Phytochemical investigation and Bioevaluation of Medicinally Important Plant: *Rheum emodi*

Iram Ayoob[1], Masood ur Rahman[2,3], Shahid Banday[4], Mohd Akbar Khuroo[1], Khursheed Ahmad Bhat[3].

Department of Chemistry, University of Kashmir, Srinagar,190006, India.

Department of Chemistry, National Institute of Technology, Srinagar, 190006, India.

BOC Division, IIIM, Santana, Srinagar, 190005, India.

Department of Biotechnology, University of Kashmir, Srinagar, 190006, India.

Abstract

Rheum emodi (Polygonate) commonly known as ''Pambchallan''(in Kashmir) is a medicinally important plant showing a wide range of biological activities. Phytochemical investigation of the roots of this plant yielded six compounds i.e. Chrysophanol, emodin, noreugenin, Physcion, aloe emodin glycoside and Chrysin. All the isolated compounds were envisaged for bio evaluation (antioxidant and antimicrobial activity). Among all tested isolates, compounds **1**, **3** and **4** were found to be better antioxidants and with respect to antimicrobial screening, varied activity was demonstrated by all the six compounds against bacterial and fungal pathogens. Compounds **1**, **4** and **5** were found to be active against both fungal strains. Compounds **2** and **3** were active against single bacterial strain E.*coli* while as compound **6** was found to be potent against bacterial pathogen *E.coli* and fungal pathogen *A. fumigatus*.

Keywords*:* Rheum Emodi, Bio Evaluation, Anti-Oxidant, Anti-Microbial, E. Coli, A. Fumigatus

1. Introduction

Rheum emodi (Polygonaceae) locally known as''Pambchallan''(in Kashmir)is a leafy perennial herb distributed in altitudes ranging from 2800 to 3800 m in the temperate and subtropical regions of Himalayas from Kashmir to Sikkim in India [1] . The plant has large woody roots, large leaves , branched leafy stems and usually the height of the plant is about 1.5-3.0 metres [2-5]. Roots and rhizomes constitute the main parts of the plant which are used as drugs. The rhizomes of the plant are collected in the month of October – November and the ones found in India are darker, inferior in aroma, coarser and untrimmed [6-7]. For the people living especially in rural and high altitudes of Kashmir, *R. emodi* in various forms constitutes an important source of food. The rhizomes of the plant are firstly cut into short pieces, threaded on a string and then dried either in the sun or by artificial heat. The dried pieces are then stored by the people and cooked during winters. In addition to it, the leaves of this plant locally called by the name of "Pambhaak" are also being cooked as a vegetable .The leaf stalks are either eaten raw or boiled, then they are sprinkled with salt and pepper by the locals of Kashmir[8]. It has also been found that the flowers of the plant are also edible. In addition to it, *R. emodi* is also used for making pies that serve a number of functions like antipyretic, antihelminthic and are used in cases of constipation, jaundice and liver disorders [9]. *R. emodi* has been used extensively in Ayurvedic and other traditional medicinal systems like homeopathic, Unani and Chinese systems [10].The extracts prepared from the roots, bark and leaves of this plant have been used as a laxative from ancient times and presently these are used in various herbal preparations[11].*R.emodi* has been used traditionally for the treatment of number of ailments like fevers, ulcers, jaundice, bacterial and fungal infections. It is also used to treat kidney stones and other liver associated disorders like gout and jaundice [12-13]. The rhizomes of the plant are used in the treatment of ailments like diabetes, atherosclerosis, ischemia. They are also used for the treatment of inflammation in Japanese and Chinese traditional medicine (Matsuda *et al*., 2001). The plant is also used to heal skin sores and scabs externally [14].In China, *R. emodi* is also used as an ulcer remedy . It is also considered as a bitter, dry herb which is used to clear heat from the liver, stomach, and blood, to expel helminthes and to treat cancer, upper intestinal bleeding (ulcers), headache and toothache in China. [15-17].

Free anthraquinones and their glycosides are the major constituents that have been isolated from this plant. Anthraquinones, both with and without carboxyl groups have been reported from this plant. Anthraquinones with carboxyl group include rhein, and without carboxyl group include chrysophanol, emodin, aloe emodin, physcion (emodin-monomethyl ether), chrysophanein and emodin glycoside [18]. In addition to it, alkyl derivatives of anthraquinones, like 6-methyl rhein and 6-methyl aloe-emodin have also been reported from this plant [19].These key constituents (i.e. anthraquinones) have been found to exhibit a number of biological activities like antioxidant, cytotoxic,antimicrobial, antifungal, antitumor, antidiabetic, antiproliferative and immunoenhancing activity[20]. Anthrone C-glucosides have also been isolated from this plant. These anthrones include 10- hydroxycascaroside C, 10-hydroxycascaroside D, 10 R-chrysaloin 1-O-β-D-glucopyranoside, cascaroside C, cascaroside D and cassialoin [21].

Apart from these constituents, different derivatives of oxanthrone have also been isolated from this plant, which include oxanthrone ether (revandchinone-4), oxanthrone esters (revandchinone-1 and revandchinone-2), and revandchinone-3 [13]. Other compounds like naphthoquinones, rutin, rheinal, rhein 11-O-β-D-glucoside, torachrysone 8- O-β-D-glucoside, epicatechin, auronols (carpusin and maesopsin), the sulfated anthraquinone glycoside sulfemodin 8-O-β-D-glucoside, β-asarone have also been reported from this plant. In addition to it, some stilbene compounds like rhaponticin etc. have also been isolated. Tannins have also been reported from R. *emodi* including hydrolysable tannins which contain ester or glycosidic bonds mainly composed of gallic acid, glucose and other monosaccharides and condensed tannins which are derived from the flavone derivatives like catechin and Leucocyanidin [22]. The petroleum ether and chloroform extracts of the rhizomes of R. *emodi* have been found to exhibit moderate antifungal and antibacterial activities. Also, the benzene extract of *R. emodi* inhibits the growth of *H. pylori* [23]. The methanolic extract of the plant shows highest DPPH radical scavenging activity than ethyl acetate and chloroform extracts. The lower IC_{50} value for methanolic extract in DPPH radical scavenging assay is attributed to the fact that the extract is rich in bioactive constituents like anthraquinones and their glucosides while as the ethyl acetate and chloroform extracts have low concentration of these anthraquinones [24]. Emodin, which is isolated from *R. emodi* has been found to inhibit MAO B. On this basis, it has been concluded that emodin can be used as a lead for the prevention and treatment of Parkinson's disease [25]. Also, various reports on emodin suggest that it is a novel anti-SARS-Cove compound and hence it can be used as a potential lead therapeutic agent in the treatment of SARS [26]. The ethyl acetate extracts prepared from the rhizomes of this plant have been found to exhibit immuno-enhancing activity on macrophage cell lines. In addition to it, the extract from the rhizomes of *R. emodi* has shown significant hepatoprotective activity against CCl_4-induced liver injury both in vitro and in vivo using 50 mg/kg, p. o. (per oral) dose of silymarin as a standard [27]. Also, it has been found that *R. emodi* rhizome extract shows significant antidiabetic activity by enhancing the peripheral utilization of glucose, by correcting impaired liver and kidney glycolysis similar to that of insulin [28]. The nephroprotective activity of both water-soluble and water-insoluble fractions of alcoholic extract of *R. emodi* has also been established. It has been suggested that this activity may be due to the tannins present in the plant [29]. In addition to this, the methanolic and aqueous extracts of the roots of *R.emodi* also show antioxidant and anticancer properties [30].In another study, it has been established that the anthraquinone derivatives, such as aloe-emodin, emodin, Rhein, corytophanid and physcion present in the plant exhibit anti-angiogenic activity, by preventing blood vessel formation in zebra-fish embryos [31]. Also the anticancer effect of aloe-emodin has been in two human cancer cell lines viz., Hep G2 and Hep 3B has been established. It has been found that aloe-emodin inhibited cell proliferation and induced apoptosis in both these cell lines by different anti proliferative mechanisms [32]. Also, Revandchinone-1, 3, and 4 isolated from *R.emodi* show significant antifungal activity against *Aspergillus niger* and *Rhizopus oryzae* . In addition to it, these also show antibacterial activity against gram positive (*Bacillus subtilis*, *Bacillus sphaericus,* and *Staphylococcus aureus*) and gram negative (*Klebsiella aerogenes*, *Chromobacterium violaceum* and *Pseudomonas aeruginosa*) bacteria [21].

2. Results and discussion

2.1. Phytochemistry

The roots of R. *emodi* were collected from the hilly areas of Dhaara , Harwan and Drang (Kahmir, India) .The air dried powdered material was then extracted with DCM:MeOH(1:1) and MeOH . The concentrated extracts were then subjected to column chromatography over silica gel. Repeated column chromatography of DCM: MeOH extract using varied solvent polarity (hexane: ethyl acetate) and recrystallization techniques afforded four compounds **(1-4).** Similarly, the repeated column chromatography of MeOH extract yielded two compounds **(5-6)**using chloroform-methanol as eluent with increasing polarity of 20 % and 30% MeOH respectively (Figure 1.1).

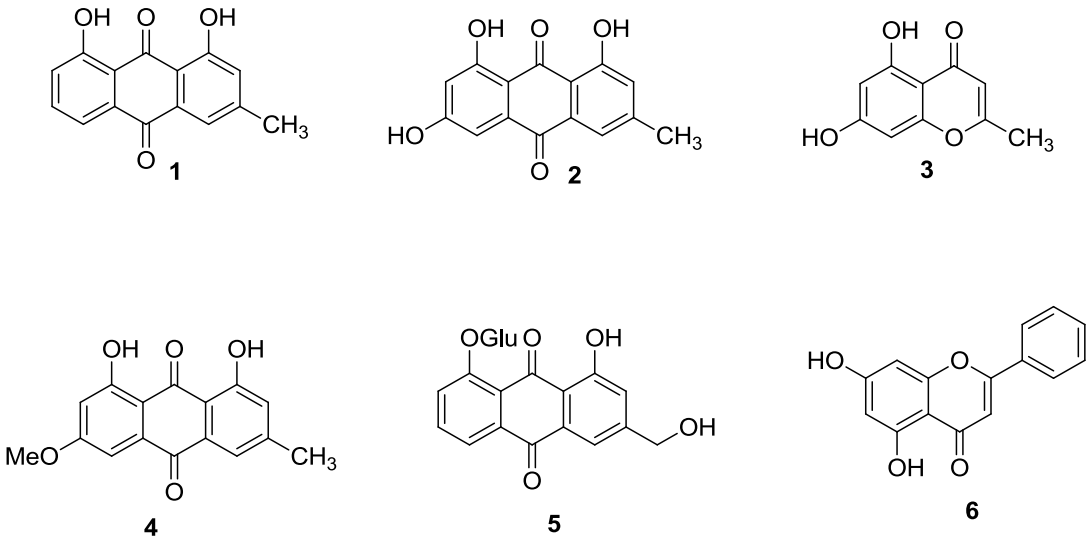

Figure 1.1. Molecular structures of the isolated compounds.

2.1.1. Compound-1

Compound **1** was obtained as orange crystalline solid with melting point of 145-147°C. Mass spectrum showed the molecular ion peak at m/z 254. From the elemental analysis, MS and other spectral data (^{1}HNMR, ^{13}CNMR) compound **1** was assigned the molecular formula $C_{15}H_{10}O_4$. The IR spectrum showed a hydroxyl band at 3469 cm^{-1} and bands at 1719 and 1633 cm^{-1}due to nonchelated and chelated –CO respectively.

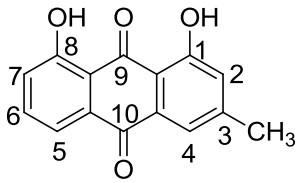

Figure 1.2. Structure of Compound-1

The ^{1}HNMR spectrum of**1**showed two sharp singlets at δ 11.91and 11.79 , corresponding to the presence of two hydroxyl groups. A singlet at δ 2.44 integrating for three protons corresponds to aromatic methyl group. Also peaks integrating for five protons were observed in the aromatic region which indicates the presence of five aromatic protons within the molecule. A double-doublet observed at δ 7.80(dd, J_1=8.4, J_2 =7.6 Hz, 1H) was assigned to H-6. Two doublets observed at δ 7.71 (d, J=8 Hz, 1H) and δ 7.38 (d, J=8 Hz, 1H) were assigned to H-5 and H-7, since J values indicate that these two protons are ortho coupled to H-6. Two singlets observed at δ :7.54 and 7.21, each integrating for one proton were assigned to H-4 and H-2 respectively. ^{13}CNMR-DEPT spectrum showed the presence of fifteen carbons including one methyl, five methines and nine quarternary

carbons. Finally, the structure of the compound **1** was characterized as Chrysophanol (3-methyl-1,8-dihydroxy anthraquinone) by comparison of its ^{1}H and $^{13}CNMR$ data with that reported in literature [33].

2.1.2 Compound-2

Compound **2** was obtained as yellowish powder with melting point of 256-257 °C. It showed the molecular ion peak at m/z 270in the mass spectrum , which corresponds to the molecular formula $C_{15}H_{10}O_5$. IR spectrum showed band at 3469 cm^{-1}due to hydroxyl group , bands at 1719 and 1633cm^{-1}due to non chelated and chelated –CO respectively.

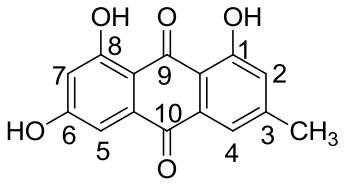

Figure 1.3. Structure of Compound-2

The $^{1}HNMR$ spectrum of **2** exhibited three singlets at *δ*:12.07,11.40 and 11.31 corresponding to the presence of three hydroxyl groups. Also a singlet observed at *δ* 2.50 integrating for three protons indicates the presence of aromatic methyl group. Further four singlets, each integrating for one proton were observed in the aromatic region which correspond to the presence of four aromatic protons in the molecule.$^{13}CNMR$-DEPT spectrum showed the signal for fifteen carbons including one methyl, four methines and ten quartenary carbons. Thus, the compound**2** was characterized as Emodin (3-methyl-1,6,8- trihydroxy anthraquinone) by comparison of its spectral data with the data from the literature[33].

2.1.3. Compound-3

This compound was obtained as light brown powder with melting point of 274-275 °C. It showed the molecular ion peak at m/z 192 in the mass spectrum corresponding to the molecular formula $C_{10}H_80_4$. IR spectrum showed bands at 3150 and 1647 cm^{-1} due to hydroxyl and carbonyl group respectively.

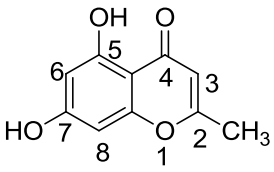

Figure 1.4. Structure of Compound-3

In the $^{1}HNMR$ spectrum of **3**, two singlets at *δ* 12.97 and 12.85 were assigned to two hydroxyl groups.The signal at δ 2.50 integrating for three protons corresponds to the presence of methyl group in the molecule. The signal at *δ* 6.27 was attributed to H-3, since this proton is dishielded by adjacent carbonyl group. Two signals at *δ*: 6.48 (s, 1H), 6.32 (s, 1H) were assigned to H-8 and H-6 respectively. The ^{13}C NMR-DEPT spectra showed ten carbon signals comprising of one methyl, three methines and and six quarternary carbons. Thus, the compound **3** was characterized as Noreugenin by comparison of its ^{1}H and $^{13}CNMR$ data with that reported in literature[34].

2.1.4. Compound-4

This compound was obtained as yellowish powder with melting point of 204-205°C.It showed molecular ion peak at m/z 284 in the mass spectrum which corresponds to the molecular formula $C_{16}H_{12}O_5$. IR spectrum showed bands at 1681 and 1628 cm^{-1} due to non chelated and chelated CO respectively.

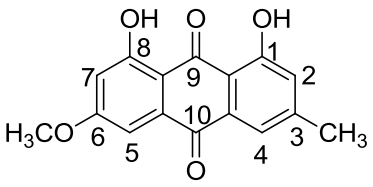

Figure 1.5. Structure of Compound-4

In the ^{1}HNMR spectrum of **4**, two singlets at δ 12.31 and 12.11 were assigned to two hydroxyl groups. Two singlets each integrating for three protons were observed at δ: 3.94 and 2.46 which corresponds to the presence of a methyl and methoxy group in the molecule . Also four protons were observed in the aromatic region from δ 7.63-6.69. Two singlets observed at δ:7.63(s,1H) and 7.08 (s,1H) were assigned to H-4 and H-2 respectively. Two more doublets observed at δ: 7.37(d, J=2.4 Hz, 1H) and 6.69(d, J=2.4Hz, 1H) were assigned to H-5 and H-7, since J values indicate these are meta coupled. The ^{13}C NMR-DEPT spectra showed sixteen carbon signals including one methyl, one methoxy, four methines and ten quarternary carbons. Finally, the compound **4** was characterized as Physcion by comparison of its ^{1}H and ^{13}CNMR data with that reported in literature [33].

2.1.5. Compound-5

This compound was obtained as a brownish powder with melting point of 224-226°C. It showed the molecular ion peak at m/z 432 in the mass spectrum corresponding to the molecular formula$C_{21}H_{20}O_{10}$. IR spectrum showed bands at 3426 cm^{-1} due to hydroxyl group , bands at 1672 and 1634 cm^{-1} due to non chelated and chelated CO respectively.

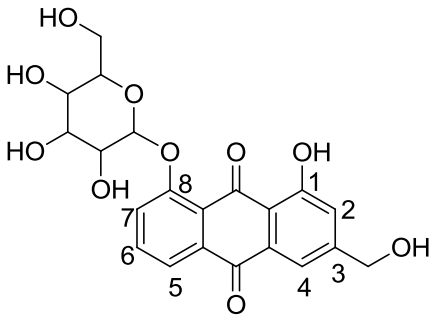

Figure 1.6. Structure of Compound-5

The ^{1}HNMR of **5**showed signals for five protons in the aromatic region. A doublet observed at δ 7.70(1H, J=8.0 Hz) was assigned to H-6. A multiplet integrating for two protons observed at δ 7.87 was assigned to H-7 and H-5. Two singlets observed at δ 7.64 and 7.26 were assigned to H-4 and H-2 respectively. Singlet integrating for two protons at δ 4.60 was assigned to hydroxyl methylene protons. A signal observed at δ 12.83 was assigned to hydroxyl group. These signals are characteristic for aloe emodin aglycone with additional signals of sugar moiety at δ 5.60 (d, J=8.0 Hz, 1H) for an anomeric proton in the β- configuration and multiplets from δ 5.10- 4.61 indicate β-D- glucose moiety. The ^{13}C NMR-DEPT spectra showed twenty one carbon signals including two methylenes, ten methines and nine quarternary carbons. Finally, the compound **5** was characterized as aloe-emodin-8-O-β-D-glucoside by comparison of its ^{1}H and ^{13}CNMR data with that reported in literature[35].

2.1.6. Compound-6

This compound was obtained as a pale yellowish powder with melting point of 206°C. It showed molecular ion peak at m/z 254 in the mass spectrum which corresponds to the molecular formula$C_{15}H_{10}O_{4}$. The ^{1}HNMR spectrum of **6** exhibited a flavonoid pattern and showed two meta coupled doublets at δ 6.45 and 6.15 (1H,

J=1.5 Hz each) which were assigned to H-8 and H-6 respectively. A singlet at δ 6.89 was assigned to H-3. Signals at δ: 8.00-7.97 (m, 2H) and 7.55-7.48 (m,3H) suggest that there is no substitution in the ring B of the flavonoid. Signals at δ: 12.77 and 10.91 were assigned to two hydroxyl groups.

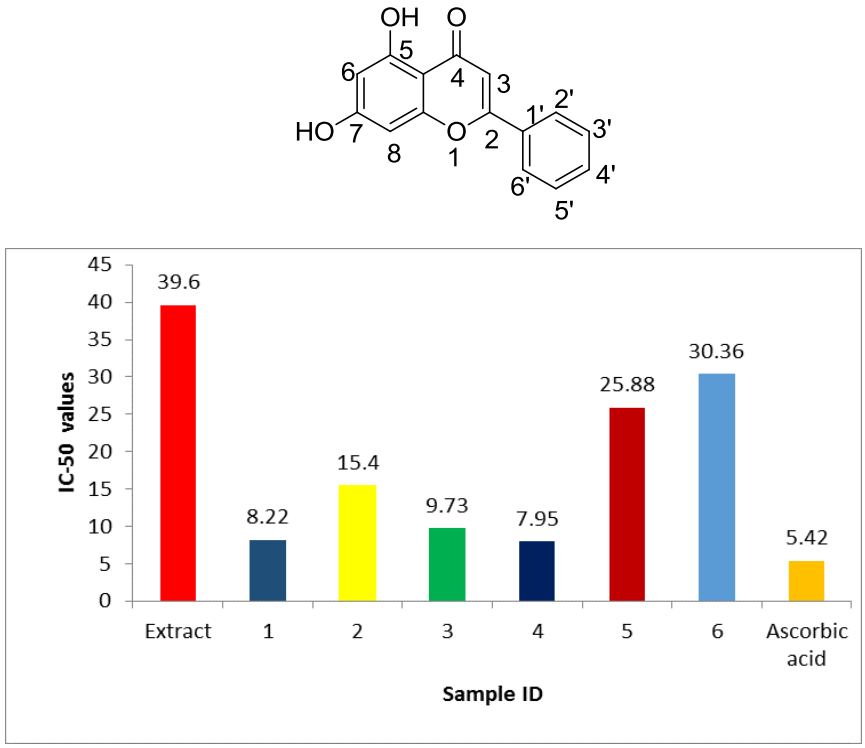

Figure 1.7. Structure of Compound-6

The ^{13}C NMR-DEPT spectra showed fifteen carbon signals including eight methines and seven quarternary carbons. Analysis of IR spectrum further supported the presence of carbonyl group (1655 cm^{-1}) and hydroxyl group (3403 cm^{-1}).Finally, the compound **6** was characterized as Chrysin by comparison of its ^{1}H and ^{13}CNMR data with that reported in literature [36].

2.2. Biological evaluation

2.2.1. Bioactivity of the isolated constituents using DPPH Radical Scavenging Assay

All the isolated constituents were studied for the possible antioxidant activity using DPPH scavenging assay .To evaluate their DPPH scavenging effect, the isolates were screened at different concentrations (µg/ml) in terms of the IC_{50} values depicted in Table 3.1. Ascorbic acid served as a positive control in this assay. From the results presented in Table 3.1 , it was found that that only **four** out of **six** tested compounds displayed broad spectrum antioxidant activity .Compound **4** showed maximum antioxidant potential with an IC_{50} value of 7.95 followed by compound **1** (Chrysophanol) with an IC_{50} value of 8.217. Compound **3** also displayed DPPH radical scavenging efficiency with IC_{50} value of 9.73. However it is clear from the activity profile that these compounds doesnot overpower the activity of positive control which in our study is Ascorbic acid. Moreover based on the activity profile of these compounds, effective SAR could be the main tool for improving the potency and efficacy of these compounds in near future.

Sample ID	Concentration (µg/ml)				
	5	10	15	25	IC_{50}
	% Inhibition				
1	34.43	54.89	67.87	76.99	8.22
2	20.75	33.45	53.79	61.99	15.4
3	32.61	52.66	55.5	59.98	9.73
4	43.88	52.87	63	68	7.95
5	20.45	28.54	42.34	48.67	25.88
6	12.38	18.50	33.90	38.90	30.36
Extract	12.4	26.74	39.98	43.78	39.6
Ascorbic acid	49.34	58.21	69.12	77.45	5.42

Table 3.1. IC_{50} of the compounds isolated from *R. emodi*

2.2.2. Antimicrobial activity

All the isolated compounds (1 to 6) were screened for antimicrobial activity against bacterial (*S.aureus* and *E.coli*) and fungal pathogens (*C.albicans* and *A.Fumigatus*) via agar tube dilution method. Preliminary antimicrobial screening of the compounds was carried out at 3mg/ml concentration and activity was determined. All the analogues were further assayed at different concentrations (0.1 – 3mg/ml) to determine the MIC values (Table-2). Ciprofloxacin and Amphotericin B, used as potent broad-spectrum antimicrobial agents were used as positive control in this method. All the compounds showed a wide range of activity towards bacterial and fungal pathogens. However some of the compounds were much active against fungal pathogens and a few against bacterial pathogens as well. Among the active ones, compounds 1, 4 and 5 were found to be active against fungal pathogens viz *C. albicans* and *A. fumigatus* with MIC values of 23,31, 29,37 and 30,35 respectively and compound 2 and 3 were most active against single bacterial strain viz *E.coli* with MIC of 21 and 17 respectively. Compound 6 was found to be active against bacterial pathogen *E.coli* and fungal pathogen *A. fumigatus* with MIC values of 33 and 27 respectively. However among all the tested compounds, all the isolated compounds were found to be least active against bacterial pathogen *S.aureus*. Moreover it is evident from the MIC values that these compounds does not overpower the efficacy of positive controls taken in this study, so it is necessary to go for their effective SAR to improve the the antimicrobial activity of these compounds in near future.

		Bacterial pathogens		Fungal pathogens	
S.No.	Compound	*S.aureus*	*E.coli*	*C.albicans*	*A.fumigatus*
		(ATCC 29213)	(ATCC 25922)	(ATCC 90028)	(ATCC 19518)
1.	1	68	40	23	31
2.	2	72	21	48	58

3.	3	52	17	64	128
4.	4	41	72	29	37
5.	5	98	38	30	35
6.	6	58	33	44	27
7.	Amphotericin B			0.5	0.5
8.	Ciprofloxacin	0.125	0.007		.

Table 2. MIC (mg/ml) determination of isolated compounds of Rheum emodi.

3. Conclusion

All the six compounds were isolated from Rheum *emodi* and were characterized via spectral techniques viz ESI-MS, H^1 NMR and C^{13}NMR. These characterized compounds were envisaged for bioevaluation (antioxidant and antimicrobial activity). Among all tested isolates, Compounds 1, 3 and 4 were found to be better antioxidants and with respect to antimicrobial screening, varied activity was demonstrated by all the six compounds against bacterial and fungal pathogens. compound 1, 4 and 5 were found to be active against both fungal strains. Compound 2 and 3 were active against single bacterial strain E.*coli* while as compound 6 was found to be potent against bacterial pathogen *E.coli* and fungal pathogen *A. fumigates*. The current study gives the preliminary idea about the antimicrobial and antioxidant potential of isolated constituents and it will be note worthy to carry out the the effective SAR of the potent and active isolates.

4. Experimental

4.1. General experimental procedures

^{1}H NMR and ^{13}C NMR spectra of the molecules along with their chemical shifts expressed in δ and their coupling constants in Hertz were recorded on Bruker DPX 500 instrument using $CDCl_3$ /MeOD/DMSO as the solvents with TMS as internal standard. Infrared spectra of the compounds were recorded as KBr pellets in cm^{-1} on a Hitachi 270-30 spectrophotometer. Melting points were being determined on a Buchi melting point apparatus. Column was run using silica gel (60-120 mesh). TLC plates were visualized under UV light and after exposure to iodine vapour in iodine chamber .The spraying reagent used was Cerric sulphate**.**

4.2. Plant Material collection

The roots of *R.emodi* were collected from from the hilly areas of Dhaara , Harwan and Drang (Kashmir, India) . The plant was then properly identified by a plant taxonomist Mr. Akhtar and the specimen voucher number **1902 KASH** was deposited in Kashmir University Herbaria.

4.3. Extraction and Isolation

Air-dried and coarsely powdered plant material (root part, 3Kg) was divided into two parts. One part of the dried roots(about 1 kg) was extracted with DCM:MeOH (1:1)for 48 hours and the other part (about 2kg) was extracted with methanol for about 48 hours. The extracts thus obtained were concentrated under reduced pressure to give crude extract of 100.0 g for DCM:MeOH (1:1) as extracting solvent and 400.0 g for MeOH as an extracting solvent. The DCM:MeOH extract was then dissolved in minimum amount of methanol and adsorbed on silica gel to form slurry. The dried slurry was subjected to column chromatography over silica gel to afford compounds **1**(800.0 mg), **2**(1. 0 g), **3** (500.0 mg) and **4** (500.0 mg) using hexane-ethyl acetate as eluent with increasing polarity of 2 %, 10 %, 16 % and 30% Et OAC respectively. After this, MeOH extract was firstly dissolved in minimum amount of methanol and was then adsorbed on silica gel to form slurry. The

dried slurry on subjecting to column chromatography over silica gel afforded compounds **5**(300.0 mg), **6**(500.0 mg) using chloroform-methanol as eluent with increasing polarity of 20 % and 30% MeOH respectively.

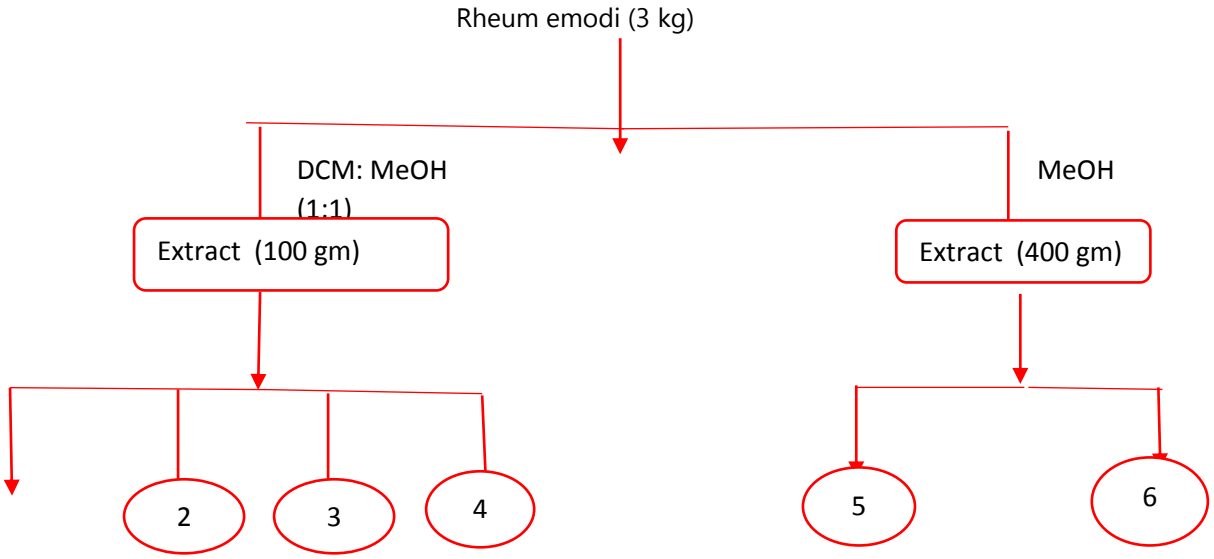

Fig.3. Flow chart depicting preparation of extracts and isolation of compounds from the roots of Rheum *emodi*.

4.4. Spectral data of the isolated constituents

Chrysophanol (1): Orange crystalline solid; IR (KBr) ν_{max} cm^{-1}:3469 ,1719 ,1633;^{1}H NMR (400 MHz, DMSO) δ:11.91(s,1H,OH), 11.79(s,1H, OH), 7.80(dd, J_1=8.4, J_2 =7.6 Hz, 1H) , 7.71 (d, J=8 Hz, 1H) , 7.54 (s, br,1H), 7.38(d, J=8 Hz, 1H), 7.21(s, br,1H), 2.44 (s,3H,CH_3) .^{13}C NMR (101 MHz, DMSO) δ: 191.62, 181.49,161.57, 161.31,149.18,137.33, 133.31, 133.01, 124.40,124.08,120.55, 119.32, 115.85, 113.76, 21.62.

Emodin (2) : Yellowish powder;IR (KBr) ν_{max} cm^{-1} :3469 ,1719 ,1633;^{1}H NMR (400 MHz, DMSO) δ:12.07(s,1H,OH), 11.40(s, 1H, OH), 11.31(s, 1H, OH) ,6.76(s,1H) ,6.39 (s, 1H,) ,6.34 (s, 1H), 5.82 (s,1H), 2.50(s, 3H, CH_3) . ^{13}C NMR (101 MHz, DMSO) δ:191.14, 182.40, 166.53, 165.73, 162.72, 149.32, 136.26, 133.99, 124.82, 121.37, 114.29, 110.0, 109.62, 108.67, 22.12 .

Noreugenin (3): Light brown powder; IR (KBr) ν_{max} cm^{-1}: 3150 , 1647 cm;^{1}H NMR (400 MHz, DMSO) δ:12.97 (s, 1H, OH), 12.85(s, 1H, OH),6.48 (s, 1H), 6.32 (s, 1H) , 6.27 (s,1H) , 2.50 (s,3H, CH_3) . ^{13}C NMR (101 MHz, DMSO) δ:181.76, 167.67, 164.08, 161.51, 157.80, 107.94, 103.43, 98.74, 93.71, 19.91.

Physcion (4) : Yellowish powder; IR (KBr) ν_{max} cm^{-1}:1681 ,1628;^{1}H NMR (400 MHz, $CDCl_3$) δ:12.31(s, 1H, OH) 1 (s, 1H, OH), 7.63(s, br, 1H), 7.37(d, J=2.4 Hz, 1H), 7.08(s, br, 1H), 6.69(d, J=2.4Hz, 1H), 3.94(s, 3H, OCH_3),2.46(s, 3H, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 190.7, 181.9,166.5, 165.1, 162.4,148.4,135.2,133.2, 124.4, 121.2, 113.7,110.2,108.2, 106.8,56.1, 22.2.

Aloe emodin glycoside (5) : Brownish powder;IR (KBr) ν_{max} cm^{-1}:3426 , 1672 ,1634 ;^{1}H NMR (400 MHz, DMSO) δ: 12.83(s, 1H, OH), 7.87(m, 2H), 7.70(d, J=8.0 Hz, 1H), 7.64(s, 1H), 7.26(s, 1H),5.60(d,J=8.0 Hz, 1H),5.10(m, 3H),4.60-4.65 (m, 3H).^{13}C NMR (101 MHz, DMSO) δ: 188.11, 182.38, 161.67, 158.34, 152.63, 136.29, 135.24,132.24, 122.59,121.54, 121.24, 120.56,116.20, 115.89,100.84, 77.79,76.74,73.43, 70.11,62.06, 60.70.

Chrysin(6) : Pale yellowish powder;IR (KBr) ν_{max} cm^{-1}:1655,3403;^{1}H NMR (400 MHz, DMSO) *δ*: 12.77(s,1H,OH), 10.91(s, 1H, OH), 8.00-7.97(m, 2H), 7.55-7.48(m,3H), 6.89(s,1H), 6.45(d, J=1.5 Hz, 1H), 6.15(d, J=1.5 Hz, 1H). ^{13}C NMR (101 MHz, DMSO) *δ*: 181.94, 164.50, 163.22, 161.53, 167.52, 131.09, 130.76, 129.20(2C), 126.46 (2C), 105.21, 104.03, 99.09, 94.20.

4.5. Evaluation of antioxidant potential using DPPH assay

DPPH free radical scavenging activity was evaluated by measuring the scavenging activity of the samples on stable 2,2-diphenyl-1-picryl hydrazyl radical (DPPH) [37].A 0.5 mM solution of DPPH in methanol was prepared. Different concentrations of each sample (5–50 µM) were added to 1.0 ml (0.5 mM DPPH) and final volume was made to 3.0 ml with methanol. The mixture was shaken vigorously and kept standing at room temperature for 15 min. Then the absorbance of the mixture was measured at 517 nm on UV spectrophotometer. The decrease in the absorbance indicates an increase in DPPH-radical scavenging activity. The percentage inhibition was calculated by the following equation:

$$\text{DPPH radical scavenging (\%)} = A_C\text{-}A_S/A_C \times 100$$

where A_C is the absorbance of control and A_S is absorbance of sample. Ascorbic acid was used as positive control. The experiment was done in triplicate and mean values were calculated. Standard deviation for the triplicate analysis was also calculated. IC_{50} value was calculated as the concentration of sample required to scavenge 50% of DPPH free radicals.

4.6. Evaluation of anti microbial activity of the isolates

a. Antibacterial activity:- Using a single sterile pipette, 0.6 ml of the broth culture of the test organism was added to 60 ml of molten agar, which had been cooled to 450C, mixed well and poured into a sterile Petri dish (for the 9 cm Petri dish, 0.2 ml of the culture was added to 20 ml of agar). Duplicates plates of each organism were prepared. The agar was allowed to set and harden and the required number of wells were dug in the medium with help of sterile metallic cork borer ensuring proper distribution of the well in the periphery and one in the center.[38] Agar Plugs were removed. Stock solutions of the test samples at a concentration of 1mg/ml were prepared in the sterile DMSO and 100 µl and 200 µl of each dilution was added to the respective wells. The control well received only 100 µl and 200 µl DMSO. Gentamycin was used as standard drug. The plates were left at room temperature to allow diffusion and then incubated at 370C for 24 hr. The Diameter of the zones of inhibition was measured to the nearest mm (the well size also being noted).

b. Anti fungal activity :-The antifungal activity of the natural product analogs were evaluated by the agar tube dilution method [38]. The samples (24 mg/ml) were dissolved in sterile DMSO, which served as a stock solution. Sabouraud dextrose agar (SDA) was prepared by mixing 32.5g sabouraud, 4% glucose agar and 4.0 g of agar-agar in 500 ml distilled water thoroughly with a magnetic stirrer. Then a 4 ml aliquot was dispensed into screw cap tubes, which were autoclaved at 120°C for 15 min and then cooled to 15°C. The non-solidified SDA media was mixed with stock solution. (66.6 µl) giving a final concentration of 400 µg of the extract per ml of the SDA. The tubes were then allowed to solidified in slanted position at room temperature and then inoculated with a piece (4 mm diameter) of an inoculum removed from a seven days old culture of fungi to determine non-mycelial growth; an agar surface streak was employed. Other media supplemented with DMSO and reference antifungal drugs served as a negative and positive control respectively. Inhibition of fungal growth was observed visually after 7 days of incubation at 28 ± 10C. Humidity (40-50%) was controlled by placing an open pan of water in the incubator.

5. References

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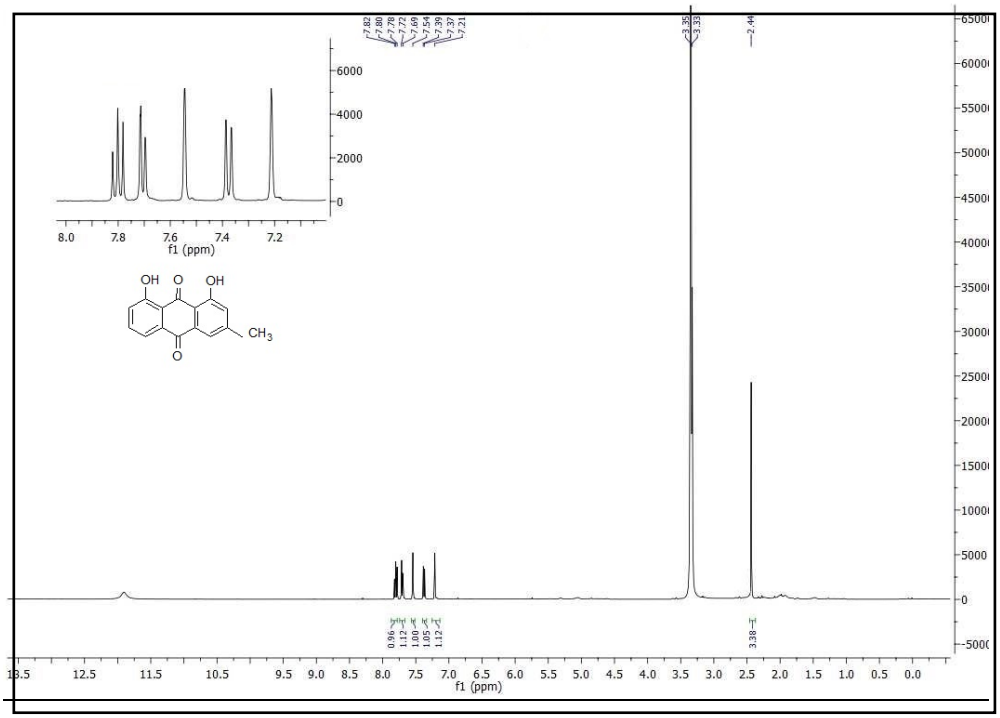
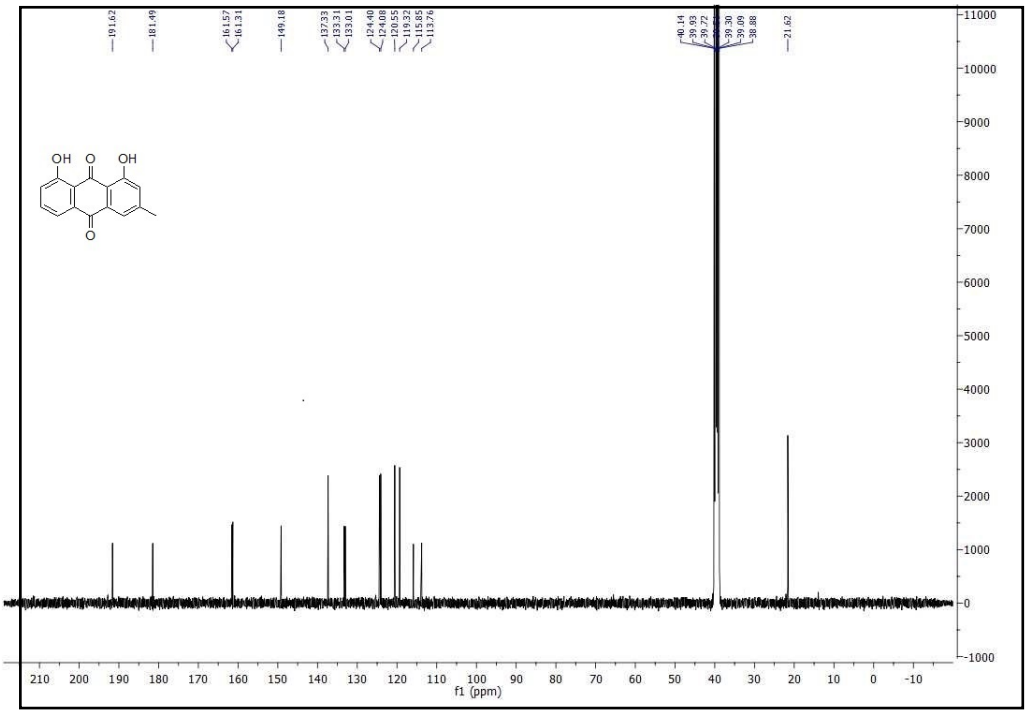
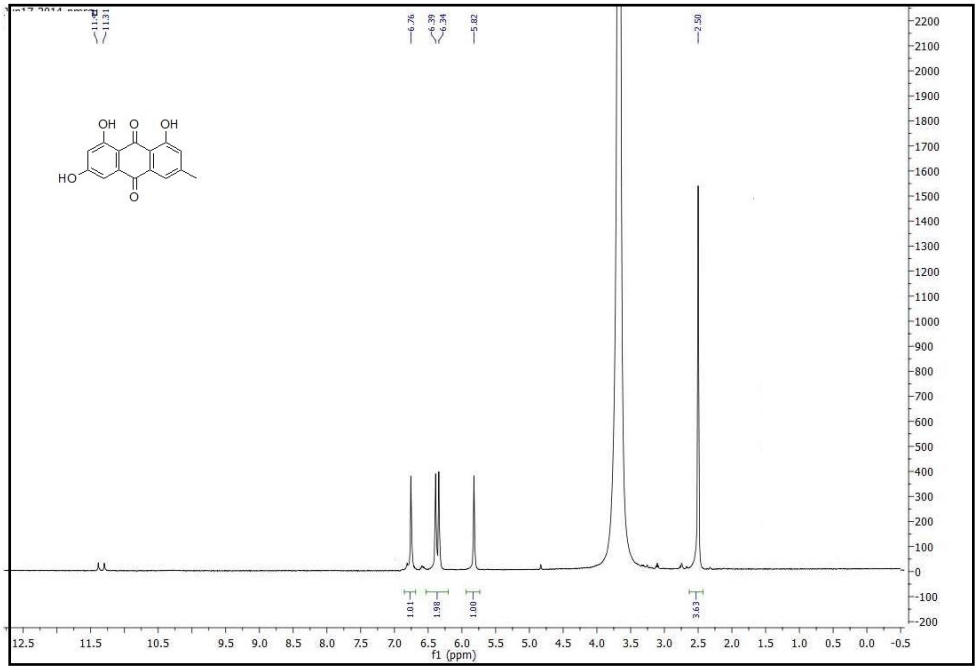
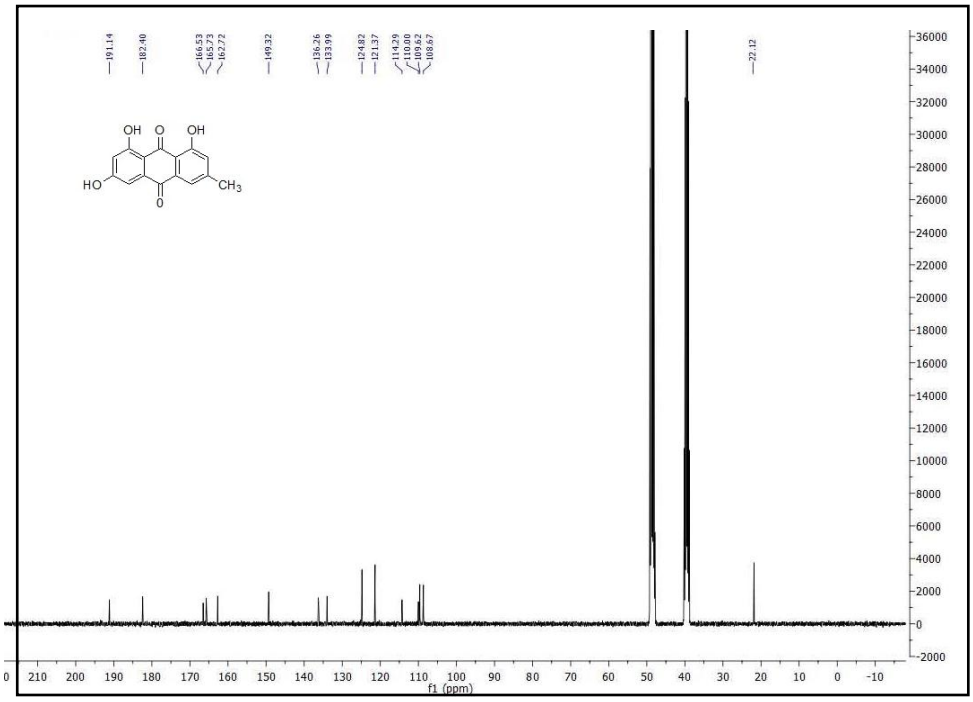
Supplementary Information





^{1}H and ^{13}C NMR of Chrysophanol





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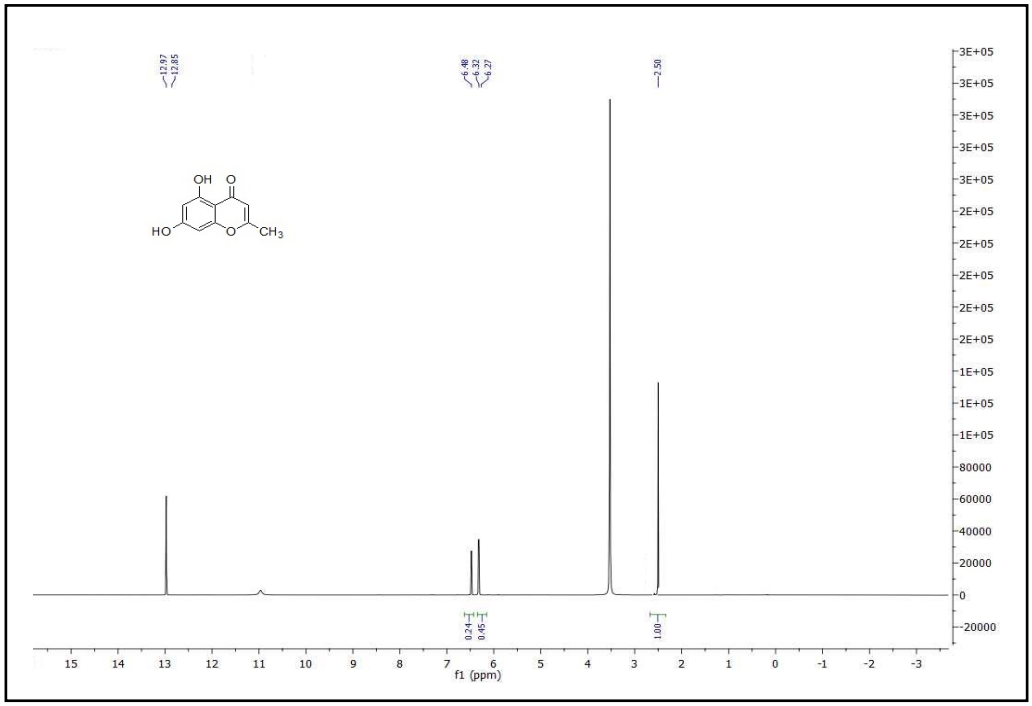

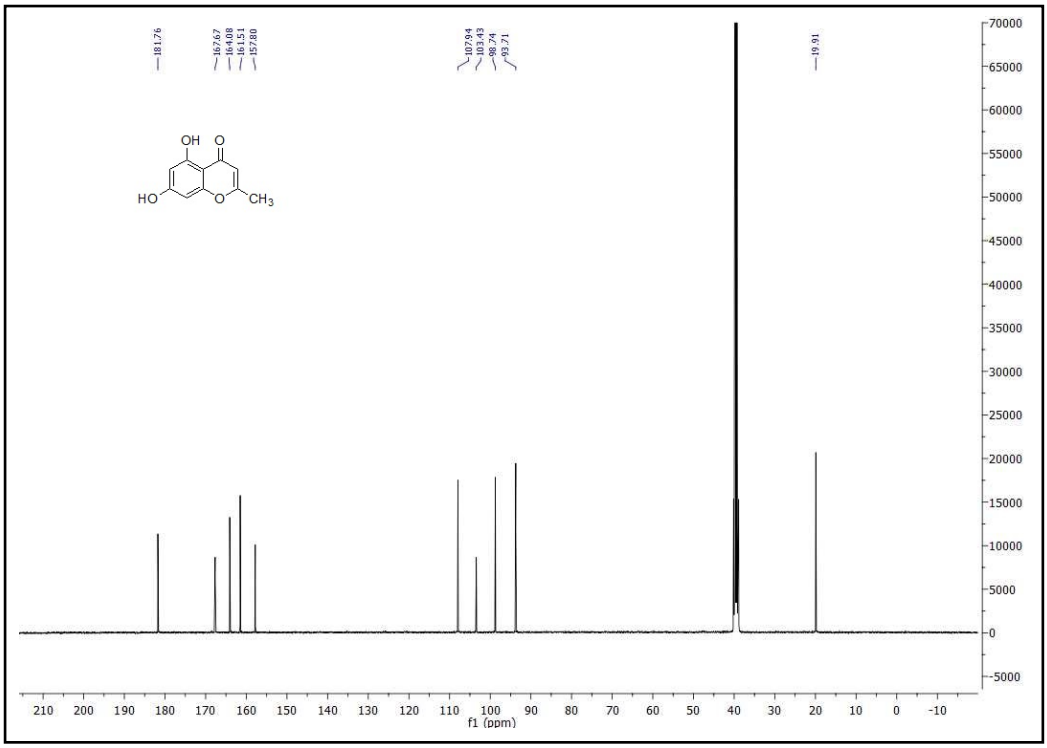

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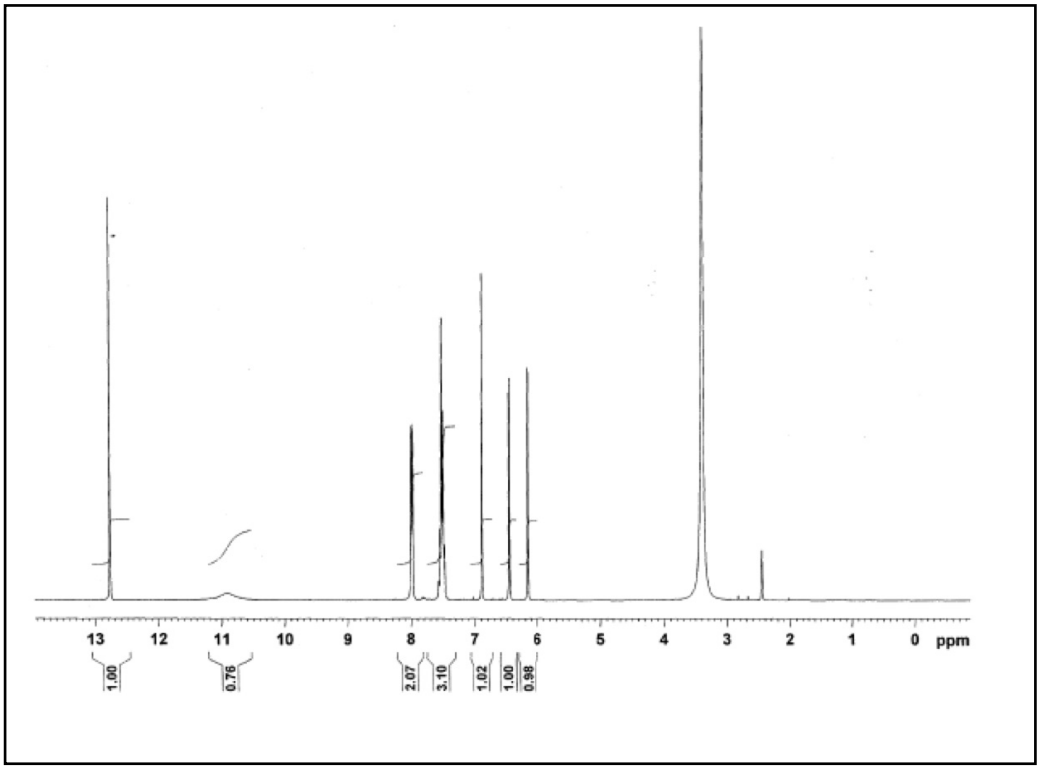

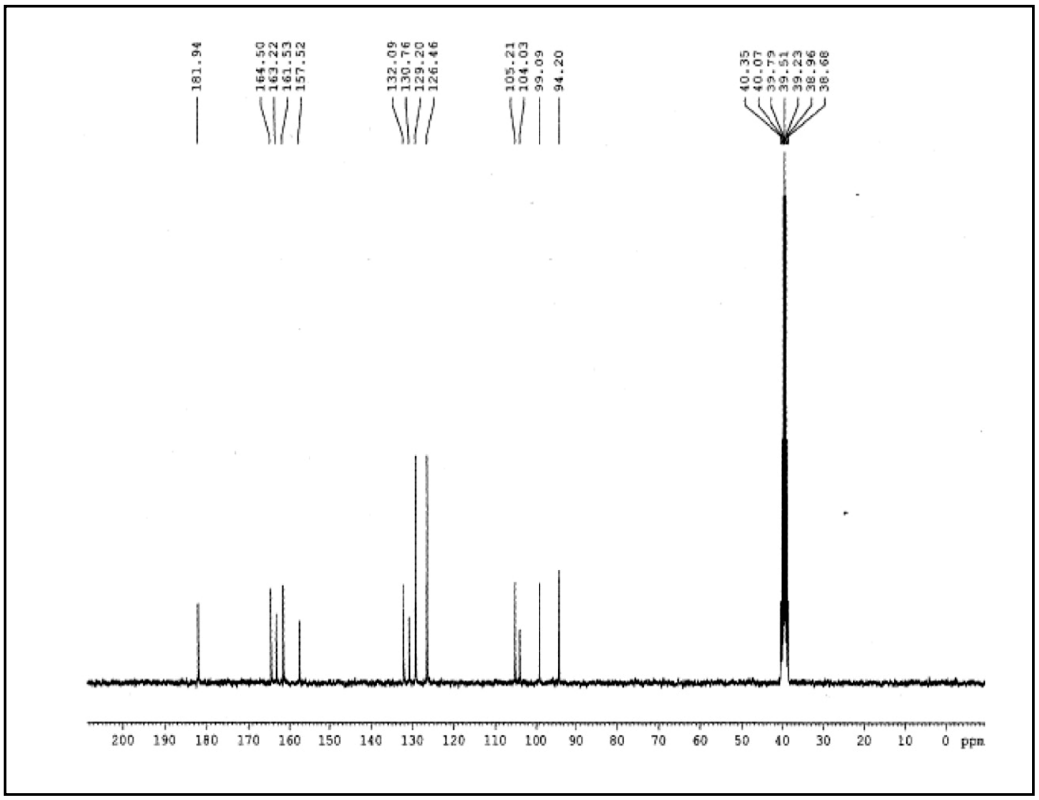

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