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BIOGUIDED FRACTIONATION AND CHARACTERISATION OF THE EXTRACTS OF *STROBILANTHES CILIATUS* NEES

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ABSTRACT

Herbalism is based on relationship between plant and human, plant and planet, human and planet. Use of herbs in the healing process means taking part in an ecological cycle. The anti-inflammatory activities of *Strobilanthes ciliatus* was evaluated by several *in vitro* methods. The petroleum ether and alcoholic extracts of leaf and total alcoholic extract of stem was taken for evaluation. The anti-inflammatory activities were evaluated by cyclooxygenase inhibition assay, 5-lipoxygenase inhibition assay, myeloperoxidase, inducible nitric oxide synthase estimation, and cellular nitrite estimation on RAW 264.7 cell line. All the three extracts showed anti-inflammatory activity, but the petroleum ether extract of leaf showed more significant activity. Thus the promising extract (LPE) was subjected to column chromatography. The isolated compound was characterised by UV, IR, NMR, HRMS spectral studies and it was identified as Betulin. The COX and 5-LOX assay of the isolated compound was further conducted to justify and support the use of the plant as anti-inflammatory agent.

KEYWORDS: Strobilanthes ciliatus, Anti-inflammatory, Column chromatography, Characterisation, Betulin.

INTRODUCTION

According to N. R. Farnswoth, an eminent scientist working in the field of medicinal plants “Each plant is unique and chemical factory capable of synthesizing an unlimited number of lightly complex and unusual chemical substances whose structure could escape imagination of synthetic chemist forever.[1] Medicinal plants by definition are any substance with one or

more of its organ contains properties that can be used for therapeutic purpose or which can be used as precursor for the synthesis of various drugs.

Strobilanthes ciliatus belongs to Acanthaceae and is the second largest genus of this family. It comprises of 300 species of tropical Asia.[2] *Strobilanthes ciliatus* is one of the endemic and potential medicinal plants. The entire plant was recognised as valuable drug and frequently used by ancient traditional medicinal system. The leaves, stem, and roots of the plant possess many therapeutic effects such as limbago, sciatica, diuretic, diaphoretic, anti-inflammatory, skin disease, jaundice, leprosy, leucoderma and in the treatment of rheumatism.[3]

Inflammation is a beneficial host response to foreign invades but it may cause tissue damage. The cause of inflammation includes infection, tissue necrosis, foreign bodies, trauma and immune response. The main mediators of inflammation are COX enzyme, 5-LOX enzyme, Nitric oxide, Myeloperoxidase, Cellular nitrate etc. The preliminary phytochemical screening showed the presence of flavonoids, phenolic compound, tannins, steroid, glycosides and triterpenoids.[4] Terpenoids and steroids such as lupeol, stigmasterol, betulin and stigmasterol glycosides are also reported from the acetone extract of the stem of *Strobilanthes ciliates.*[5] Betulin is a pharmacologically active compound. It is a naturally occurring pentacyclic lupane type triterpenoid which exhibit a variety of biological and medicinal properties such as inhibition of human immune deficiency (HIV) virus, anti-bacterial, anti-inflammatory, anthelmintic, anti-cancer and anti-tubercular activity. An attempt was made to correlate the bioactivity of the plant extract with isolated constituents by bioguided fractionation.

The three extracts of the plant namely LPE (Pet ether extract of leaf), LAE (Alcoholic extract of leaf) and SAE (stem alcoholic extract) was subjected to 5 *in vitro* models of anti-inflammatory studies on RAW 264.7 cell lines. The promising extract was selected for column chromatography to isolate the compound from the extract. COX and 5-LOX assay was further conducted in the isolated compound.

MATERIALS AND METHODS

Collection of plant material: *Strobilanthes ciliatus* was collected from Kaduthuruthi and Palai region of Kottayam district in the month of April to July 2013. It was authenticated by Mr. Rogimon P. Thomas, Asst. professor, Department of Botany, CMS College Kottayam. Specimen voucher No.268 and is preserved in the herbarium of CMS College Kottayam for future reference.

Extraction of the plant material: About 500gm of the dried and powdered leaf and stem of *Strobilanthes ciliatus* was extracted. The leaf was extracted with petroleum ether (60^0-80^0 C) by soxhlation. After the complete extraction the solvent was distilled off. The resulting semisolid residue was dried. The marc was then extracted with 95% ethanol by hot soxhlet extraction process. The residue thus obtained was dried.

The powdered stem was extracted with 95% ethanol and followed the same process as above. The three extract obtained was named as LPE (Petroleum ether Extract of Leaf), LAE (Alcoholic Extract of Leaf)), SAE (Alcoholic extract of Stem).

***In vitro* anti-inflammatory activity studies**

1. Cytotoxicity Assay by MTT Method

The cytotoxicity assay by MTT method was carried out[6] on RAW 264.7 Cell line.

2. Cyclooxygenase (COX) activity

The COX activity was assayed by the method of Walker and Giers.[7]

3. Lipoxygenase (5-LOX) activity

The determination of LOX activity was as per the method described by Axelrod et al.[8]

4. Myeloperoxidase (MPO) activity

Myeloperoxide activity was determined by the method described by Bradley 1982.[9]

5. Inducible nitric oxide synthase (iNOS) estimation

Nitric oxide synthase was determined by the method described by Salter et al.[10]

6. Estimation of Cellular Nitrite Levels

The level of nitrite level was estimated by the method of Lepoivre et al.[11]

Chromatographic studies were conducted to identify the components present in the biologically active extract using column chromatography. IR, H1NMR, C13NMR, HRMS were performed for the spectral identification of compound.

1. Column chromatography

a) Filling of the column for column chromatography

b) Preparation and loading of the sample

c) Column chromatographic profile

2. Structure elucidation and characterization of the column isolated fractions.

3. Determination of COX and 5-LOX activity of isolated compound- The COX activity was assayed by the method of Walker and Gierse and 5-LOX activity as per Axelrod et al.

Statistical analysis: - All experiments were carried out in triplicate and the triplicate and the absorbance value expressed are mean ± SD. IC_{50} values were determined by using software sigma plot 12.2 version. Significance is found out using one way ANOVA- Tukey multiple comparison test.

RESULTS AND DISCUSSION

Cytotoxicity Assay by MTT Method: The cytotoxicity of three extracts towards RAW 264.7 cell lines were determined by MTT assay. The data indicates that the rate of cell viability decreased with increasing concentration of all examined extracts compared to that of control. The results of MTT assay of the three extracts were given in table no. 1.

Cyclooxygenase inhibition: All the extracts exhibited cyclooxygenase inhibition and the highest was with LPE followed by SAE and LAE. The results obtained from the COX assay is depicted in table no.2.

5- Lipoxygenase inhibition: Comparison of 5-LOX inhibition studies is exhibited in table no.3 and the results shows that 5-LOX inhibition exhibited by LPE is highest among the three extracts and was followed by LAE and the least by SAE. Inhibition of 5-LOX by the extracts and standard is expressed in table no.3.

Estimation of Myeloperoxidase activity: All the extracts showed decrease in enzyme level with increase in concentration. This indicates a dose dependent MPO inhibition by the extracts and standard. LPE showed the highest activity and least activity by SAE. The results are given table no. 4.

Estimation of Cellular nitrate level: All the extract showed a decrease in nitrite level with increase in concentration. This indicated dose dependent nitric oxide inhibition by the extract and the standard. LAE showed the highest activity. Results obtained from estimation Cellular nitrate level are tabulated in table no.5.

Estimation of iNOS: All the extract shows a decrease in delta OD with increasing in concentration of drug and standard. Inducible nitric oxide synthase is activated in response to cytokines and overexpression of iNOS leads to increased production of nitric oxide and subsequent inflammatory pathways. From our results the iNOS activity which was expressed in terms of OD units was found to be increased by activation with LPS whereas compound treatment decreases the iNOS levels in a dose dependent manner. Due to the multiple mechanisms of action, it is suggested that these extract can be used to develop safer and newer drugs to treat inflammatory conditions. Result obtained from iNOS estimation is tabulated in table no.6.

Spectral identification using IR, NMR, and MS

Column chromatographic studies of the petroleum ether extract of leaf yielded a crystalline solid.

Appearance: White crystalline solid.

Solubility: Soluble in DMF, 1-butanol, acetone, methyl acetate, chloroform

Melting point- 245° C

λ_{max} of compound -324.57 nm (methanol)

Chemical test: The compound gave a positive (violet coloration) Lieberman Burchard test, and was suggested to be a Triterpenoid.

IR Spectroscopy of isolated compound

The result obtained from IR spectroscopy of the isolated compound is tabulated in table no.7 and IR spectrum is given in as fig.no.1.

NMR studies: The C_{13} and H_1 NMR of the isolated compound were performed and the results were illustrated in table no.9 and the spectrums were given as figure no. 2 and 3.

The H_1 NMR spectrum of the compound showed five methyl singlet at δ^H 0.75, 0.86, 0.90, 0.92 and 0.95, a vinyl methyl group at δ^H 1.68 (H-30, S), a doublet of one oxygenated methine proton at δ^H 3.13(H-3, dd 3.128, 3.115). Two doublets of one oxygenated methylene group at δ^H 3.30 (H-28 b,d) and two broad singlet of a terminal double bond, one proton each at δ^H 4.565 (H-29 a) and 4.569(H-29 b) (Table No.). The C_{13} NMR revealed 30 carbon signals which included 6 methyl groups, 12 methylene groups, 6 methine carbon and 6 quaternary carbons (table no.7). The oxygenated carbon signals appear at δ_C 58.98 (C-28) and 78.26(C-3) and double bonds at δ_C 108.81 (C-29) and 150.48 (C-20), characteristic of

triterpene of the lup 20 (29)-ene type.[12] Thus the compound was identified as Betulin, a lupane type triterpenoid previously reported from other sources.

HRMS Spectrum of Isolated compound: The HRMS spectrum of isolated compound (Betulin) is showed as fig.no.4. The compound was positive to Liebermann Buchard Test and therefore it was suggested to be a triterpenoid. HRMS gave a molecular ion peak at 513.3581 which corresponds to (M+3 Na) +2H. The molecular weight of the isolated compound was found to be 442.7168 and the molecular formula is $C_{30}H_{50}O_2$. The IR spectrum showed vibration bands corresponding to hydroxyl group (3362 cm^{-1}) and a terminal double bond (2938,1643,881 cm^{-1}).

Determination of COX and lox activity of the isolated compound: The results obtained from Determination of COX and 5- LOX activity of Betulin is given in table no.9.

Cytotoxicity Assay by MTT Method

Table No. 1: MTT assay of the extracts.

Extract	Sample conc. (µg/mL)	Absorbance ± SD	% Viability
Control		0.6906±0.0050	100
LPE	25	0.6469±0.0053	93.63
	50	0.5697±0.0316	82.46
	75	0.3848±0.0407	55.70
	100	0.3468±0.0334	50.20
SAE	25	0.3844±0.0089	55.63
	50	0.3623±0.0118	52.44
	75	0.3226±0.0118	46.69
	100	0.2679±0.0271	38.78
LAE	25	0.4112±0.0183	59.52
	50	0.3853±0.0185	55.77
	75	0.2779±0.0234	40.23
	100	0.1971±0.0364	28.52

Cyclooxygenase inhibition

Table No. 2: COX inhibition by the extracts using RAW264.7 cell lines.

Extract	Conc. (µg/mL)	Absorbance± SD	% Inhibition	IC 50
STD (diclofenac sodium)	control	0.3181±0.0240	-	8.803
	25	0.0890 ±0.05474	72.02±7.19	
	50	0.0630 ±0.02128	80.19±6.69	
	75	0.0540 ±0.02193	82.23±6.89	
	100	0.0480 ±0.01452	84.91±4.57	
LPE	control	0.0019±0.0019	-	82.3016
	25	0.1312 ± 0.00779	13.85±4.29***	
	50	0.0987 ± 0.00593	35.19±4.01***	
	75	0.0781 ± 0.00574	48.71±3.87***	
	100	0.0675 ±0.00521	55.67±3.95***	
	125	0.0590 ± 0.00435	61.26±3.32	
	150	0.0320 ± 0.00619	78.92±3.74	
SAE	25	0.1271 ±0.00210	16.55±0.78***	117.77
	50	0.1196 ±0.00406	21.47±2.85***	
	75	0.0997 ±0.00148	34.53±1.65***	
	100	0.0884 ±0.00537	41.95±4.15***	
	125	0.0723 ±0.00249	52.52±1.39	
	150	0.0582 ±0.00429	61.78±3.19	
LAE	25	0.1326 ± 0.00502	12.93±2.19***	129.17
	50	0.1244 ±0.00417	18.32±3.68***	
	75	0.1102 ±0.00453	27.64±2.09***	
	100	0.0927 ±0.00335	39.13±2.83***	
	125	0.0811 ±0.00227	46.74±2.03	
	150	0.0623 ±0.00101	59.09±1.13	

ANOVA followed by Tukey's multiple comparison tests was used. All values are expressed as mean ± SD; ***p< 0.0001.

5- Lipoxygenase inhibition

Table No.3: 5-LOX inhibition by the extract on LPS induced RAW 264.7C cell lines.

Extract	Conc. (µg/mL)	OD at 234 nm	% Inhibition	IC_{50} (µg/mL)
STD (diclofenac sodium)	Control	0.2530±0.0172	-	12.14
	25	0.0710 ±0.0147	71.93±5.82	
	50	0.0340 ±0.0141	86.56±5.34	
	75	0.0288 ±0.0021	93.01±0.60	
	100	0.0050 ±0.0022	98.02±0.88	
LPE	Control	0.0824±0.0045	-	76.616
	25	0.0784 ± 0.0056	4.85±2.10***	
	50	0.0699 ± 0.0043	15.17±5.33***	
	75	0.0431 ± 0.0016	47.69±2.04***	
	100	0.0199 ±0.0013	75.84±1.63***	
SAE	Control	0.4785±0.0309	-	243.7
	25	0.3448 ± 0.0032	25.39±0.42***	
	50	0.3322 ± 0.0071	28.03±0.73***	
	75	0.3287 ± 0.0025	30.61±1.46***	
	100	0.4785 ± 0.0309	31.38±0.52***	

LAE	Control	0.4785±0.0309	-	111.113
	25	0.3401 ± 0.0115	28.92±2.41***	
	50	0.3173 ± 0.0068	33.81±1.28***	
	75	0.2980 ± 0.0049	37.79±1.03***	
	100	0.2571 ± 0.0059	46.43±1.18***	

Absorbance value expressed as mean± SD, n=3.

ANOVA followed by Tukey's multiple comparison tests was used. All values are expressed as mean ± SD; ***p< 0.0001.

Estimation of Myeloperoxidase

Table No. 4: Myeloperoxidase estimation of extracts.

Extract	Conc. (µg/mL)	OD± SD	Enzyme U/mL
STD (diclofenac sodium)	Control	0.0164±0.0022	-
	25	0.0043 ±0.0019	0.0014±0.0001
	50	0.0030 ± 0.0014	0.0009±0.0010
	75	0.0026 ± 0.0019	0.0008±0.0001
	100	0.0023 ± 0.0010	0.0007±0.0001
LPE	Control	0.0170±0.007	-
	25	0.0100±0.0055	0.0033±0.0007^{ns}
	50	0.0064 ±0.0011	0.0021±0.0003^{ns}
	75	0.0044 ±0.0011	0.0014±0.0003^{ns}
	100	0.0022 ±0.0017	0.0007±0.0002^{ns}
SAE	Control	0.1750±0.0050	-
	25	0.1526± 0.0036	0.0503±0.0010***
	50	0.0771 ±0.0026	0.0254±0.0040***
	75	0.0120 ± 0.0019	0.0039±0.0008^{ns}
	100	0.0088 ± 0.0036	0.0029±0.0080^{ns}
LAE	Control	0.1750±0.0050	-
	25	0.1034 ± 0.0037	0.0341±0.0019***
	50	0.0210 ± 0.0032	0.0069±0.0002***
	75	0.0041 ± 0.0009	0.0013±0.0001^{ns}
	100	0.0032 ± 0.0005	0.0010±0.0001^{ns}

ANOVA followed by Tukey's multiple comparison tests was used. All values are expressed as mean ± SD; ***p< 0.0001; ns >0.05

Estimation of Cellular nitrate level

Table No.5: Cellular nitrite estimation of extracts

Extract	Conc. (µg/mL)	OD± SD	Nitrite concentration (µg)
STD (Diclofenac sodium)	Control	0.1875 ±0.0094	
	25	0.1468 ± 0.0112	729.59±16.11
	50	0.1184 ± 0.0073	588.44±14.47
	75	0.0920 ± 0.0086	485.04±15.32
	100	0.0684 ± 0.0114	339.94±19.75
LPE	Control	0.1875 ±0.0094	
	25	0.1782 ±0.0202	882.09±24.29***
	50	0.1519 ±0.0098	751.90±18.15***
	75	0.1382 ±0.0116	682.89±11.37***
	100	0.1290 ±0.0096	638.55±11.86***
SAE	Control	0.1761 ±0.0130	
	25	0.1644 ± 0.0151	813.78±16.46***
	50	0.1430 ± 0.0167	707.85±12.56***
	75	0.1319 ± 0.0116	652.90±18.97***
	100	0.1309 ±0.0069	647.95±09.29***
LAE	Control	0.1761 ±0.0130	
	25	0.1497 ±0.0156	741.01±09.87^{ns}
	50	0.1397 ± 0.0120	691.51±17.67***
	75	0.1290 ± 0.0134	638.55±14.36***
	100	0.1241 ± 0.0101	614.29±15.74***

ANOVA followed by Tukey’s multiple comparison test used. All values are expressed as mean ± SD; ***p< 0.0001; ns >0.05.

Estimation of iNOS

Table no. 6: estimation of iNOS activity of extracts

Extract	Sample concentration (µg/ml)	OD 1	OD 2	Difference in OD
STD (Diclofenac Sodium)	Control	-	-	0.074
	25	0.075±0.0289	0.046±0.0134	0.029
	50	0.053±0.0117	0.043±0.0142	0.010
	75	0.041±0.0268	0.034±0.0159	0.007
	100	0.022±0.0085	0.016±0.007	0.006
LPE	Control	0.069±0.0167	0.026±0.0134	0.0430
	25	0.089±0.0205	0.0633±0.011	0.0257
	50	0.0527±0.0115	0.0366±0.006	0.0161
	75	0.0403±0.0108	0.0262±0.0114	0.0141
	100	0.0274±0.0108	0.0163±0.0072	0.0111
LAE	Control	0.223±0.0477	0.102±0.0226	0.1210
	25	0.8434±0.024	0.7715±0.026	0.0719
	50	0.1647±0.0143	0.1497±0.0114	0.0150
	75	0.1575±0.0100	0.1499±0.0056	0.0076
	100	0.2655±0.008	0.2629±0.0027	0.0026
SAE	Control	0.223±0.0477	0.102±0.0226	0.1210
	25	0.652±0.039	0.5505±0.033	0.1015

	50 75 100	0.7677±0.038 0.6789±0.048 0.2966±0.018	0.7557±0.054 0.6717±0.159 0.2922±0.052	0.0126 0.0072 0.0044

Absorbance value expressed as mean± SD, n=3

IR Spectrum of LPE-1

Table No.7: IR values of LPE-1.

Sl. No.	Wave Number of Compound (CM^{-1})	Referance Wave Number (CM^{-1})	Functional Group
1	3362	3456	O-H Stretching
2	2938	2925	C-H stretching of aliphatic compound
3	1643	1639	C=O stretching
4	1453	1460	OH bending
5	1375	1345	CH_2 – CH_3 bending
6	881	885	Wagging vibration of CH_2 in the terminal group.-

NMR studies

Table No. 8: NMR values of isolated compound.[12]

C/H Position	Cδ 400MHZ		Hδ 400 MHZ	
	(Kengne et al., 2016)	Me OD	(Kengne et al., 2016)	Me OD
1	38.7	38.65	-	-
2	27.4	26.63	-	-
3	79	78.26	3.18 dd	3.138
4	38.9	38.55	-	-
5	55.3	55.42	0.67 m	0.71
6	18.3	18.04	1.38m,1.52m	1.390,1.534
7	34	34.07	1.39,1.39	1.4,1.4
8	40.5	40.75	-	-
9	50.4	47.76	1.27	1.18
10	37.2	36.88	-	-
11	20.8	20.57	1.21m,1.42	1.71,1.2
12	25	25.22	1.02m1.63	1.007,1.6
13	37.5	37.31	1.6m	1.602
14	45.2	42.42	-	-
15	27	26.78	1.05m,1.67m	1.071,1.626
16	29.7	28.97	1.21m,1.9m	1.2,1.92
17	47.8	47.42	-	-
18	48.9	47.76	1.57m	1.567
19	47.8	47.42	2.38	2.45
20	150.5	150.48	-	-
21	29.7	29.46	1.42,1.95 m	1.427,1.949
22	34.6	33.96	1.02,1.83	1.007,1.94
23	27.4	27.2	0.93 S	0.92

24	15.9	15.13	0.76 S	0.753S
25	16.3	17.96	0.82 S	0.860 S
26	16.1	15.28	0.98 S	0.95 S
27	15.4	14.7	0.90 S	0.9 S
28	60.6	58.98	3.33 d ,3.80 d	3.308 , 3.726
29	109.7	108.81	4..58 brs 4.66 brs	4.565,4.569
30	19.1	20.57	1.68 S	1.684 S

Determination of COX and 5-LOX activity of the isolated compound

Table No.9: COX assay of isolated compound.

Sample	Conc. (µg/mL)	OD±SD	% Inhibition	IC_{50}
LPE-1 (Betulin)	Control	0.1492±0.0021	-	99.18
	25	0.1201± 0.0053	19.5±3.59***	
	50	0.1083 ±0.0036	27.4±2.42***	
	100	0.0740 ±0.0160	50.4±11.40***	
STD	Control	0.3181±0.0240	-	8.80
	25	0.0890±0.5474	72.02±7.19	
	50	0.0630±0.0212	80.19±6.69	
	100	0.0480±0.0145	84.91±4.57	

ANOVA followed by Tukey's multiple comparison test used. All values are expressed as mean ± SD; ***p< 0.0001.

Table No. 10: 5-LOX assay of isolated compound.

Extract.	Conc. (µg/mL)	OD±SD	% Inhibition	IC_{50}
LPE-1 (Betulin)	Control	0.2273±0.0014		133.85
	25	0.2062 ±0.0063	9.35±2.77***	
	50	0.1951 ±0.0030	14.16±1.35***	
	100	0.1396 ±0.0047	38.58±2.09***	
STD	Control	0.2530±0.0172	-	12.14
	25	0.0710±0.0147	71.93±5.82	
	50	0.0340±0.0141	86.56±5.34	
	100	0.0050±0.0022	98.02±0.88	

ANOVA followed by Tukey's multiple comparison test used. All values are expressed as mean ± SD; ***p< 0.0001.

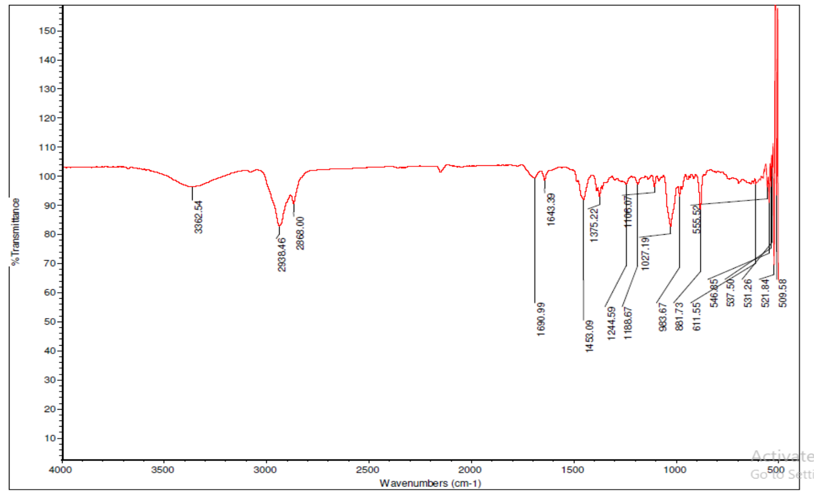

Fig. No. 1: IR spectrum of LPE.

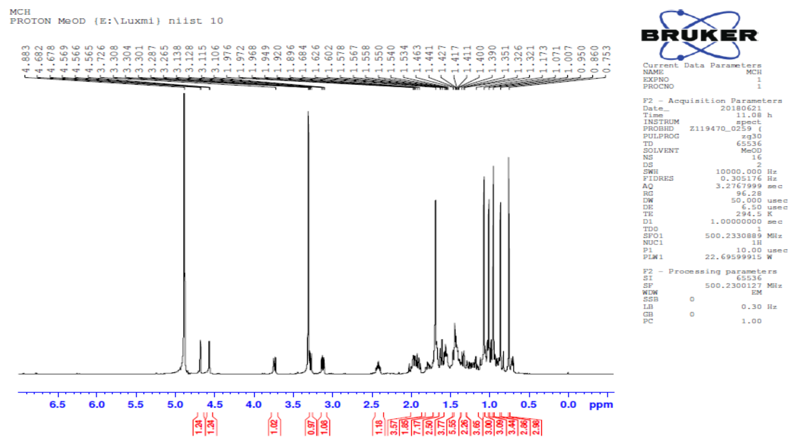

Fig. No. 2: H^1 NMR of isolated compound.

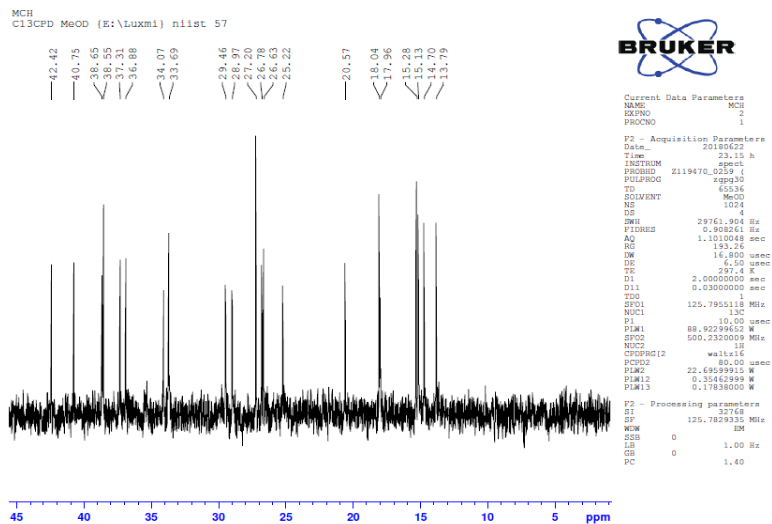

Fig. no.3: C_{13} NMR of isolated compound.

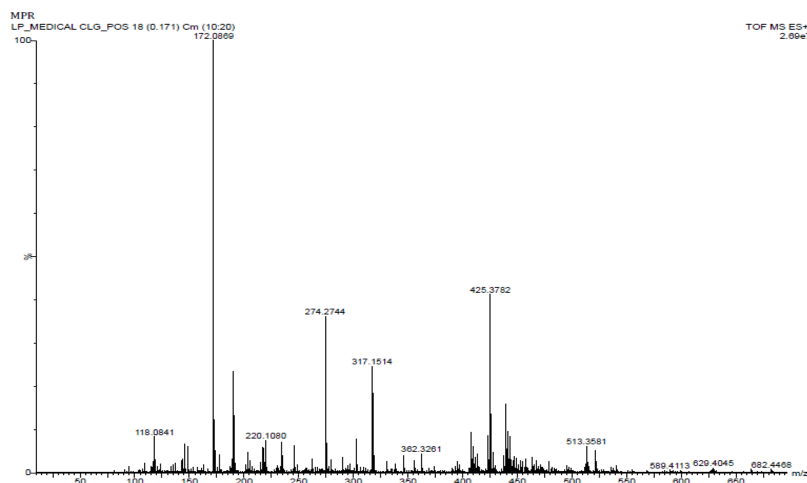

Fig. no.4: HRMS spectrum of isolated compound (LP Positive).

CONCLUSION

In-vitro anti-inflammatory studies were done on RAW 264.7 cell lines. The studies were done to evaluate the mechanism behind the anti-inflammatory activity of the three extracts. All the extracts inhibited COX, 5-LOX enzymes and also showed significant decrease in enzyme level of MPO, cellular nitrate and iNOS. MTT assay done on RAW 264.7 cell line the cell viability at 80µg/ml. LPE showed the highest activity of the three extract.

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