the chloroform crude extract.

Materials and Methods
^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker NMR spectrometer. The chemical shifts were given in ppm (δ) and were referenced relative to $CDCl_3$ (δ 7.28 and 76-77 ppm for ^{1}H and ^{13}C-NMR respectively) and the chemical shifts were expressed in (ppm) values with TMS as an internal reference. The IR spectra were recorded on a Bruker FT-IR spectrometer.

The IR spectra were recorded on a Bruker FT-IR spectrometer. Structural elucidation was done by comparing the values with the reported data in the literature.

Experimental section

The different crude extracts were prepared by standard procedure and subject to antibacterial activity test, chloroform extract showed relatively superior antibacterial activity was subjected to column chromatography for the isolation of lead bioactive molecules. Silica gel (60-120 mm mesh size) was used as a stationary phase in column and it was dried at 100^0C for 2h to activate, glass column was packed with the activated silica gel slurry in chloroform. The slurry was added to the column gradually to avoid cracks. This process was continued till a uniform column of desired length was obtained. 4 g of the crude extract was mixed with 12 g of silica gel and mix with minimum amount of chloroform to obtain homogenous mixture. Then the solvent was carefully evaporated under vacuum and loaded to the column that was already packed with silica gel. The solvent system selected for isolation of compounds was made by carrying out TLC analysis of the crude extract in various combinations of solvents with increasing polarity. Chloroform and ethyl acetate mixture were found to show good separation of compounds on the TLC plate. The column was eluted with different solvent ratio of chloroform: ethyl acetate (in the ratio 100:0, 95:5, 90:10, 85:15, 80:20, 75:25, upto 50:50, 45:55, upto 25:75, 20:80, 15:85, 10:90, 5:90, 0:100) and various fractions were collected. Successive elution of column with increasing polarity of chloroform and ethyl acetate resulted in 162 fractions.

A total of 162 fractions were collected and further checked by TLC. The spots developed were visualized under UV light at 254nm and 365nm and then by exposure to iodine vapor. Fractions which have similar spot were collected and combined together in a conical flask. Totally 23 combined fractions were collected and labeled by letters from A-W. Fractions 1-6(A), 7-9(B), 10-14(C), 15-18(D), 19-24(E), 25-27(F), 28-33(G), 34-37(H), 38-41(I), 42-57(J), 58-67(K), 68-70(L), 71-82(M), 83-88(N), 89-92(O), 93-106(P), 107-118(Q), 119-126(R), 127-138(S), 139-148(T), 149-154(U), 155-159(V) and 160-162(W) were combined based on TLC profile. Further the two fractions B and K which shows one spot on TLC at different solvent system.

However, the other fractions has more than one spot, based on TLC profile the fractions J, K, M, and P were selected for further purification by column chromatography pure compounds (SP-1, SP-2, SP-3 and SP-4) were obtained with the solvent system chloroform: ethyl acetate.

Table 1: Isolated compounds

Fractions	Code	Weight of isolated compound
J 42-57	SP-1	1.2 g
K 58-67	SP-2	530 mg
M 71-82	SP-3	620 mg
P 93-106	SP-4	140 mg

Structural elucidation of isolated compounds

The structures of the compounds (SP-1, SP-2) that were isolated from chloroform extract of *Spirogyra sp.* were proposed based on the spectroscopic data (IR, ^{1}H NMR, ^{13}C NMR, and DEPT-135) and comparing with the reported data in literature. SP-1 compound found to be Monomethyl Lutein, where as SP-2 is γ-sito sterol and matches with spectrum of γ-sito sterol.

Structural elucidation of compound SP-1

Rf value of Mono methyl Lutein is 0.25

The IR spectrum (Appendix 1) of SP-1 revealed the presence of hydroxyl (3400 cm^{-1}) and alkene (1661 cm^{-1}) functional groups. The ^{13}C-NMR spectrum of SP-1 showed carbon resonances in the olefinic (C=C double bond) region (124.48 to 138.50 ppm) indicating double bonds. In the ^{13}C-NMR spectrum, the signals of double bonds in the cyclic alkene systems were observed at δ126.2 (C-5) and 137.5 (C-6) as well as δ124.8 (C-4') and 137.7 (C-5'), and two oxygenated carbon signals were identified at δ 65.0 (C-3) and 65.9 (C-3'). These facts suggested that compound SP-1 was of a carotenoid derivative. The ^{1}H-NMR spectrum of SP-1 displayed ten methyl protons at δ0.9-2.04, two carbinol protons at δ4.06 and 4.30, and fifteen olefinic protons at δ5.51-6.72. The ^{1}H NMR data displayed two methyl groups bound to aliphatic carbons at δH 0.97 (3H, s, H-31) and δH 0.89 (3H, s, H-16) and an overcrowded olefinic signals, which appeared between 5 and 7 ppm, characteristic of polyenes, SP-1 Information from ^{13}C-NMR, as well as ^{1}H-NMR and other spectral data led to the identification of the structure of compound SP-1 displayed high structural similarity to lutein [21].

Table 2: ^{13}C-NMR, DEPT and ^{1}H spectral data of compound SP-1 along with the data of lutein

No.	^{13}C-NMR of SP-1 (ppm)	^{13}C-NMR of Lutein (ppm)	^{1}H NMR of SP-1	^{1}H NMR of Lutein	δ DEPT SP-1	Nature of the carbon
1	37.12	37.13				C
2	48.44	48.45	1.75, 1.64	1.78, 1.50	48.44	CH2
3	65.1	65.1	4.03	4.03	65.1	CH
4	42.55	42.56	2.32, 2.44	2.05, 2.42	42.55	CH2
5	125.6	126.17				C
6	138	138				C
7	128.73	128.73	6.14	6.12	128.72	CH
8	130.82	130.81	6.66	6.65	130.81	CH
9	135.7	135.07				C
10	130.05	130.04	6.14	6.07	130.09	CH
11	124.48	124.49	6.66	6.73	124.48	CH
12	137.56	137.57	6.28	6.28	137.56	CH
13	136.5	136.42				C
14	132.57	132.58	6.26	6.23	132.57	CH
15	130.06	130.09	6.64	6.63	130.09	CH
16	30.26	30.26	1.02	1.08	30.26	CH3
17	28.73	28.73	1.09	1.09	28.73	CH3
18	21.61	21.62	1.75	1.78	21.61	CH3
19	29.69	29.7	1.93	1.97	29.69	CH3

20	12.81	12.81	1.98	1.97	12.81	CH3
1'	34.03	34.04				C
2'	44.63	44.64	1.27, 1.98	1.37, 1.85	44.63	CH2
3'	65.93	65.93	4.25	4.25	65.93	CH
4'	128.54	128.81	5.46	5.5	128.72	CH
5'	137.77	137.77				C
6'	54.98	54.97	2.44	2.47	54.98	CH
7'	131.31	131.3	5.48	5.47	131.31	CH
8'	130.1	130.09	6.66	6.65	130.09	CH
9'	135.7	135.7				C
10'	125.59	125.6	6.14	6.05	125.59	CH
11'	124.93	124.94	6.66	6.74	124.94	CH
12'	138.5	138.5	6.28	6.28	138.5	CH
13'	136.7	136.49				C
14'	137.77	137.73	6.26	6.23	137.73	CH
15'	130.82	130.81	6.64	6.63	130.81	CH
16'	29.5	29.5	0.87	0.85	29.5	CH3
17'	24.29	24.29	1.02	1.03	24.28	CH3
18'	22.85	22.86	1.64	1.64	22.85	CH3
19'	12.77	12.76	1.92	1.9	12.75	CH3
20'	13.1	13.11	1.99	1.95	13.11	CH3

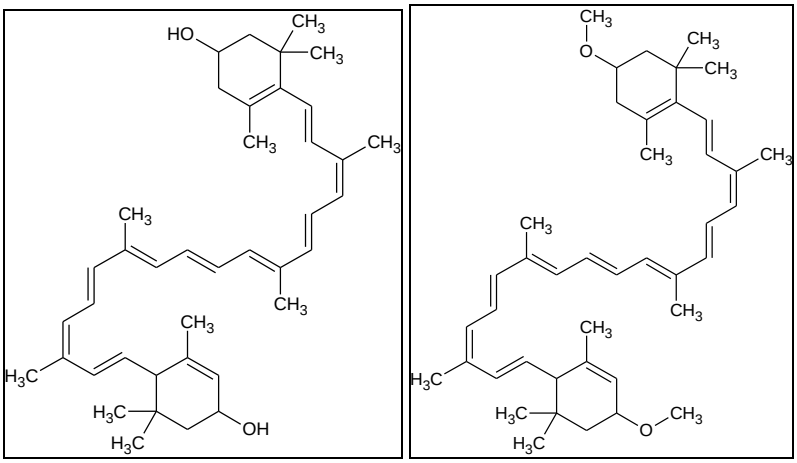


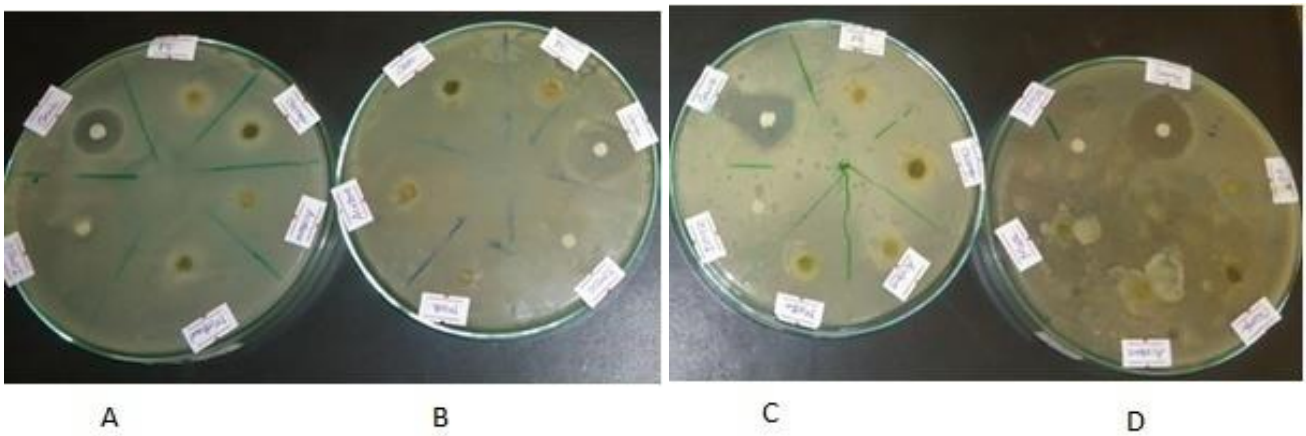
Fig 1: Structure of compound Lutein and Structure of Methyl derivative of Lutein (SP-1)

Structural elucidation of compound SP-2

The spectral data (IR, ^{1}H NMR, ^{13}C NMR and DEPT-135) of SP-2 compound is carefully analyzed and it is confirmed that γ-sito sterol, in comparision with the reported data in literature. The spectral data of SP-2 matches with that of γ-sito sterol.

Antibacterial activities of SP-1, SP-2

The isolated compounds of *S.rhizopus* as SP-1 and SP-2 were tested for antibacterial activity against four human bacterial pathogens *S.thyphimurium, P.aeruginosa, S. aureus and E. coli.* results were presented in the Table; degree of activity was higher than crude extracts. From the Table, SP-2 isolate was found to show the maximum zone of inhibition against *S.thyphimurium* and *P.aeruginosa.* SP-1 isolate showed the maximum zone of inhibition against *P.aeruginosa* and *S.aureus*, followed by *E.coli*. Similarly, SP-4 isolate showed the maximum zone of inhibition against *S.thyphimurium*, followed by *P.aeruginosa*. However, SP-3 isolates were not inhibiting all bacteria strains.



A: *Salmonella typhimurium* B: *Pseudomonas aeruginosa* C: *Staphylococcus aureus* D: *Escherichia coli*

Table 3: Antibacterial Activity of *isolated compounds*

Compound / sample	*Escherichia coli*	*Salmonella thyphimurium*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
SP-1	17	18	20	20
SP-2	20	21	18	28
Gentamicine*	27	23	25	29
DMSO*#	-	-	-	-

Values are in mm; *(+)ve control; *# (-)ve control, No inhibition zone

According to previous reports in literature the isolated and characterized compound Lutein is an antioxidant which is believed to be an essential nutrient for normal vision[22]. Studies have also indicated that Lutein and methyl derivative improves heart health, protects our skin against UV damage, reduces diabetes induced oxidative stress, and possesses anti-inflammatory and anti-cancer properties [23]. Lutein and its derivatives offers eye protection by lowering the risk of both age related vision loss, gradual loss of central vision [24]. In the present investigation, mono methyl derivative of Lutein isolated from green algae *S.rhizopus* was investigated for its antibacterial activity against pathogenic bacteria.

Conclusion

Based on the results, it can be concluded that green algae *S.rhizopus* has economical value, chloroform crude extract possess a significant antimicrobial activity due to the presence of bioactive compounds. The isolated pure compound SP-1 shows greater activity than the extract, the other fractions SP-3, SP-4 needed further purification. The present study concluded that the green algae *spirogyra sp.* collected from Jimma town, Ethiopia are potential sources of bioactive compounds and can be used in drug discovery program. Characterization and antimicrobial studies of compound SP-3 and SP-4 is under progress and result will publish in the due course of time. Further research is needed in which the extract could possibly be exploited for pharmaceutical and nutritional use.

Acknowledgement

We are grateful to Ministry of Higher Education and Research (MHER), Ethiopia for the research funding. We express our sincere thanks to Dr.Jemal Abafita, President of Jimma University for the constant encouragement and we also thank Dr.Gemechis File**,** Dean, College of Natural Sciences (CNS), Jimma University and University of Addis Ababa for providing the chemicals, solvents and for spectral data.

Conflict of interest

No conflict of interest and the authors authenticate that there are no known conflicts of interest affiliated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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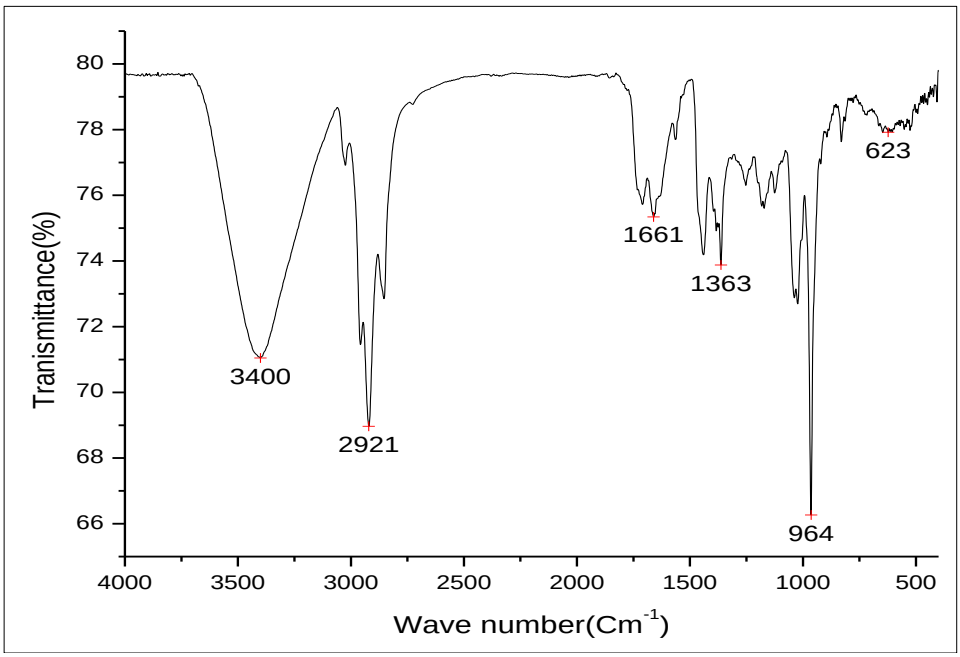
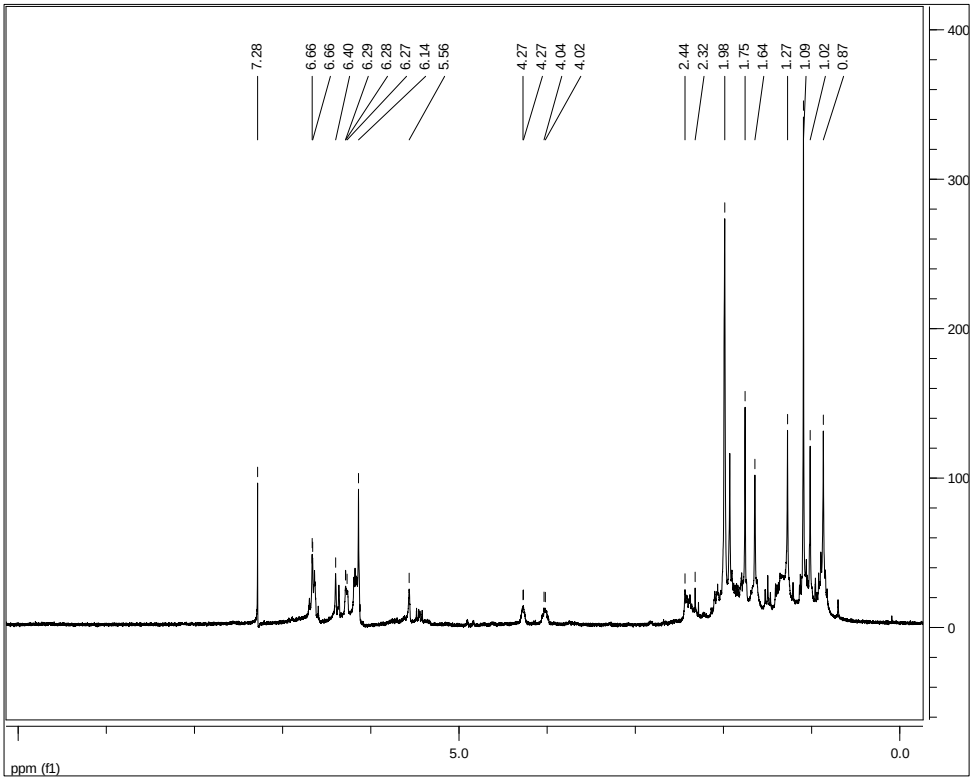
Appendixes

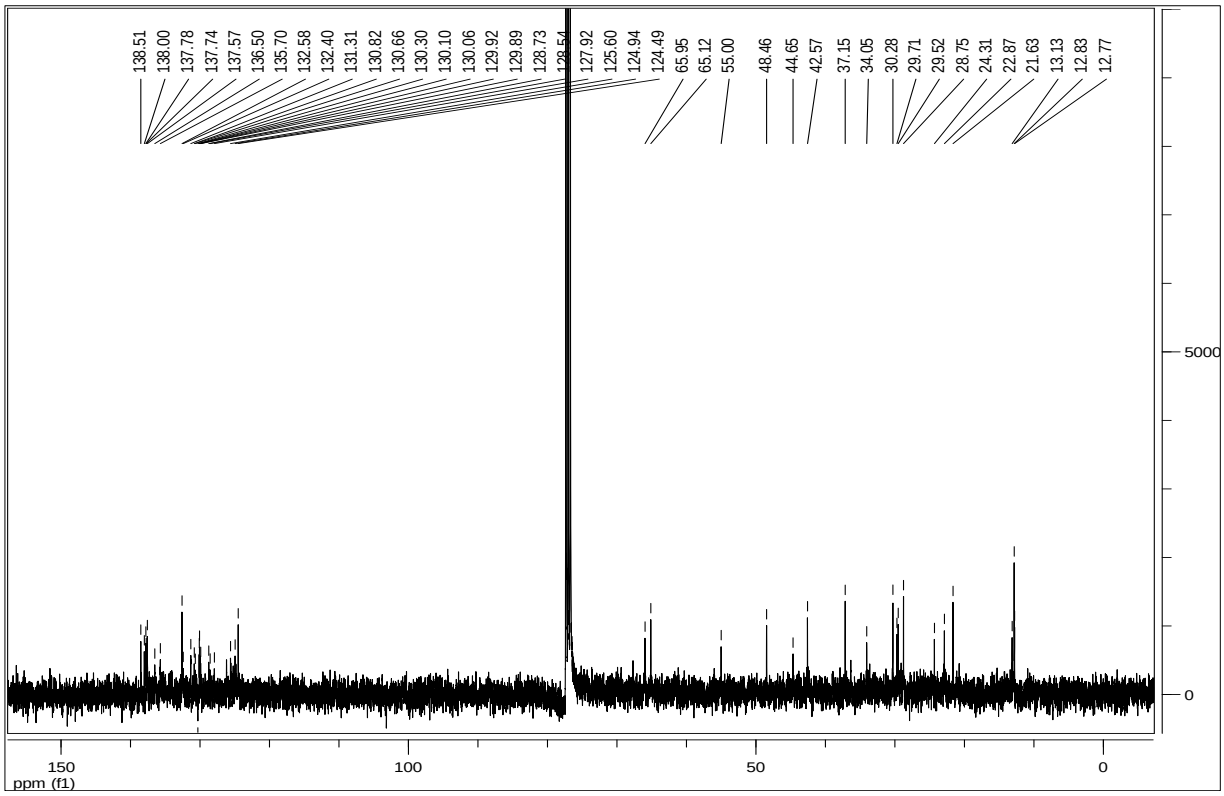
Appendix 1 IR spectrum of compound SP-1



Appendix 2 1H-NMR spectrum of compound SP-1



Appendix 3 13C-NMR spectrum of compound SP-1



Appendix 4 DEPT-135 spectrum of compound SP-1