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Original Research Paper

ISOLATION, CHARACTERIZATION OF BIOACTIVE MOLECULES FROM *XANTHIUM SPINOSIUM* AND THEIR BIOLOGICAL EVALUATION

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ABSTRACT

Two sesquiterpene lactones; xanthanolides, isolated and their structures were established by spectroscopic methods (IR, ^{1}H – NMR, ^{13}C – NMR and Lc-Ms spectra). Antisuppresent activity and antimicrobial study, Were carried out the isolated isoxanthanol has got significant antisuppresent activity as compared to control and standard were as xanthanol has got moderate activity compared to control and standard. But the isolated compounds does not show any significant antimicrobial activity compared to control and standard.

Keywords: *Xanthium spinosum*, Compositae, Sesquiterpene lactones, Xanthanolides, Isolation, antisuppresent activity and Antimicrobial study.

INTRODUCTION

Xanthium spinosium L. (Compositae), is a gregarious weed found abundantly throughout India. The chemistry of this genus is quite homogeneous, sesquiterpene lactones being detected in all cases.[1-4] The occurrence of some toxic kaurene glycosides has been reported.[5,6] These compounds inhibit mitochondrial ADP/ATP translocation [7] and produce nephrotoxic effects.[8, 9] In Bolivia the roots decoction is used for the treatment of arteriosclerosis and hypertension, the leaf decoction is used in some inflammatory conditions such as oophoritis "inflammation of the ovaries", hepatitis, odontalgia, cystitis, nephritis and gastritis[10 ,11] while in North America the Cherokee also use this herbal drug for lung problems and snake bites.[12] However, it has spread all over the world and now all parts of the plants are used for a diverse range of medicinal purposes in many different countries. For instance, in Spain the leaves are used as contraceptive drug[13], the fruits to treat kidney malfunction and hyperglycemia[14] and in Italy the seeds decoction is used to treat diarrhoea.[15,16] The present study deals with an isolation, preliminary study on anti suppressant or anti-oxidant and antimicrobial activity.

MATERIALS AND METHODS

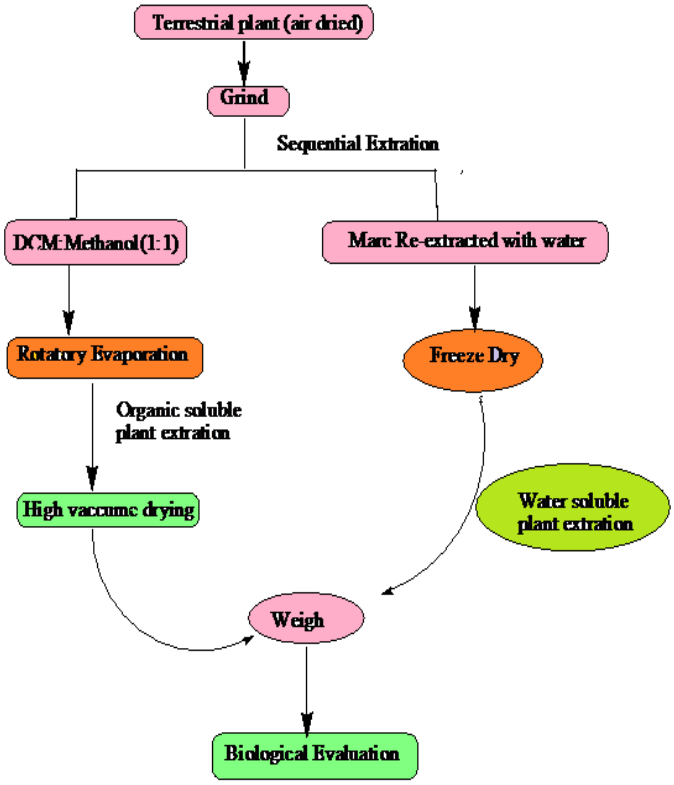
Plant materials were collected in December 2011 from Gulmarg (J&K), and identified by Dr. J P. Sharma (Department of Plant Biology, CSIR-IIIM JAMMU). Voucher specimens (578) are deposited in the Herbarium of the Department of Natural product. The plant materials were dried at room temperature, yielding 11.5 K g of whole plant from 35 kg of the wet material.

Extraction

The dried whole plant (11.5K g) of plant was crushed with Warning CB-6 blender (model 33BL13). The raw material was percolated with 1: 1 MeOH - DCM at room temperature. After that material was placed for vacuum drying which contain organic soluble plant extract. March was Re-extracted with water (contained water soluble

plant extract) followed by freeze drying then final weight of the extract was taken.

NCI Protocol for Extration[17]



Isolation and Purification of the Compounds

Methods used for the isolation of the compounds[18-21]

Optimization of the TLC System

Different solvent systems were tried to develop a TLC system for proper identification of the compound-spots present in the extract. The following gradient solvent systems were used:

Ethyl Acetate: Hexane -10%, 15%, 20%, 25%, 30%, 50%, 70%

DCM: Hexane -10%, 30%, 50%, 80%, 100%

Ethyl Acetate: DCM -2%, 5%, 7%, 10%, 12%, 15%, 20%, 25%, 30%, 50%

Methanol: DCM -2%, 5%, 10%

The developed TLCs were observed under UV-spectrophotometer and then sprayed with cerric reagent (cerric ammonium sulphate). Ethyl Acetate: DCM showed maximum separation.

Flash Column Chromatography (Fractionation)

Versa flash was used. For FCC, silica gel 790 gm (230- 400 mesh, Merck 11677), extract material 60 gm and the following experimental parameters

Column Chromatography [18-21]: (Isolation)

Column:

Column length: 90 cm

Column diameter: 2.5cm

Stationary phase: silica gel (60-120 mesh)

Mobile phase: Pet. Ether, DCM, Ethyl Acetate.

Charged material: Concentrated Chloroform extracts (4.354g)

Volume of each fraction: 20-25 m

The column was first run with pure DCM (100%) and then the polarity of the mobile phase was gradually increased by Ethyl Acetate. Each fraction of 20-25ml was collected and simultaneously TLC of the column fractions (in EtOAc : DCM) were taken. Accordingly the Ethyl Acetate concentration was gradually increased. As shown in the table II and III. On the basis of TLC pattern, fractions 85-120 were added together and concentrated (0.373g) in rota-evaporator and it was charged for the second column for further purification. Similarly Fractions 101-200 were added together and concentrated (96 mg) in rota-evaporator. This concentrate compound was further purified by crystallization using MeOH-Hexane solvent system.

Crystallization of Isolated Compound

Crystallization was done by adding small amount of methanol and hexane in excess amount to the isolated compound. Then after decanting the hexane layer in another fresh and clean empty flask and kept it for overnight. Pale yellow colour needle shaped crystals were formed which are again washed with Hexane or 1% (DCM : Hexane) obtain compound XS-cr.

Visualization

UV light at 254 nm, spraying with cerric ammonium sulphate, and then heating at 145 °C for 5-8 min.

Physical properties of the compound XS-cr:

Physical status : Crystalline mass

Colour : Yellowish white

Odour : None

Solubility : Chloroform, MeOH

Melting Point : 101-102^{0}C

High Performance Liquid Chromatography 18-21:

HPLC analysis of the isolated compound XS-cr:

The purity assessment of the compound was determined through the HPLC.

Instrument : Thermo
Software : Chromquest
Detector : PDA detect
Column : E-Merck RP – C_{18}, 5µm, (4 mm x 250 mm)
Flow Ra : 0.5 mL/min
Detector : PDA, 220 nm
Mobile Phase (Isocratic Flow) : Pump – A ACETONITRILE 90%
Pump – B Water 10%
Column Temperature : 30^0C
Injected Volum : 5 µL
Sample Preparation : 1 mg in 1.5 mL of HPLC grade Methanol

Detection was carried out at 220 nm. RP-HPLC was carried out on a LiChrospher 100 RP-18 (10 µm, 250×4 mm) reversed-phase (RP) column (Merck) using the following elutions:

Biological Activity Studies

Immunosuppressant Activity [22-24]

200 µl of sodium thio glycollate is injected in the intra peritoneal Cavity of the mice. After 48 hrs, macrophages are isolated from peritoneal excaudate cells*(PECs). Macrophages were obtained from mice euthanized by cervical dislocation in a laminar flow chamber to ensure sterile conditions. The abdomen of the rat was soaked with 70% ethanol for disinfection, a midline incision was then made with scissors, and the abdominal skin retracted. 30 ml de Hanks' balanced salt solution (HBSS) was then injected into the peritoneal cavity using a syringe with a 19-G needle horizontally in the upper part of the abdomen and extracted the media containing macrophages. The media containing macrophage were Centrifuge at 1800 rpm, 4^0c for 10 min. discarded the supernatant and added 2 ml of RBC lysis buffer to the pellet and incubated the samples on ice for 15 min. Centrifuged again at 1800 rpm, 4^0c for 10 min. discarded the supernatant and re-suspended the pellet in 2 ml RBC lysis buffer. Trypan blue staining method was use to Check, the viability of cells. To the micro culture plate, added 100 µl of cells, 50 µl media and 50 µl drugs to the wells labeled un-stimulated and the wells labeled stimulated added 100 µl cells, 50 µl drug and 50 µl LPS. the plate were Incubated, at 37^0c in 5% CO_2 for 12-24 hrs.

The Estimation of Release of Nitric Oxide(NO)* [22-24]

Nitrite estimation was done by Griesse reagent (1% sulphanilamide, 2% H3PO4 and 0.1% napthylethylenediamine dihydrochloride) for assaying the macrophages function in the supernatant of un-stimulated and LPS stimulated macrophages. The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine was read at 450 nm and referred to the absorbance of standard solutions of sodium nitrite in the same way with Griess reagent. The absorbance so recorded gives the amount of NO produced in accordance with Beer Lambert's Law.

Antimicrobial Activity [25-32]

Antimicrobial activity of the isolated compounds xs-cr and xs- cr 2 were evaluated for their antibacterial (*S. cerevisiae* , *S. aureus*, *E. coli*) and antifungal (*C. albican,* BS (*Bacillus subtilis*), ML (*M.luteus*) activities by serial plate dilution method at 512 µg/mL, 256 µg/mL, 128 µg/mL, 64 µg/mL, 32 µg/mL, 16 µgmL, 8 µg/mL, 4 µg/mL, 2 µg/mL, 1 µg/mL, 0.5 µg/mL, 0.25 µg/mL, 0.125 µg/mL, 0.0625 µg/mL, 0.0312 µg/mL and 0.0312 µg/mL and compared with reference drugs ciprofloxacin and *Amphotericin B*, respectively.

Xs-cr showed MIC of 256 µg/mL against *E. coli* , MIC of 128 µg/mL against *S. cerevisiae* and MIC of 128 µg/mL, against *S. aureus,* compared with standard drug ciprofloxacin having MIC of 0.032 µg/mL against *E. coli* , MIC of 0.12 µg/mL against *S. cerevisiae* and MIC of 0.25 µg/mL, against *S. aureus* Similarly Xs-cr 2 showed MIC of 256 µg/mL against *E. coli* , MIC of 256 µg/mL against *S. cerevisiae* and MIC of 256 µg/mL, against *S. aureus,* compared with standard drug ciprofloxacin having MIC of 0.032 µg/mL against *E. coli* , MIC of 0.12 µg/mL against *S. cerevisiae* and MIC of 0.25 µg/mL, against *S. aureus.* Xs-cr showed MIC of 128 µg/mL against Ca, MIC of

128 µg/mL against B*S.* and MIC of 128 µg/mL, against ML, compared with standard drug *Amphotericin B* having MIC of 0.5 µg/mL against *CA* , MIC of 0.5 µg/mL against B*S* and MIC of 0.5 µg/mL, against *ML.* Xs-cr 2 showed MIC of 256 µg/mL against Ca, MIC of 256 µg/mL against B*S.* and MIC of 256 µg/mL, against ML, compared with standard drug *Amphotericin B* having MIC of 0.5 µg/mL against *CA*, MIC of 0.5 µg/mL against B*S* and MIC of 0.5 µg/mL, against *ML. The result is shown in table V*

RESULTS AND DISCUSSION

Isolation of Xanthanolid from *X. spinosum* Species

The plant of *Xanthium spinosum* belonging to the family Asteraceae was selected for the islotaion of xanthanolides. Whole plant was extracted with Methanol: DCM following NCI protocol. An optimized TLC system was developed for the extract to get proper resolution of the compounds present in the extract. EtOAc: DCM combination showed maximum separation. The extract was subjected to column chromatography systematically on the basis of observed TLC pattern. Successively two columns were run and from that one pure compound was isolated (96 mg) which was designated as XS-cr. Further the purity of the isolated compound XS-cr was determined by HPLC. HPLC chromatogram showed that the compound was more than 98% pure with the Retention time 4.305 min. XS-cr was characterized by different spectroscopic method like IR, NMR and Mass spectroscopy.

Characterization of Xanthanolide from *X. spinosum* Species

Structure of iso-xanthanol

Iso-xanthanol (XS-cr) was obtained as a pale yellow crystalline solid. The IR spectral data revealed presence of lactone carbonyl at 1761.85 cm^{-1}, OC=O of acetate at 1247.16 cm^{-1} and a free hydroxyl group at 3467.67cm^{-1}. Two doublet at δH = 6.27 (H-13a) and δH = 5.53 (H-13b), doublets of doublets of quadrate at δH = 2.7 (H-10) and δH =4.9(H-4), and two doublets of CH3 at δH = 1.16 (H-14), δH = 1.23 (H-15) in the ^{1}H-NMR spectrum suggested that the compound was an α,β-unsaturated-γ-lactone with a side chain. Signals at δC = 41.365 (C-7), 73.618 (C-8), 30.965 (C-11), 170.1 (C-12), in the ^{13}C-NMR spectrum further supported this notation. The positive HRMS of XS-cr indicated a molecular ion at *m/z* = 331.1495 [M $^{+}$], in agreement with the molecular formula of $C_{17}H_{24}O_5$.

Determination of the Absolute Configuration of Isoxanthanol

Literature Confirmation of the Absolute Configuration of Iso-xanthanol (XS-cr). Iso-xanthanol (XS-cr) is isomeric with xanthanol (XS-cr2), and it was clear from the spectra of XS-cr2 and XS-cr that they were closely related in structure. The nrnr spectra of both XS-cr and XS-cr2 show signals for two secondary methyl groups (δH= 1.16, *J* = 7.4 cps; δH =1.23, *J* = 6.2 cps,), but the complex signal at δH= 3.7 present in the spectrum of XS-cr2 is absent in XS-cr, being replaced by a well-defined triplet at δH= 4.9. This suggested that XS-cr2 and XS-cr are related by an interchange of the hydroxyl and acetoxyl groups and this was confirmed by the preparation of the acetate of XS-cr. The spectral data of XS-cr acetate was in agreement with the data reported in the literature.

Biological Activity of Isolated Compounds

Immunosuppressant Activity [22-24]

Were carried out the isolated iso- xanthanol has got significant anti suppresent activity (40.82%)as compared to control and standard (28.99%)were as xanthanol has got moderate activity (32.45%) compared to control and standard (28.99%). As shown in table V. The X compound having Immunosuppressive activity as compared to the BMS (Betmethasone) standard .There is inc. in the % inhibition in X compound.

Antimicrobial Activity [26-33]

Xs-cr showed MIC of 256 µg/mL against *E. coli* , MIC of 128 µg/mL against *S. cerevisiae* and MIC of 128 µg/mL, against *S. aureus,* compared with standard drug ciprofloxacin having MIC of 0.032 µg/mL against *E. coli* , MIC of 0.12 µg/mL against *S. cerevisiae* and MIC of 0.25 µg/mL, against *S. aureus* Similarly Xs-cr 2 showed MIC of 256 µg/mL against *E. coli* , MIC of 256 µg/mL

against *S. cerevisiae* and MIC of 256 µg/mL, against *S. aureus,* compared with standard drug ciprofloxacin having MIC of 0.032 µg/mL against *E. coli* , MIC of 0.12 µg/mL against *S. cerevisiae* and MIC of 0.25 µg/mL, against *S. aureus*. Xs-cr showed MIC of 128 µg/mL against Ca , MIC of 128 µg/mL against B*S.* and MIC of 128 µg/mL, against ML, compared with standard drug ***Amphotericin B*** having MIC of 0.5 µg/mL against *CA* , MIC of 0.5 µg/mL against B*S* and MIC of 0.5 µg/mL, against *ML*. Xs-cr 2 showed MIC of 256 µg/mL against Ca, MIC of 256 µg/mL against B*S.* and MIC of 256 µg/mL, against ML, compared with standard drug ***Amphotericin B*** having MIC of 0.5 µg/mL against *CA*, MIC of 0.5 µg/mL against B*S* and MIC of 0.5 µg/mL, against *ML*.

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Table I: Flash column chromatography (Fractionation)

S. No.	Solvent system (2500 ml each)	Volume of the fractions	Weight of the fractions
1.	*n*-hexane	(100 ml)	2.103 g
2.	*n*-hexane–DCM	(100 ml)	0.280 g
3.	*DCM*	(100 ml)	3.280 g
4.	DCM–EtOH	(100 ml)	4.354g
5.	EtOH	(100 ml)	4.00 g
6.	EtOH - MeOH	(100 ml)	8.656 g
7.	MeOH	(100 ml)	18.279 g

Table II: Column Chromatography

Sl. No.	Eluting solvent (mobile phase)	Number of Fractions (20-25 ml each)	Number of TLC spots observed
1	Petroleum Ether	1- 40	No spot
2	DCM	41-42	No spot
3	-	43-50	Mixtures
4	-	51-60	Mixtures
5	-	61-70	Mixtures
6	-	71-75	Mixtures
7	2%EtOAc:DCM	76-84	Mixtures
8	-	85-91	Mixture of 3
9	-	92-120	Mixture of 3

TABLE III: Column Chromatography

Sl. No.	Eluting solvent (mobile phase)	Number of Fractions (20-25 ml each)	Number of TLC spots observed
1	-	101 - 120	2
2	4%- Ethyl Acetate DCM	121 - 140	2
3	-	141 - 160	2
4	6% Ethyl Acetate DCM	161 - 180	2
5	-	181 - 200	2
6	100% Ethyl Acetate	-	Column Elution

Table IV: HPLC

Pk#	R_t	Area	Area%
1	1.823	719	0.061
2	1.967	16938	1.435
3	4.305	1162917	98.504

Table V: Tested microorganisms

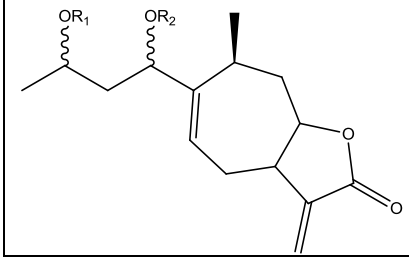
Tested Samples	Tested Microorganisms					
	Bacterial Strains			Fungal Strains		
	SA	*SC*	*EC*	*CA*	*BS*	*ML*
XS-cr	128	128	256	128	128	256
XS-cr 2	256	256	>256	256	256	>256
Amphotericin B				*0.5*	*0.5*	*0.5*
Ciprofloxacin	*0.25*	*0.12*	*<0.03*			

Ciprofloxacin served as control drug for bacterial strains, while as amphotericin B was used as control drug for fungal strains**.** SA (*Staphylococcus aureus*), SC (*Staphylococcus cerevisiae*), EC (*Enterococcus coli*), CA (*Candida albicans*), BS (*Bacillus subtilis*), ML (*M.luteus*).

Table VI: Immunosuppressant Activity

Treatment+Doses	Unstimulated (Mean±S.E)	Stimulated (Mean±S.E)	% Inhibition
Control	.2957±.0016	.338±.011	--
Std.(BMS)	.2253±.0021	.240±.045	28.99%
XS-cr	.1893±.0069	.2±.0087	40.82%
XS-cr 2	.1534±.0056	.12±.0067	32.45%

Table VII: Summary of the isolated compounds

Structure	Name	Code
	R1=H, R2=Ac, Xanthanol R1= Ac, R2= H, Isoxanthanol	XS-cr2 XS-cr

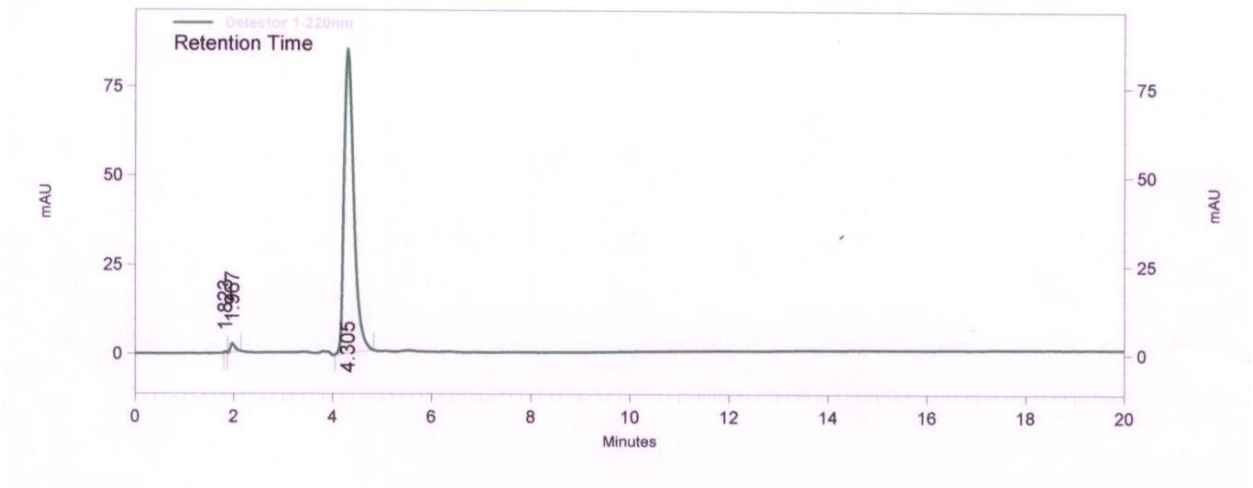


Figure 1: HPLC

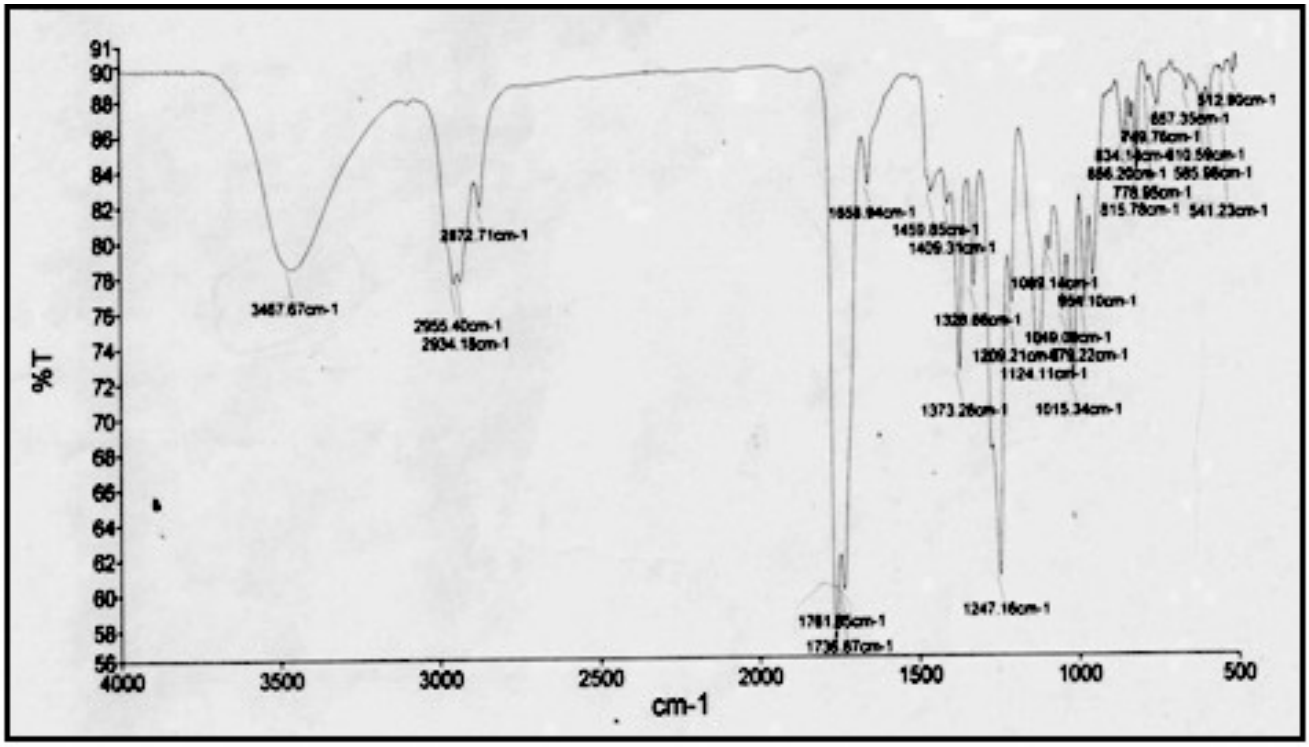


Figure : Infrared spectra(IR Spectra of XS-cr taken in $CHCl_3$)

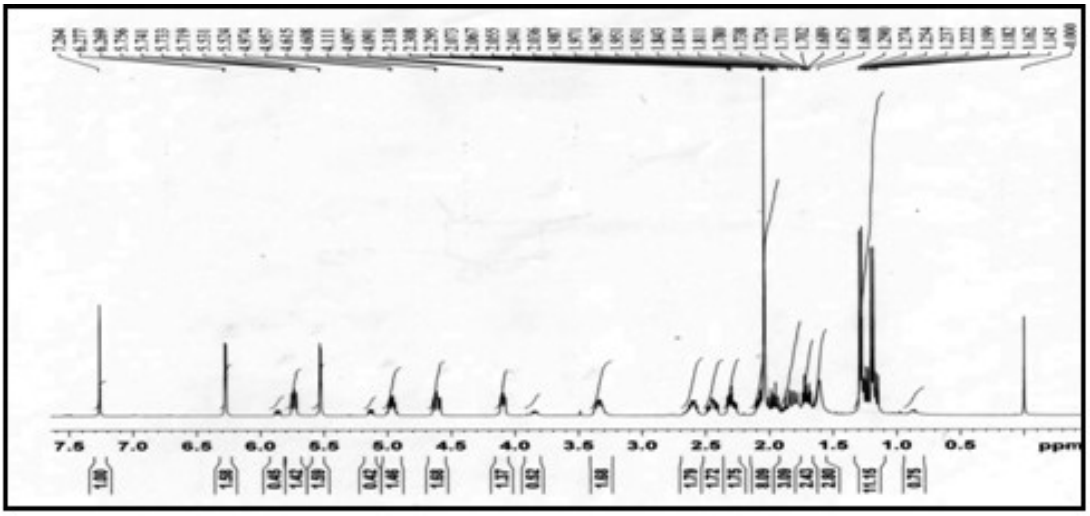


Figure 3 a: Spectrum of H[1]NMR of XS-cr

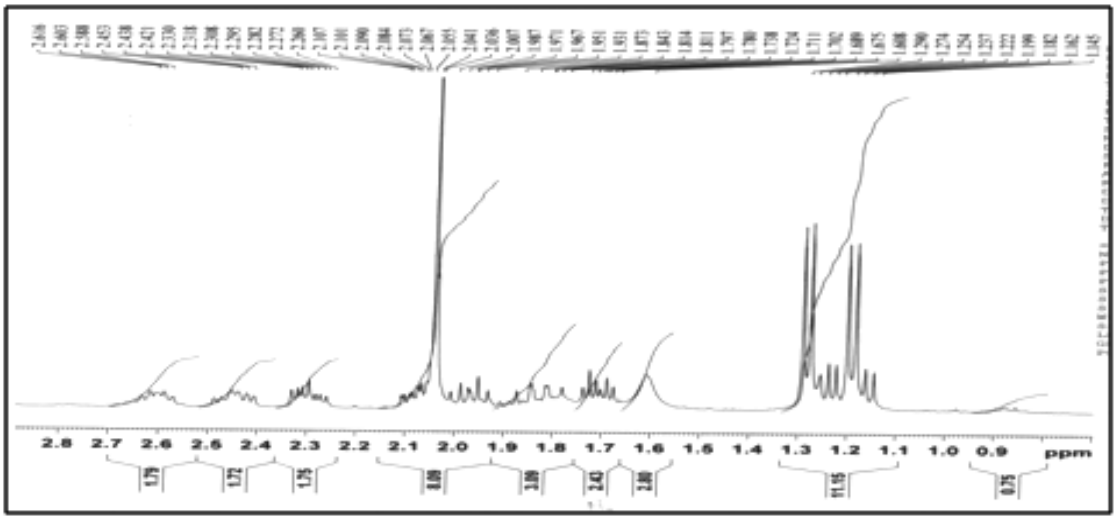


Figure 3 b: [1]H – NMR spectrum taken in ,400 MHz; with expansion of the region 1-3 ppm.

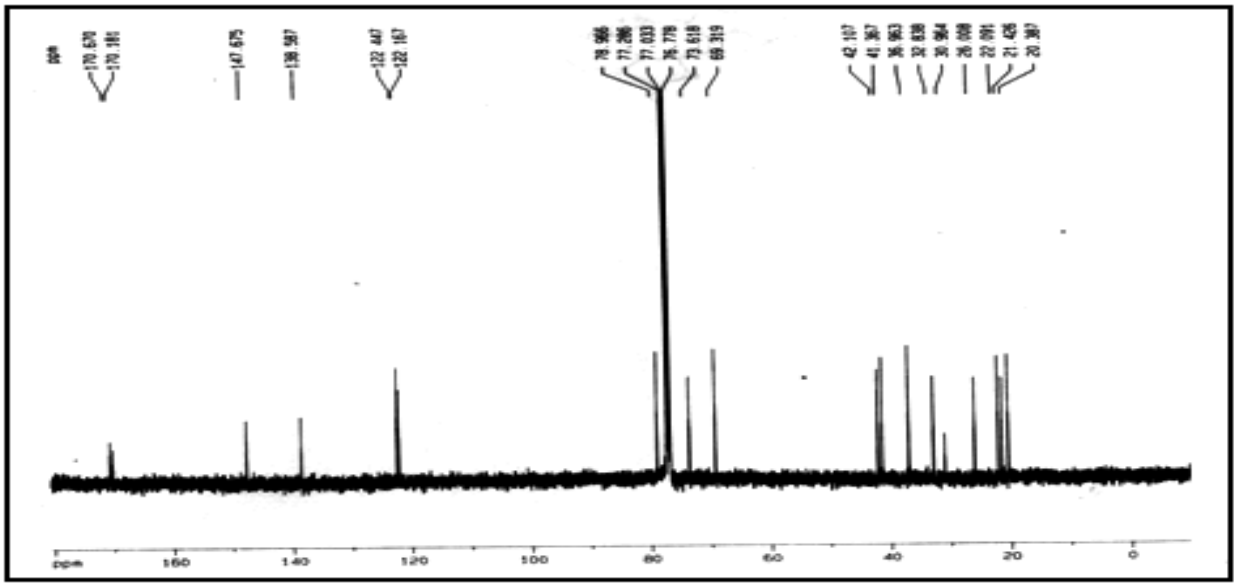


Figure 3 c: Spectrum of [13]C – NMR of XS-cr

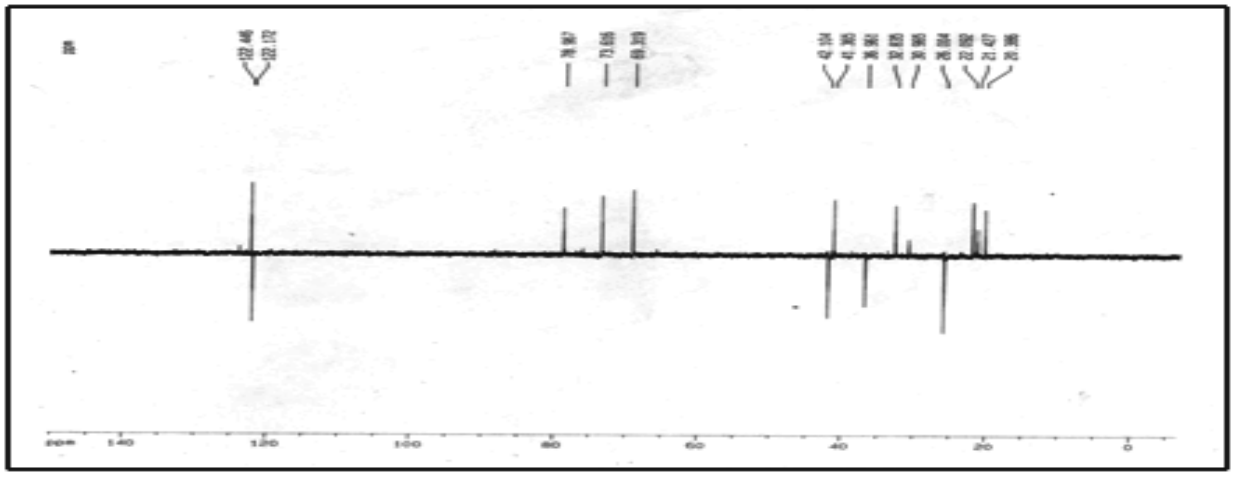


Figure 3 d: Spectrum of ^{13}C – NMR

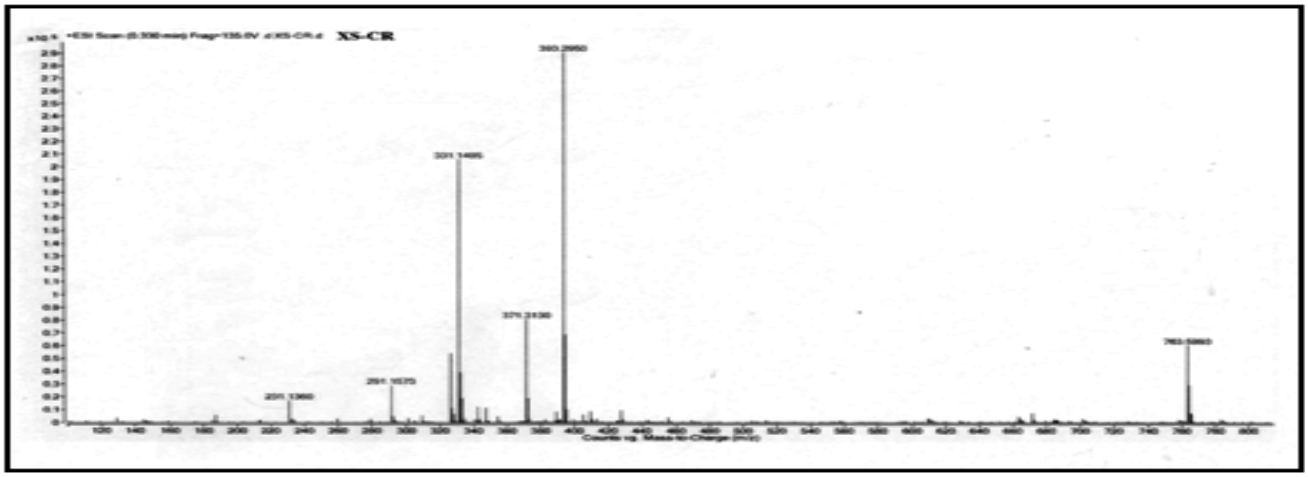


Figure 4: Mass Spectra (HRMS Mass Spectra of XS-cr taken in Methanol)

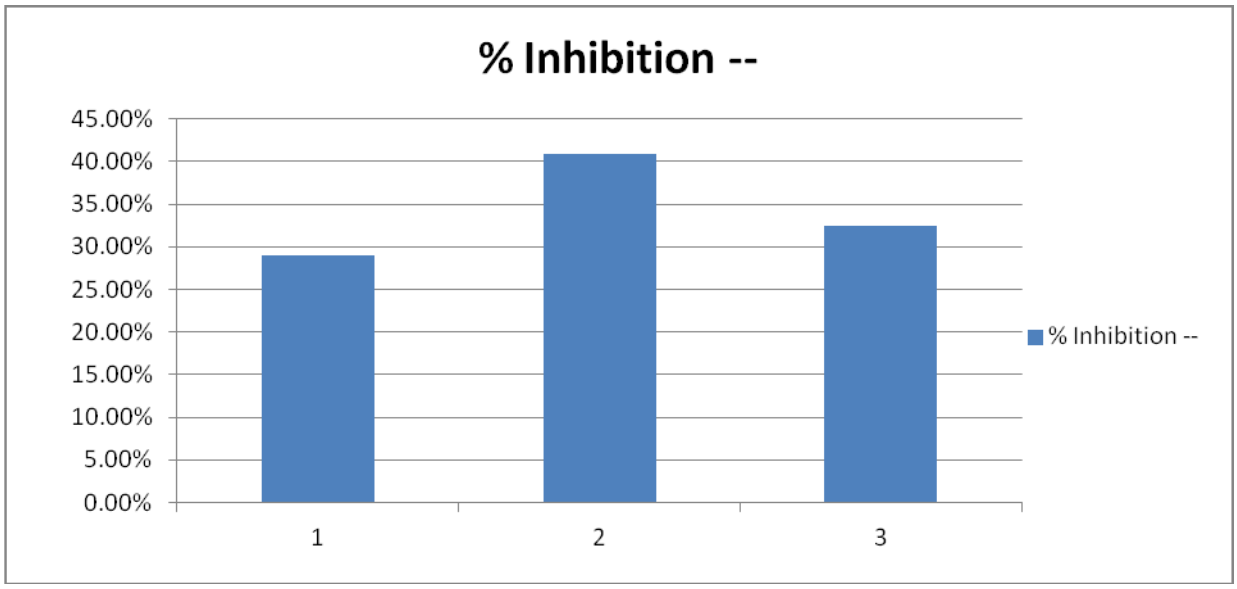


Figure 5(Graph 1): Immunosuppressant activity

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