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Chalcones as promising therapeutic agents against tubulin protein –a preliminary investigation.

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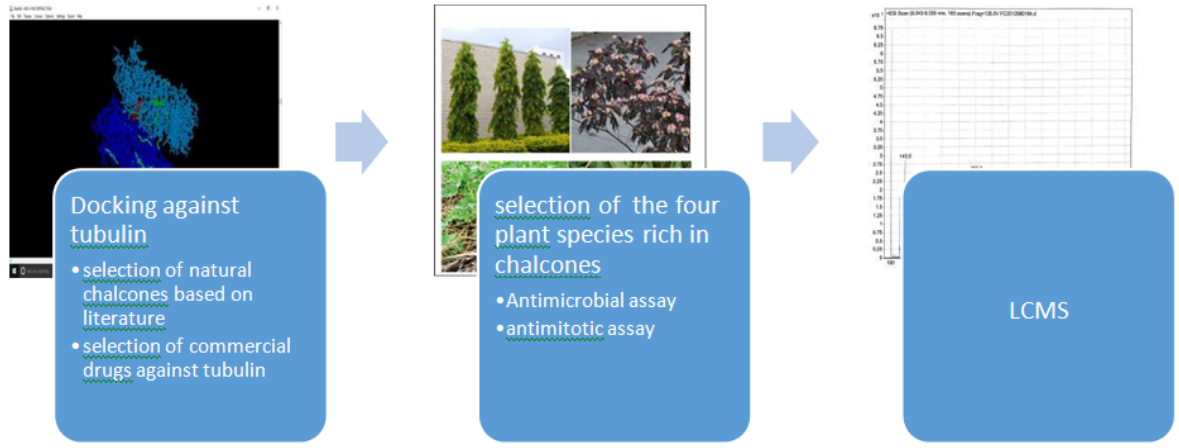
Graphical Abstract



Abstract:
Chalcones represent an important group of the polyphenolic family. This family possesses an interesting spectrum of biological activities like anti -oxidative, anti-bacterial, antiinflammatory, anti-cancer, cytotoxic and immunosuppressive potential. Compounds of this family have shown to interfere with several steps of carcinogenesis, including initiation, promotion and progression.
In the current study, the binding efficiency against tubulin of fourteen commercially available anti-mitotic drugs and fifteen natural chalcones selected based on literature (Toshihiro et.al, 2003&Alias Y et.al, 1995)were compared using Hex docking software. The results indicate that chalcones have a binding energy similar to that of established drugs and could be used as an alternative. Further, anti -microbial , anti-mitotic activities and LCMS profiling of methanolic extracts of four plant sources reported to be rich in chalcones was carried out . The plants selected were Glycyrrhiza inflata(mulethi); Mimosa pudica(touch me not); Polyalthia longifolia (ashok tree) and Bridelia stipularis .The methanolic extracts Bridelia and Polyalthia showed good antimitotic activity.
***Key words**: Chalcones;Hex ;tubulin*

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I. Introduction

Most commercially available anti-cancer drugs are targeted at inhibiting the functioning of the mitotic spindle apparatus. However, since microtubules fulfil important functions in resting and differentiated cells by mediating, e.g., intracellular transport processes, antimicrotubule drugs exhibit a plethora of unwanted side effects including severe peripheral neuropathies. Therefore, novel drug targets that spare microtubules, but inhibit the progression of mitosis are highly desired and are being exploited for the development of novel anti-mitotic drugs. Thus, current drug development programs focus on improved novel anti microtubule drugs from natural sources.

Many compounds from plants such as vinca alkaloids, taxanes, epothilones, discodermolide (Mathias *et al., 2007*) and colchicine (Zhou &Giannakakou *2005*) have been found to be anti-mitotic by binding and inhibiting microtubule function. While vinca alkaloids and colchicine show microtubule depolymerizing activity taxanes (Larkin& Kaye 2006), epothilones and discodermolides suppress dynamic instability of microtubules

resulting in a failure of chromosome alignment causing mitotic arrest and subsequent apoptosis. Chalcones are one amongst the promising group of phytochemicals which have been relatively less studied so far. Few reports are available indicating their anti-cancer properties (*Ducki et al., 2009*) .Here, we report the screening of fifteen chalcones from natural sources against tubulin .The binding efficiencies to tubulin of these compounds was compared with that of the commercially available drugs using HEX. Software**.** Further, anti -microbial , anti-mitotic activities and LCMS profiling of methanolic extracts of four plant sources reported to be rich in chalcones was also carried out.

II. Material And Methods

2.1 In silico studies:

2.1.1 Selection of natural chalcones: Families were listed out based on available literature, on their ability to produce chalcones. The following families (Table 1) were selected and the different plants under these were screened for chalcones. The structure of these chalcones was obtained from RCSB website in .pdb format.

Table 1: Details of chalcones selected

Chalcones	Source	Family	References
2'4'dihydroxy 3' methoxy chalcone	*Polyalthia cauliflora*	*Annonceae*	*Ghani N.A. et.al.2011*
6'4'dihydroxy 3' propen chalcone	*Bridelia Ferruginea benth*	*Euphorbiaceae*	*Donatus Ebere Okwu, Nneke Ukanwa, 2010*
Flavokawain A	*Alpinia pricei*	*Zingiberaceae*	*Yu feng et. al, 2010*
Flavokawain B	*Alpinia pricei*	*Zingiberaceae*	*Yu feng et. al, 2010*
Flavokawain C	*Alpinia pricei*	*Zingiberaceae*	*Yu feng et. al, 2010*
Fissistin	*Fissistigma lanuginosum*	*Annonceae*	*Alias Y et.al, 1995*
Iso fissistin	*Fissistigma lanuginosum*	*Annonceae*	*Alias Y et.al, 1995*
Pedicin	*Fissistigma lanuginosum*	*Annonceae*	*Alias Y et.al, 1995*
Isobava chalcone	*Angelica keiskei*	*Umbelliferae*	*Toshihiro et.al, 2003*
Xantho gelol	*Angelica keiskei*	*Umbelliferae*	*Toshihiro et.al, 2003*
Xantho gelol B	*Angelica keiskei*	*Umbelliferae*	*Toshihiro et.al, 2003*
Xantho gelol F	*Angelica keiskei*	*Umbelliferae*	*Toshihiro et.al, 2003*
4' hydroxy derricin	*Angelica keiskei*	*Umbelliferae*	*Toshihiro et.al, 2003*
Iso liquiritigenin	*Glycyrrhiza inflata*	*Fabaceae*	*Shoji shibata, 1994*
Lico chalcone A	*Glycyrrhiza inflata*	*Fabaceae*	*Shoji shibata, 1994*
Lico chalcone B	*Glycyrrhiza inflata*	*Fabaceae*	*Shoji shibata, 1994*

2.1.2 Selection of commercially available drugs:

For our studies, the lead compounds or drugs used in the treatment of breast cancer were searched in breastcancer.org. (Table 2)The structures of these compounds were obtained in 2D (*Images 1*) and 3D format (in .sdf format) from the Pubchem website. These were then converted to .pdb format using OpenBabelGUI software.

Table 2: list of commercially available drugs used in this study.

Commercially available drugs
1. Abraxane
2. Docetaxel
3. Halaven
4. Ixempra
5. Ixempra analogue 1
6. Paclitaxel
7. Vincristine
8. Vindesine
9. Vinflunine
10. Vinorelbine

11. Vinorelbine analogue 1
12. Vinorelbine analogue 3
13. Vinorelbine analogue 4
14. Vinorelbine – exelbine

2.1.3 Tools Used

2.1.3.1 Molinspiration:

This online tool offers broad range of cheminformatics solutions which enable molecule manipulation and processing, including SMILES and SD file conversion, molecule fragmentation, normalization of molecules, generation of tautomers, calculation of various molecular properties needed in QSAR, molecular modeling and drug design, high quality molecule depiction, molecular database tools supporting substructure and similarity searches. It works on any computer platform which has Java installed in it. It takes the input in SMILES format.

2.1.3.2 Hex:

It is an interactive protein docking and molecular superposition program. The input is mostly in PDB format for proteins and DNA, but it can also read small molecules in SDF format.

Procedure for Hex Docking

- The structure of all the established drug molecules and their analogues were obtained.
- Open Hex
- File – Open – Receptor – Select the required protein file in .pdb
- File – Open – Receptor – Select the required drug/chalcone molecule file in .pdb
- Controls– Docking – Correlation type – Shape + electrostatistics – Activate
- Save the docking summary
- Table of e-values is created and the molecule with lowest e-value is deemed as most stable.

Docking studies were carried out on both drug and chalcone molecules. The e – values thus obtained for established drugs and chalcone molecules were compared.

2.2 Preparation of plant materials

Four plants viz., *Glycyrrhiza inflata(mulethi); Mimosa pudica(touch me not); Polyalthia longifolia (ashok tree)* and *Bridelia stipularis* were selected based on their ease of availability ..The leaves of Bridelia and the bark of Polyalthia were collected from the BMSCE campus.The roots of Glycyrrhiza were purchased from a local shop.The Mimosa plant was collected from college grounds of Don Bosco Engineering College, Kumbalgodu near Kengeri.The plant materials were cleaned, dried in hot air oven for 24 hours at a temperature below 50^0C. They were ground to a fine powder and extracted by soxhlet using methanol solvent system for 16-18hrs extraction. The extract was further concentrated using rotary evaporator.

2.2.1 Assay of anti-mitotic activity

The effect of the methanolic extracts (5x, 10x, 20x) on mitosis was studied using onion root tips as per the protocol reported by Ozmen *et al*, (2007).The mitotic index was calculated after 48, 96, 120 hour interval. Mitotic index is a measure for the proliferation status of a cell population. It is defined as the ratio between the number of cells in mitosis and the total number of cells. If the Mitotic index=1, it implies normal cell division. Lower values of mitotic index denote possible anti mitotic effects.In addition the root length measurements were taken .Measurement of roots is of critical importance to understand plant growth. Root length is one of the important parameters required to understand plant performance. The lengths of the roots kept in the methanolic extracts were measured over a period of 6-7 days to observe for growth retarding effects. The lengths of these roots were compared with the lengths of the onion roots which were placed in water as control.

2.2.2 Anti-microbial activity

Assay of anti -bacterial & antifungal activity of the methanolic extracts of the four plant materials was done by disc-diffusion method .The extracts were tested against Gram +ve bacteria (*Staphlyococcus aureus)* ,Gram -ve bacteria *(E.coli*) and fungal cultures of *Fusarium.* The diameter of the zone of inhibition beyond the disc was recorded. The experiment was carried out in triplicate and the mean of the diameter of the inhibition zones is reported.

2.2.3 LC – MS Profiling

LC MS of themethanolic extracts was carried out.A gradient solvent system was used with 40 – 80% methanol and methanol (10% water, 10% acetonitrile). Injection volume was set to 50µl.

III. Results:

3.1 In silico studies:

3.1.1 Physicochemical properties

The physicochemical properties of chalcones analysed on the Molinspiration software are shown in the table 3 . The software predicts the bioavailability and bio absorption of the chalcones selected. From table 3, it can be observed that all of the chalcones has molecular weight (MW) under 500 kDa which is necessary for easier absorption/diffusion/transportation across membrane.

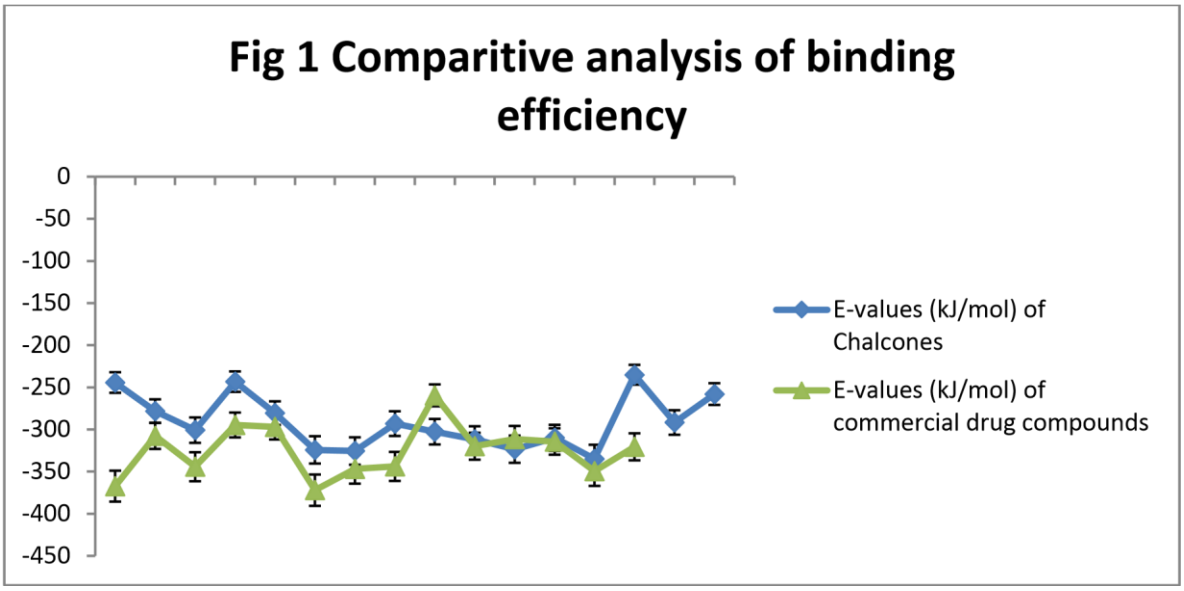
We can also infer that most of the chalcones does not violate the "Lipinski' rule of five" except for Fissistin, Isofissistin, Xanthogelol, Xanthogelol B and Xanthogelol F. This is because miLogP of these compounds, that provides molecular hydrophobicity or lipophilicity of the compound and in turn good permeability across cell membrane, should have been less than 5. Topological polar surface area (TPSA) that is a measure of oral bioavailability is also well within the requirement. Number of hydrogen bond acceptors (n-ON) and number of hydrogen bond donors (n-OHNH) of all the compounds are less than 10 and 5 respectively, fulfilling Lipinski's rule of five. The results indicate that chalcones are commendable drug candidates that satisfy most of the parameters of drug likeliness.

Table 3: Physico Chemical Properties of Chalcone

Name	mi log p	TPSA	N atoms	MW	n ON	n OHNH	n Violations	n rotb	Volume
2,4 dihydroxychalcone	2.91	60.68	18	242.27	3	3	0	3	223.75
4 Hydroxyderricin	4.88	66.76	25	338.4	4	2	0	6	320.77
Fissistin	7.61	85.23	34	466.57	6	2	1	9	445.08
flavochalcone A	3.83	65	23	314.34	5	1	0	6	286.51
flavochalcone B	3.77	55.77	21	284.31	4	1	0	5	260.96
flavochalcone C	3.29	76	22	300.31	5	2	0	5	268.98
Hydroxyderricin	4.88	66.76	25	338.4	4	2	0	6	320.77
Isofissistin	6.66	85.23	34	466.57	6	2	1	9	445.08
lycochalcone A	4.85	66.76	25	338.4	4	2	0	6	320.79
Liriobenine	3.3	48.43	21	275.26	4	0	0	0	227.36
Obava Chalcone	4.81	77.75	24	324.38	4	3	0	5	303.25
Pedicin	3.25	85.23	24	330.34	6	2	0	6	294.52
Xanthogelol	6.66	77.75	29	392.5	4	3	1	8	380.83
Xanthogelol B	5.29	97.98	30	408.49	5	4	1	9	389.43
Xanthogelol F	6.73	66.76	30	406.52	4	2	1	9	398.36

3.1.2 Docking studies

The results of docking of various commercially available drugs and natural chalcones with tubulin in Hex is given with respect to their binding energies (Fig. 1). The lower the binding energies greater is their binding affinities which indicates greater interaction. There is no significant differences in binding energies of the chalcones and the commercial drugs. The results indicate that Paclitaxel (**-372.04** kJ/**mol)** gave the lowest free energy value. Of the natural chalcones 4' hyroxyderricin isolated from the plant *Angelica keiskei* showed binding energy of **(-334.97 kJ/mol).**



3.2 Assay of anti-mitotic activity

3.2.1 Mitotic index

The mitotic index of the onion root tips incubated with the methanolic extracts was measured at 48, 96, 120 hour intervals. It was calculated using the formula given below.

Calculation: Mitotic index = I +P + M + A + T/ Total cells

I –Interphase cells;P- Prophase cells;A-Anaphase cells;M-Metaphase cells;T-Telophase cells.

The results are shown in table 4 below.

Table 4: The mitotic index of the onion roots kept in the crude methanolic extracts

Plant species	48 hours	96 hours	120 hours	Remarks
Control	1	.98	.97	*Abnormality in cells was seen
Bridelia 10x	.956	.38	.13	
Bridelia 5x	~1	.82	.706	
Polyalthia 10x	.978	.61	.38	
Polyalthia 5x	~1	0.3	.101	
*Mimosa 5x**	~1	.636	.110	

It is be observed that the mitotic index decreases with increasing concentrations of the extracts.

Further, abnormalities in cell structure was observed in the root tips immersed in methanolic extracts of Mimosa and *Glycyrrhiza (Fig 2a-c).* Hence mitotic index couldn't be measured. The abnormalities seen in Fig2. (a&b) shows elongated cells and indicate the presence of certain compounds in the plant extracts which are affecting the cells. . However, further analysis needs to be carried out, where in the specific compounds and their effects need to be determined.

Fig 2a Abnormal cells observed under microscope with methanolic extracts of Mimosa

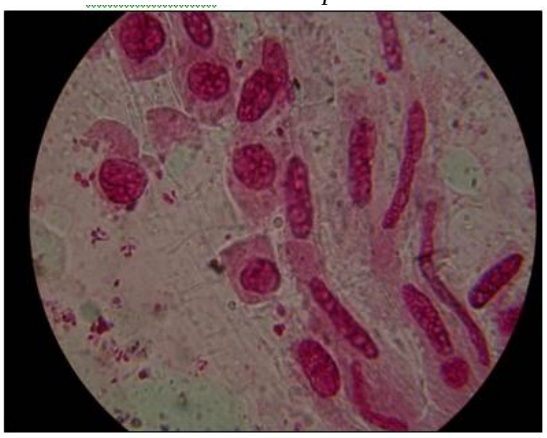


Fig 2b Abnormal cells observed under microscope with methanolic extracts of Glycyrrhiza

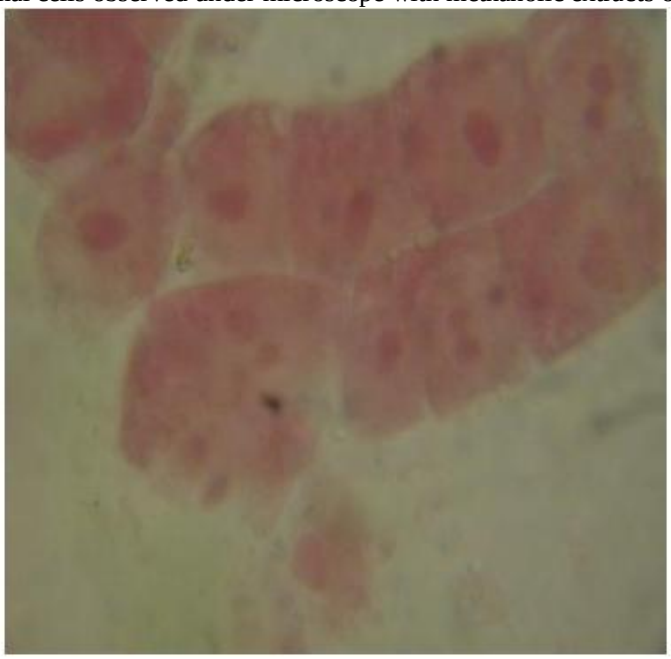
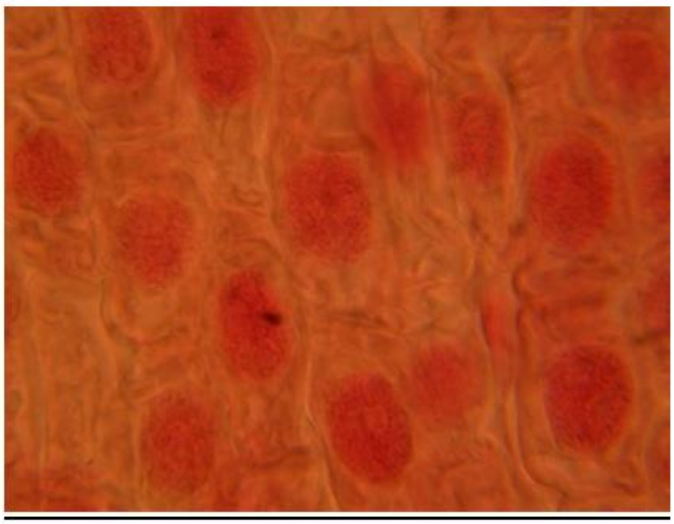


Fig 2c normal cells observed under microscope with control



3.2.2 Measurement of length

The lengths of the roots kept in the methanolic extracts were measured over a period of 6-7 days to observe rate of growth/elongation of the roots.

The average length for all the plant extracts was calculated as follows:

Plant extract	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
Control	5.3	5.6	5.7	5.9	6.0	6.1	6.1	6.4	6.7
Bridelia	5.55	5.55	5.55	5.5	5.5	5.5	5.5	5.5	5.5
Polyalthia	4.45	4.45	4.45	4.45	4.45	4.45	4.45	4.5	4.5
Glychrrhiza	7.7	7.7	7.7	7.7	7.7	7.7	7.7	7.7	7.7
Mimosa	8.83	9.1	9.16	9.16	9.16	9.16	9.16	9.16	9.16

The table shows that except for the *Mimosa* extract all the other extracts did not show any growth in the root length resulting from the inhibitory effects of the extract.

3.3 Anti-bacterial activity

Assay of anti -bacterial & antifungal activity of the methanolic extracts of the four plant materials was done by disc-diffusion method .The extracts were tested against Gram +ve bacteria (*Staphlyococcus aureus)* ,Gram -ve bacteria *(E.coli*) and fungal cultures of *Fusarium.* The diameter of the zone of inhibition beyond the disc was recorded. The results are shown in Table 4.

Table 4: Antimicrobial and anti fungal activity of the extracts based on zone of inhibition obtained

Extract	Diameter of zone (cm)		
Control (all plates)	No zone – culture growth all over		
	Gm+ve	Gm-ve	Fungal
Bridelia	1.17	1	1.1 cm
Polyalthia	2.15	No zone	No zone
Mimosa	No zone	No zone	No zone
Glychrrhiza	1.65	No zone	No zone

It is observed that only the methanolic extract of *Bridelia* showed inhibitory activity with both gram positive and gram negative bacteria and fungi. Whereas the extract of Mimosa showed no inhibitory activity .

3.4 LC-MS

LC-MS was carried out and ESI-TIC scan, multi fragment scan was taken.

Sample 1: *Bridelia*

6 distinct peaks were obtained in the TIC scan for a time range of 19 minutes.

The individual peaks obtained in the TIC were further analysed by multi fragment mm scan and a scan of the counts vs mass to charge ratio was obtained. m/z ratio of 283.2 and 305.2 were obtained from peak 1, m/z ratio of 305.2 were obtained from peak 2, m/z ratio of 353.2 were obtained from peak 3, m/z ratio of 303.1 were obtained from peak 4.

Sample 2: *Polyalthia*

5 distinct peaks were obtained in the TIC scan for a time range of 11 minutes.

The individual peaks obtained in the TIC were further analysed by multi fragment mm scan and a scan of the counts vs mass to charge ratio was obtained. m/z ratio of 341.1 were obtained from peak 3, m/z ratio of 261.1 were obtained from peak 2.

Sample 3: *Glychrrhiza*

7 distinct peaks were obtained in the TIC scan for a time range of 19 minutes.

The individual peaks obtained in the TIC were further analysed by multi fragment mm scan and a scan of the counts vs mass to charge ratio was obtained. m/z ratio of 325.1 were obtained from peak 1, m/z ratio of 325.1 were obtained from peak 2, m/z ratio of 325.1 were obtained from peak 3, m/z ratio of 249.1 and 317.1 were obtained from peak 4.

Sample 4: *Mimosa*

5 distinct peaks were obtained in the TIC scan for a time range of 13 minutes.

The individual peaks obtained in the TIC were further analysed by multi fragment mm scan and a scan of the counts vs mass to charge ratio was obtained. m/z ratio of 202.1 was obtained from peak 1

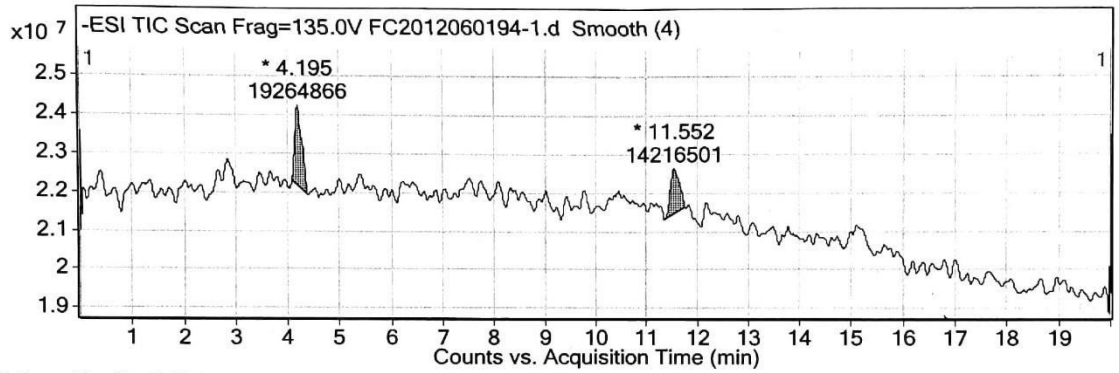
Since the m/z values fall in the range of chalcones (*Matsuda F. et al, 2009*), it can be inferred that these unknown compounds are chalcones. Maximum concentration of these compounds can be seen in *Bridelia* and *Polyalthia* extract. However, further confirmation can be obtained by NMR studies.

LC-MS results:

Sample 1: *Bridelia*

User Chromatograms

Fragmentor Voltage 135 **Collision Energy** 0 **Ionization Mode** ESI

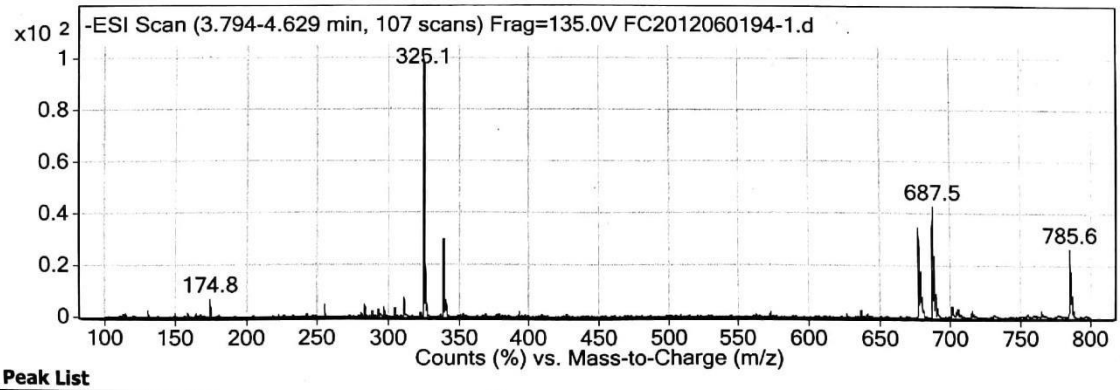


Integration Peak List

Peak	Start	RT	End	Height	Area	Area %
1	4.086	4.195	4.384	2071569	19264866	100
2	11.355	11.552	11.764	1170169	14216501	73.79

User Spectra

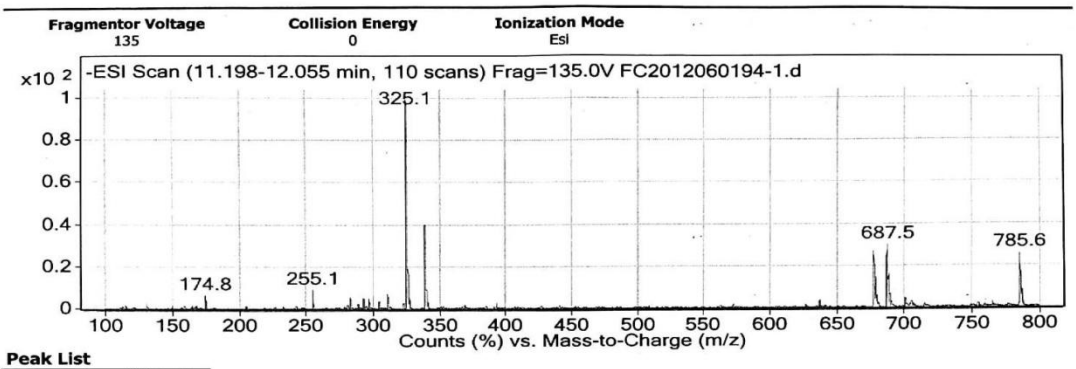
Fragmentor Voltage 135 **Collision Energy** 0 **Ionization Mode** Esi



Peak List

m/z	z	Abund
325.1	1	509306
326.1	1	107166
339.1	1	152400
677.5	1	177001
678.5	1	94303
679.5	1	91944

m/z	z	Abund
687.5	1	219125
688.6	1	120039
785.6	1	134662
786.6	1	90601



Peak List

m/z	z	Abund
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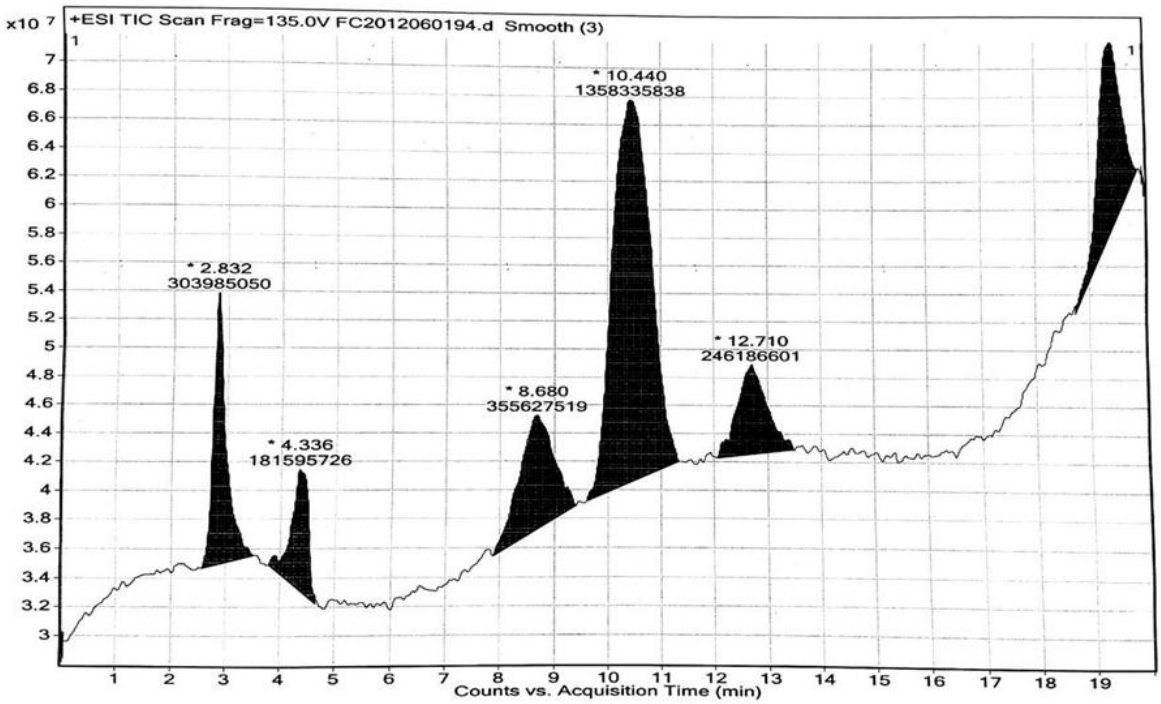
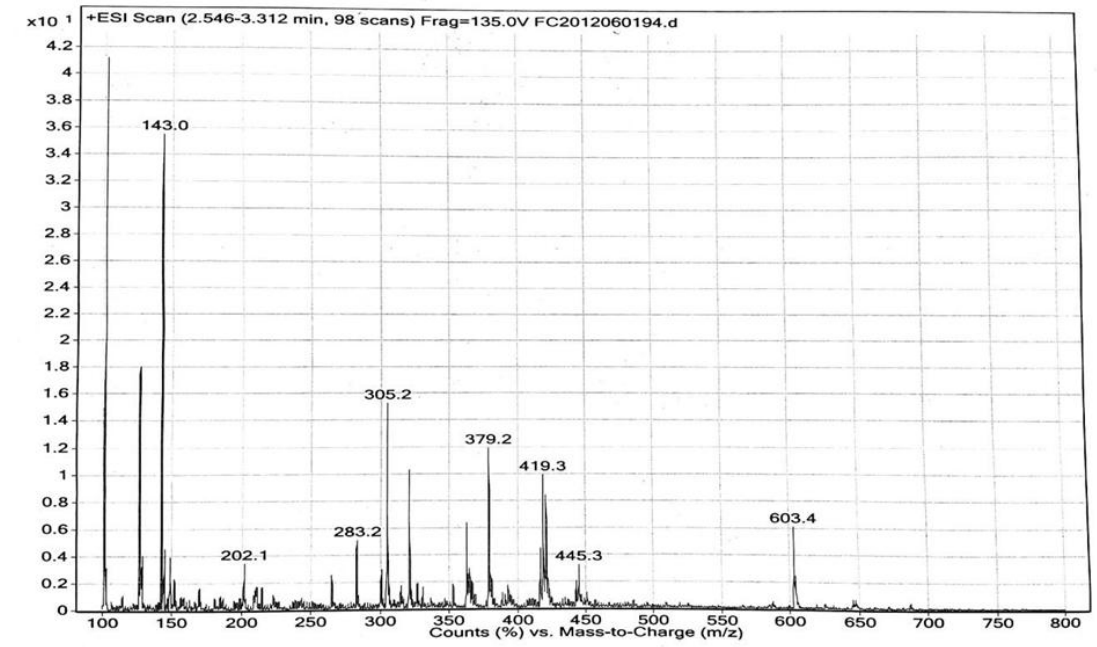
326.1	1	103586
339.1	1	203546
677.5	1	136788
678.5	1	71735
679.5	1	73133
687.5	1	153391
688.5	1	82754
785.6	1	132044
786.6	1	86280

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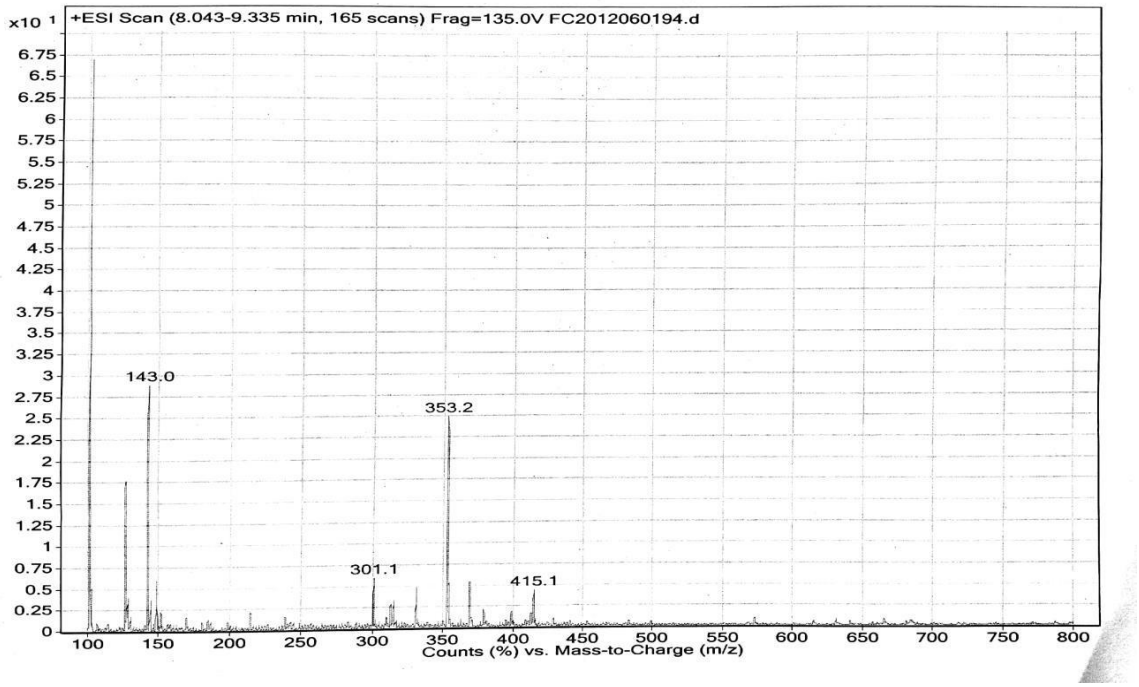
379.2
419.3
603.4
283.2
202.1
445.3
Counts (%) vs. Mass-to-Charge (m/z)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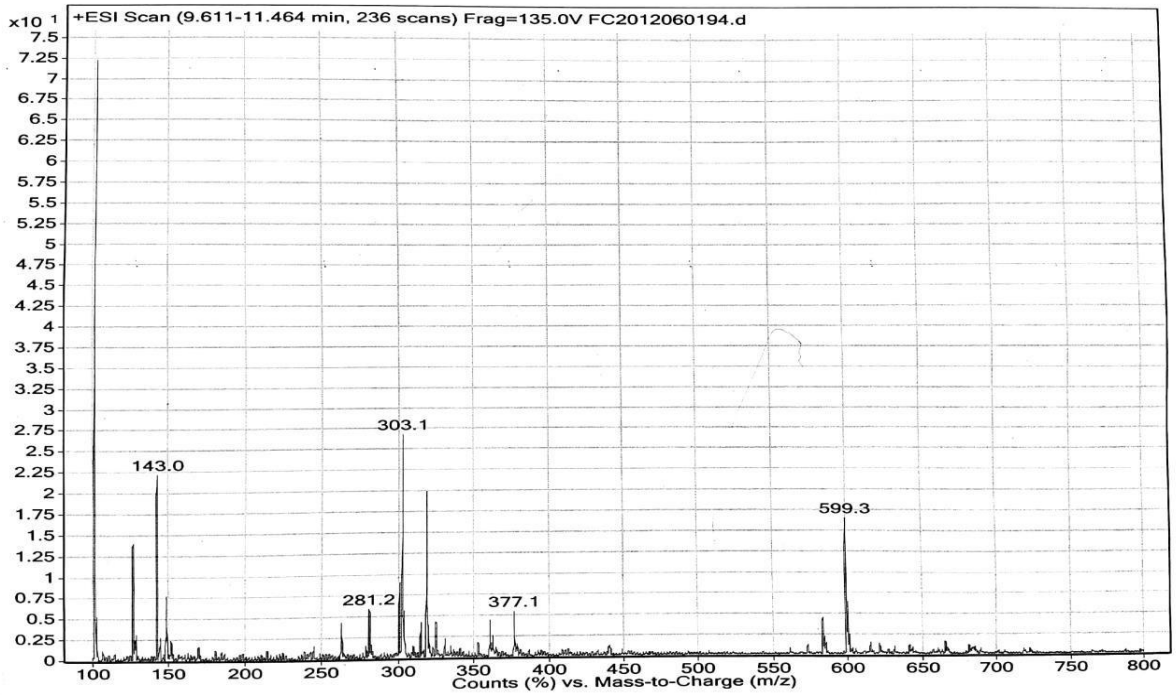
301.1
415.1
Counts (%) vs. Mass-to-Charge (m/z)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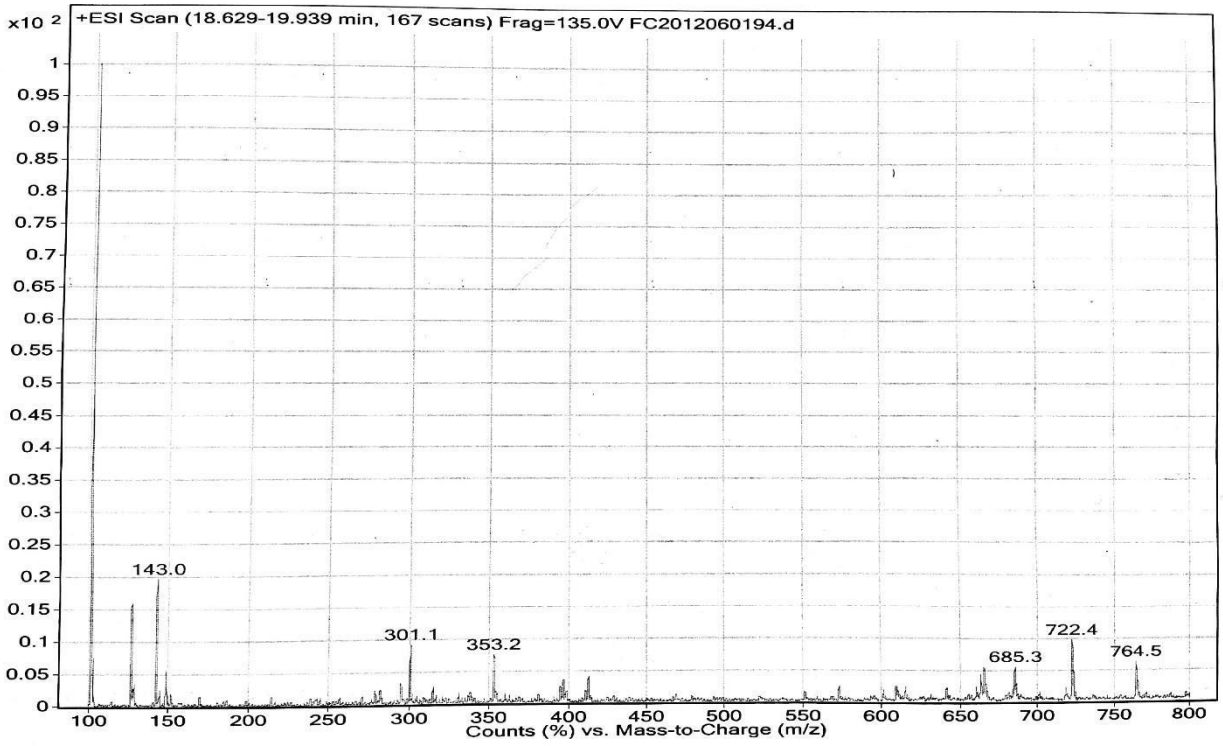
599.3
281.2
377.1
Counts (%) vs. Mass-to-Charge (m/z)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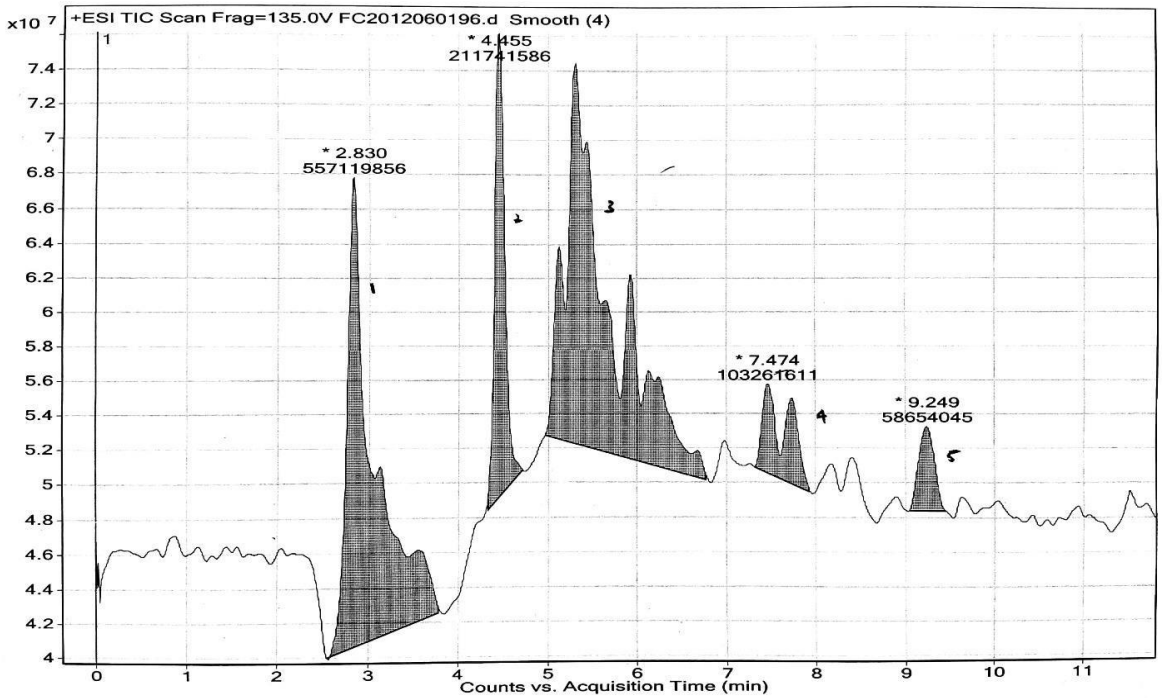
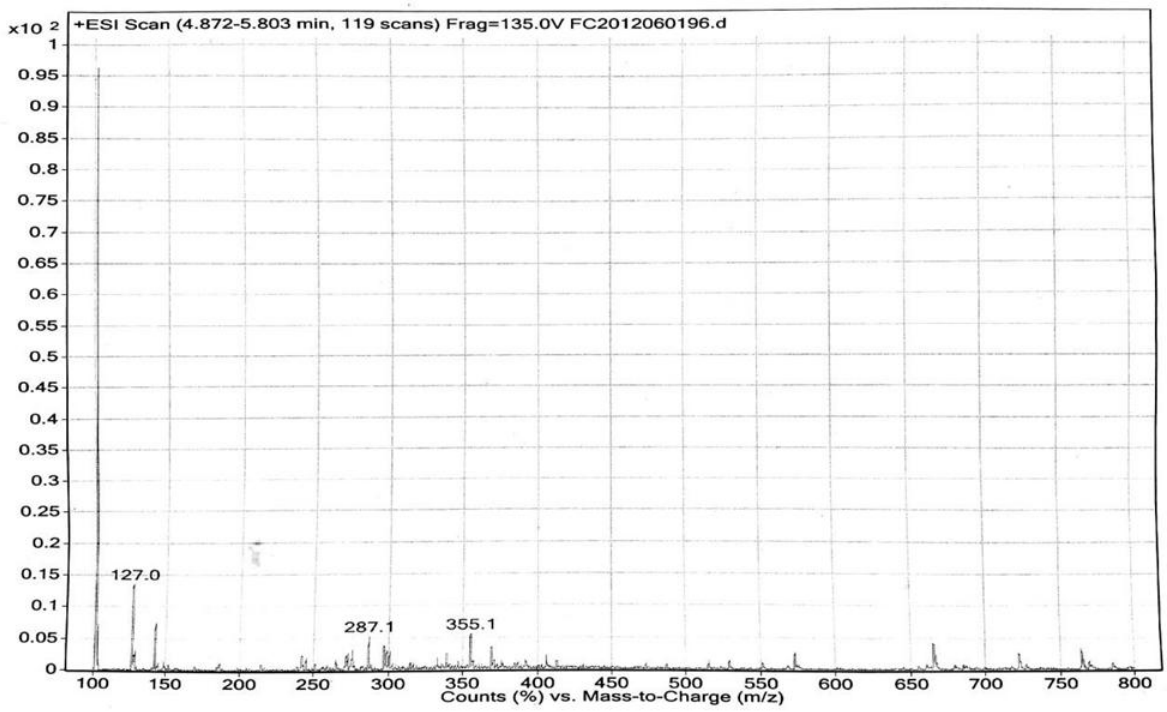
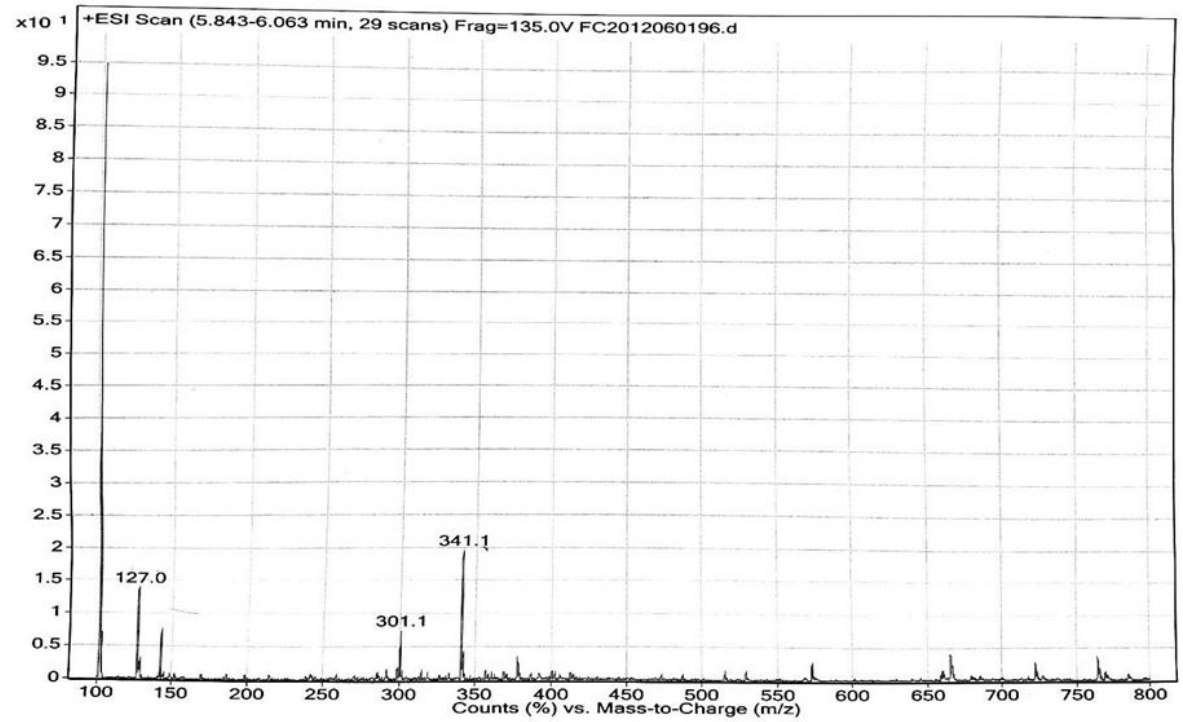
301.1
353.2
722.4
685.3
764.5
Counts (%) vs. Mass-to-Charge (m/z)

Sample 2: *Polyalthia*



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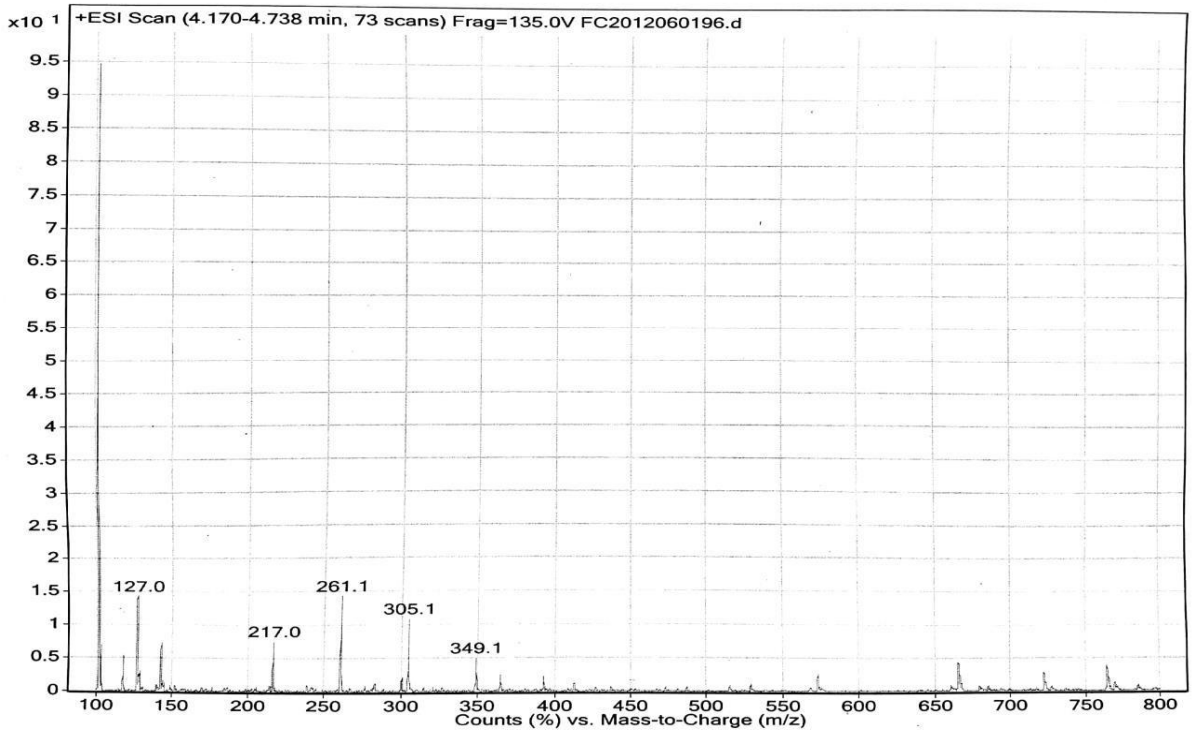
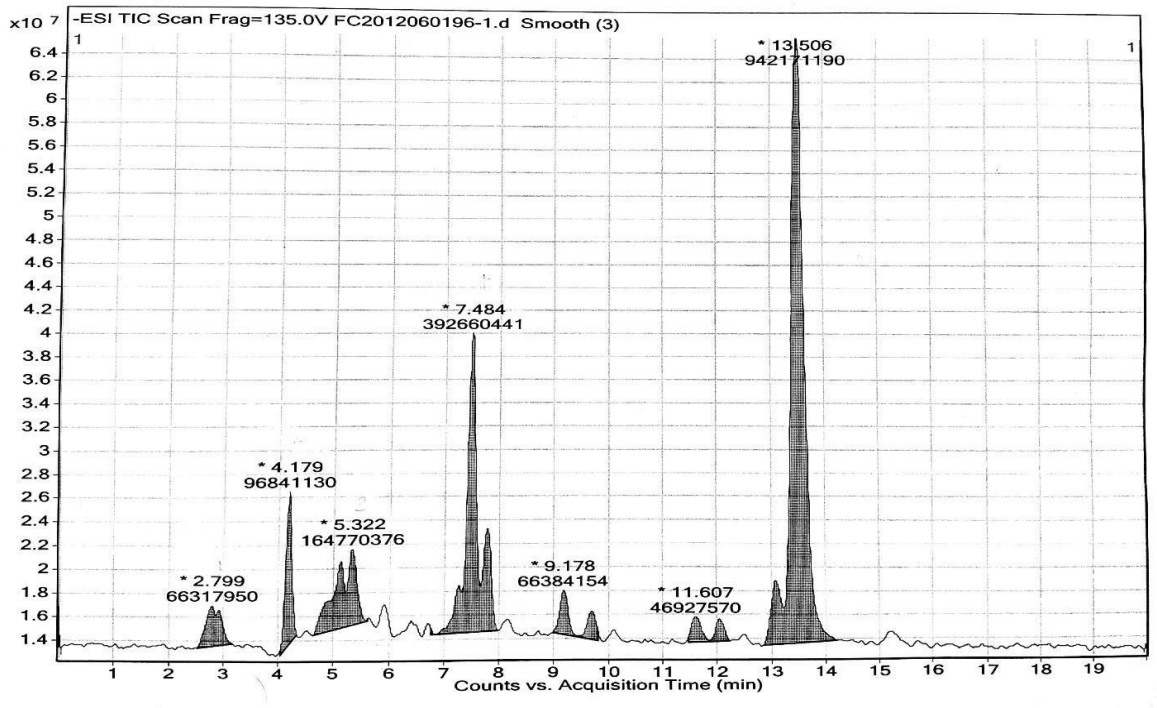
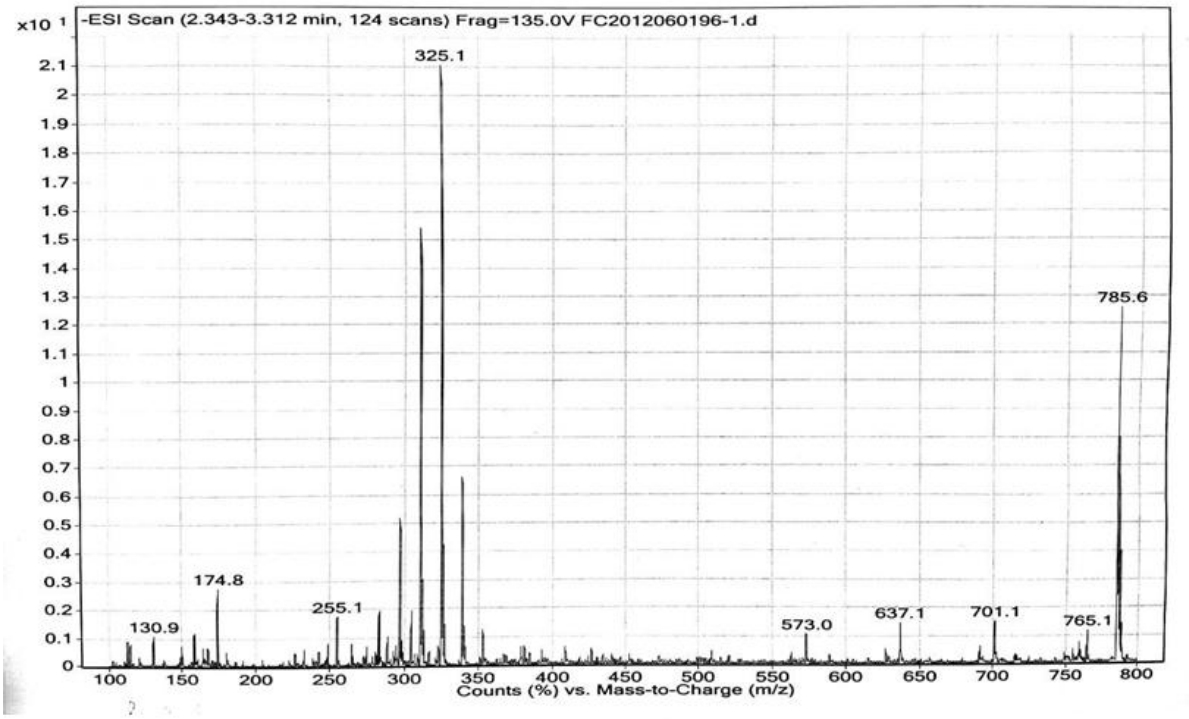
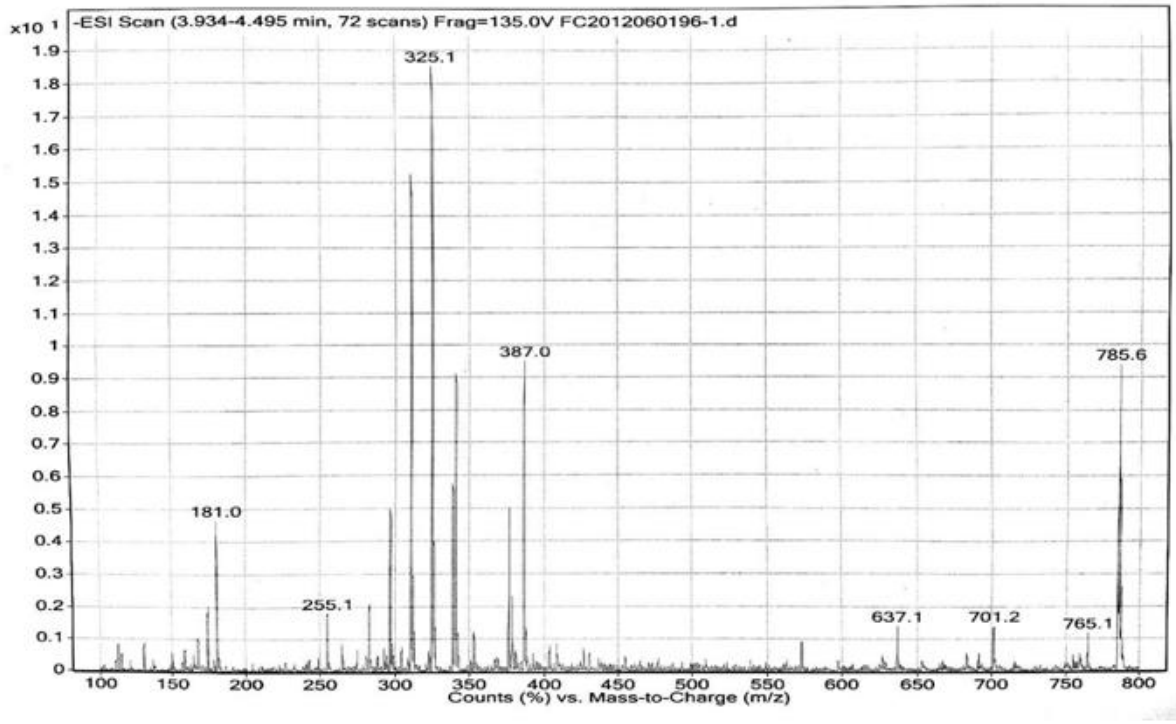
341.1
127.0
301.1
Counts (%) vs. Mass-to-Charge (m/z)

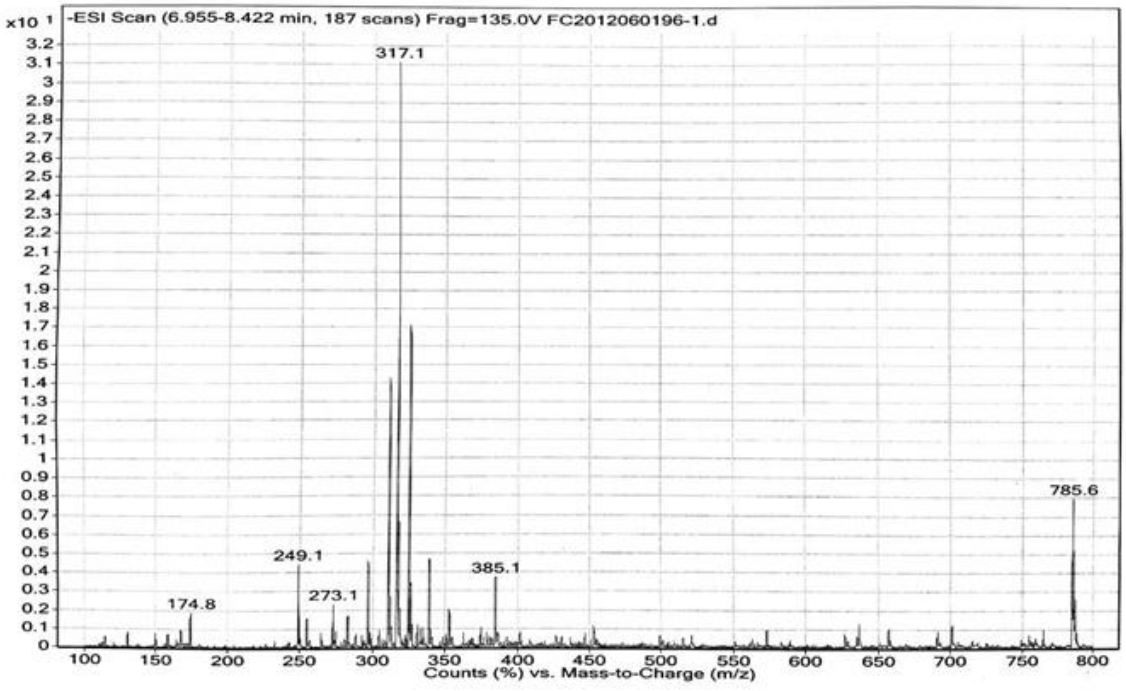

x10 1
+ESI Scan (4.170-4.738 min, 73 scans) Frag=135.0V FC2012060196.d
127.0
261.1
305.1
217.0
349.1
Counts (%) vs. Mass-to-Charge (m/z)

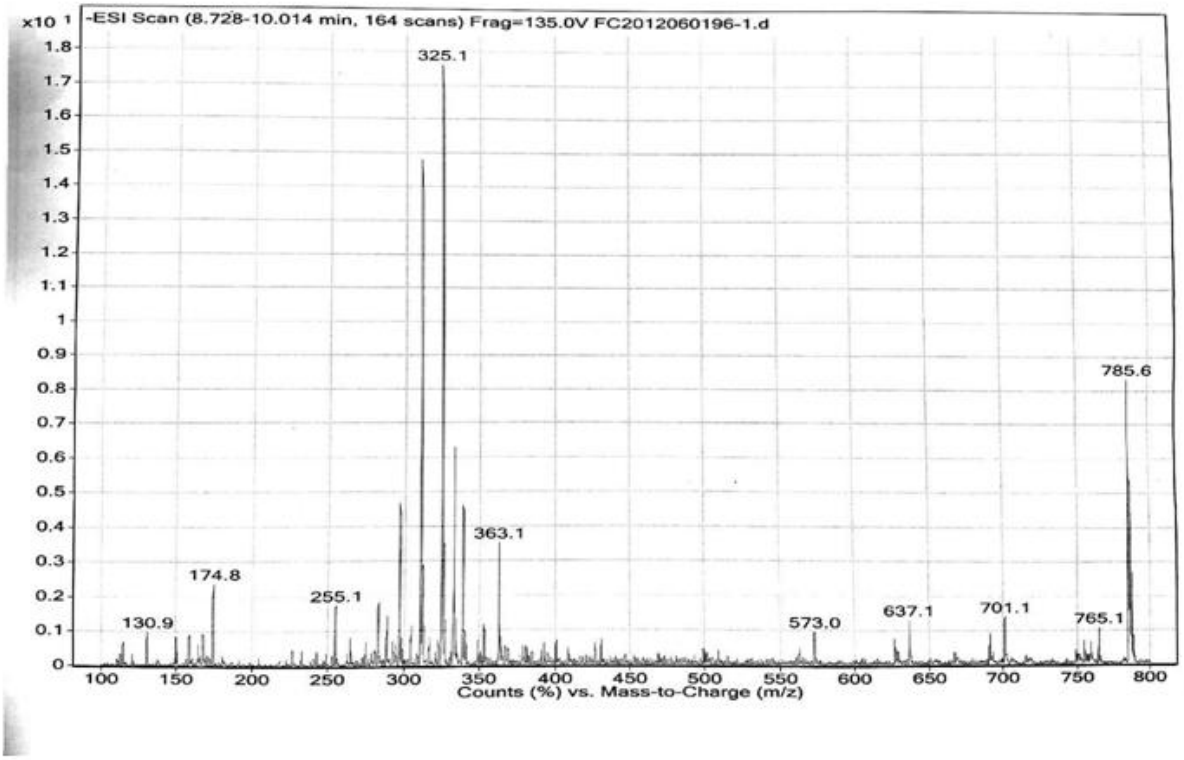
Sample 3 *Glycyrrhiza*

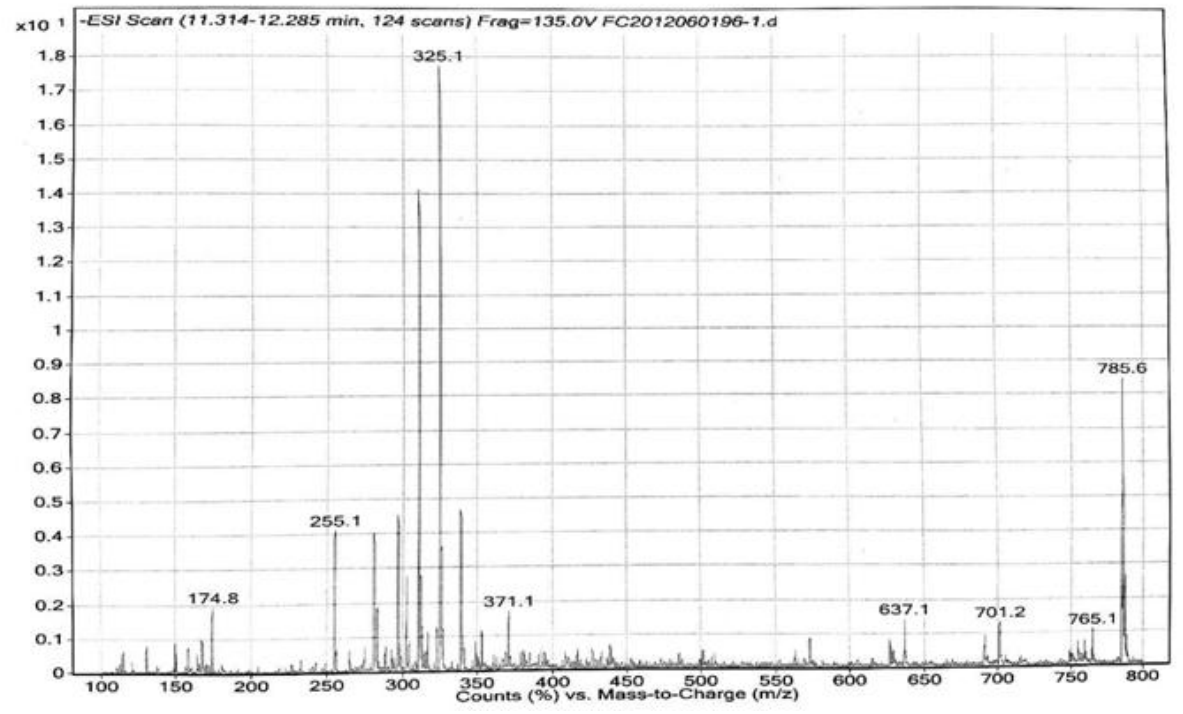


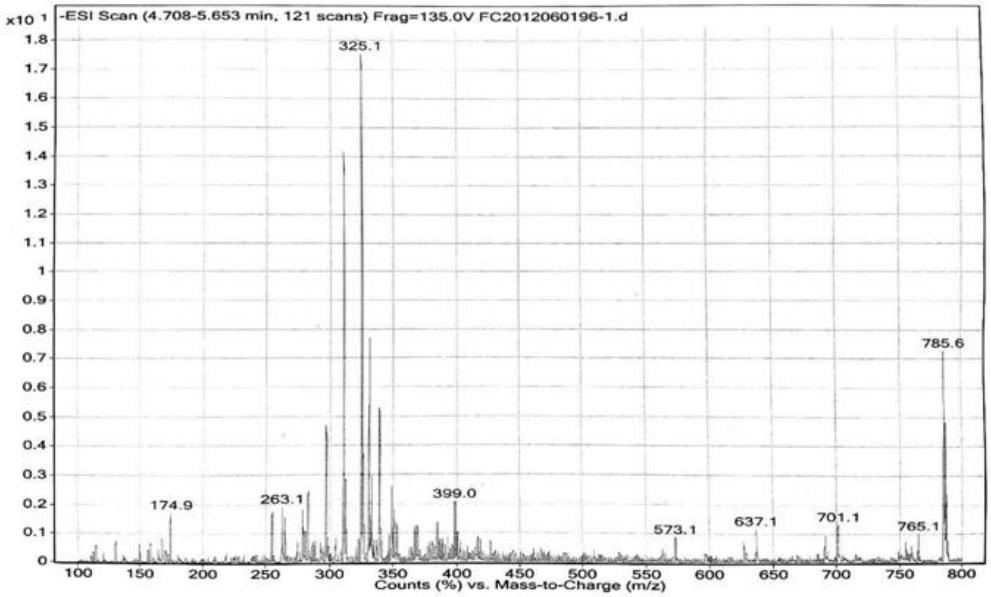



-ESI Scan (3.934-4.495 min, 72 scans) Frag=135.0V FC2012060196-1.d
x10 1
325.1
387.0
785.6
181.0
255.1
637.1
701.2
765.1
Counts (%) vs. Mass-to-Charge (m/z)


-ESI Scan (6.955-8.422 min, 187 scans) Frag=135.0V FC2012060196-1.d
x10 1
317.1
785.6
249.1
385.1
273.1
174.8
Counts (%) vs. Mass-to-Charge (m/z)


x10 1
-ESI Scan (8.728-10.014 min, 164 scans) Frag=135.0V FC2012060196-1.d
325.1
785.6
363.1
174.8
255.1
130.9
573.0
637.1
701.1
765.1
Counts (%) vs. Mass-to-Charge (m/z)

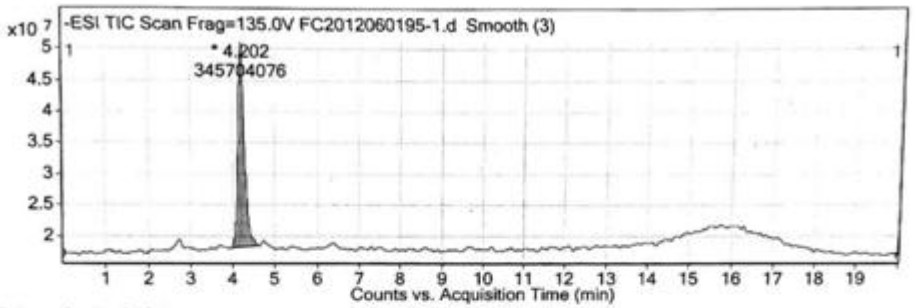

x10 1
-ESI Scan (11.314-12.285 min, 124 scans) Frag=135.0V FC2012060196-1.d
325.1
785.6
255.1
174.8
371.1
637.1
701.2
765.1
Counts (%) vs. Mass-to-Charge (m/z)



Sample 4: *Mimosa*

User Chromatograms

Fragmentor Voltage 135 **Collision Energy** 0 **Ionization Mode** ESI

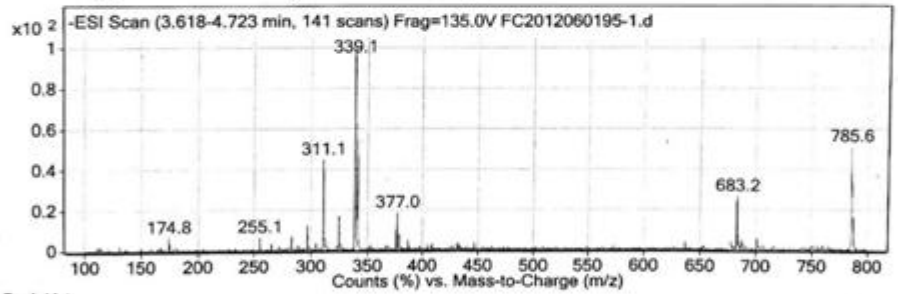


Integration Peak List

Peak	Start	RT	End	Height	Area	Area %
1	3.973	4.202	4.589	32912546	345704076	100

User Spectra

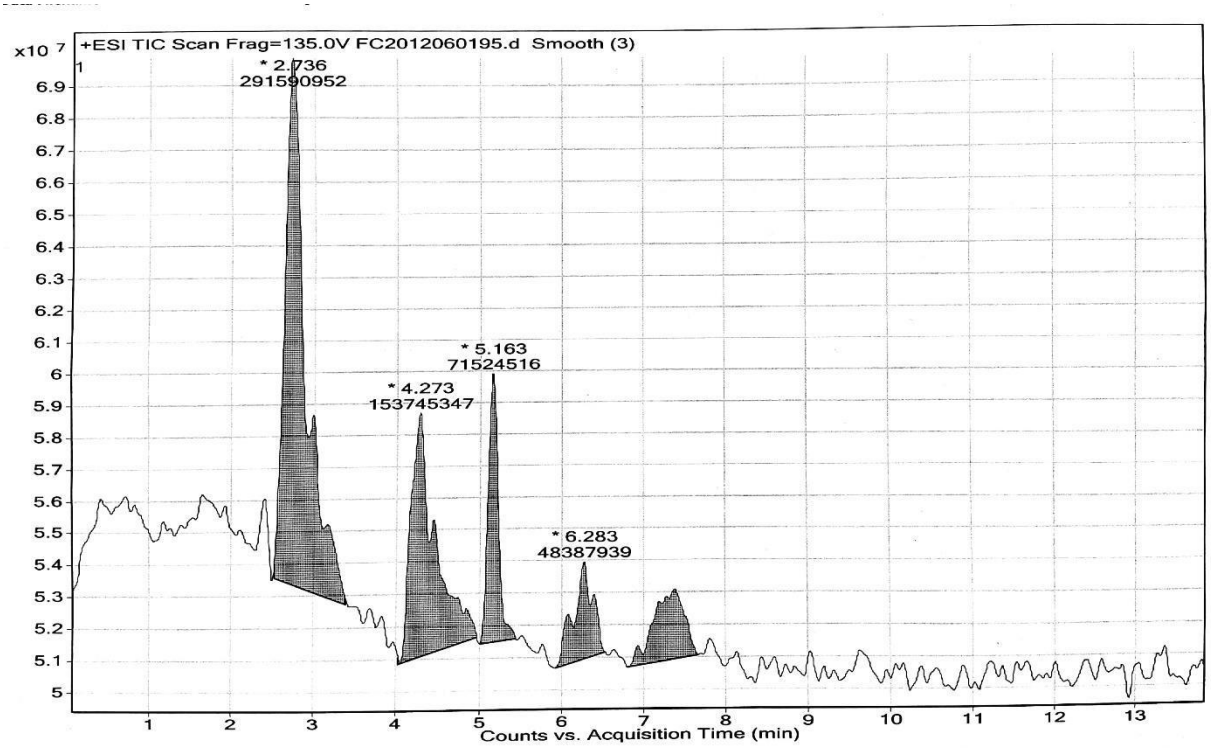
Fragmentor Voltage 135 **Collision Energy** 0 **Ionization Mode** Esi

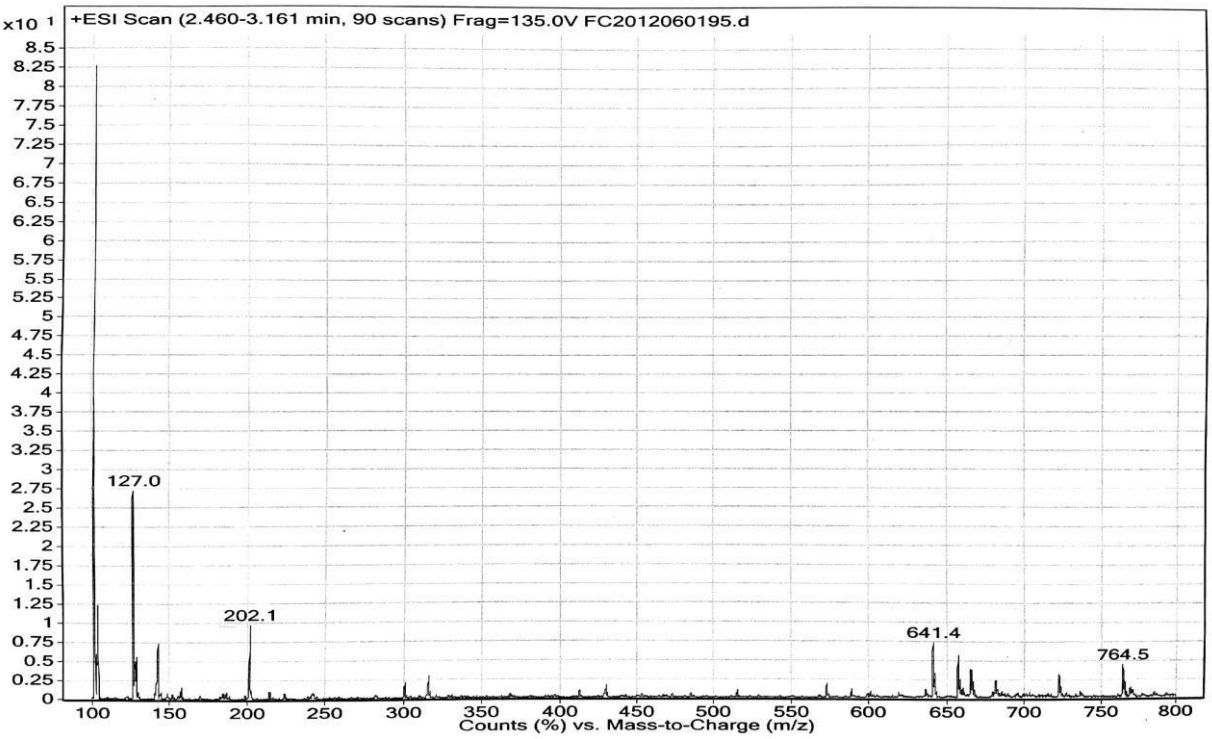


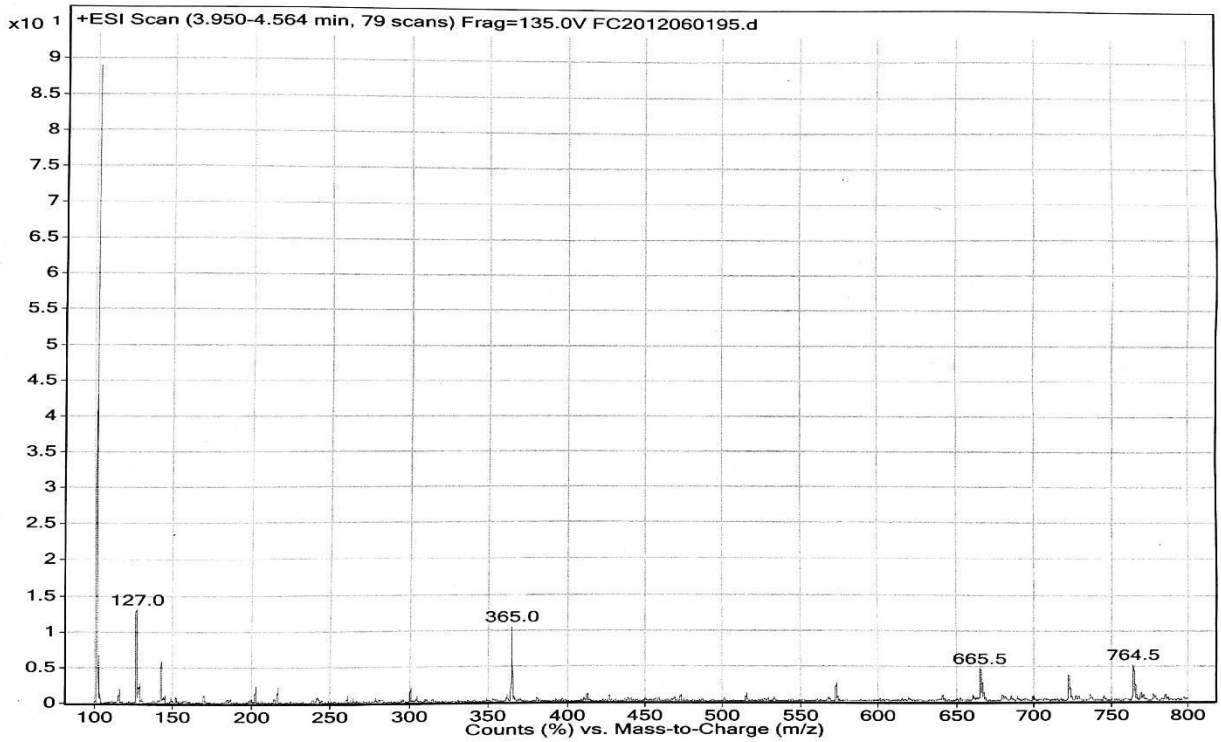
Peak List

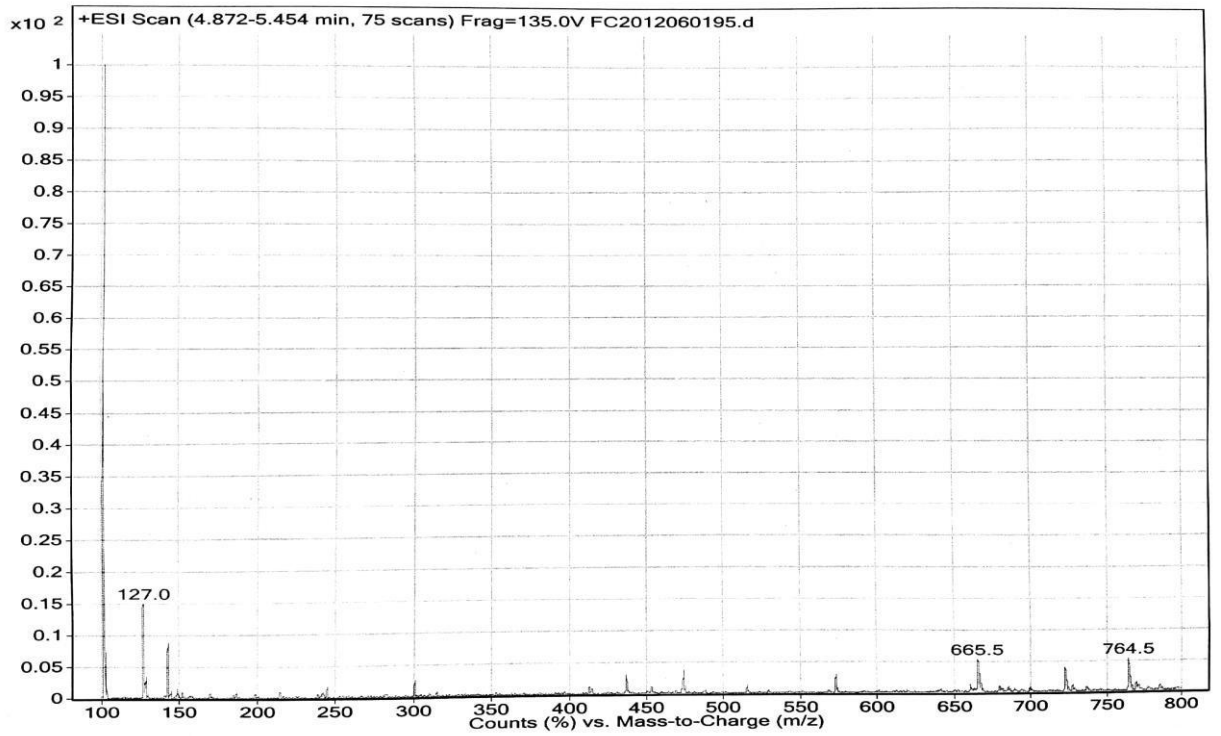
m/z	z	Abund
311.1	1	177317
325.1		66692
339.1	1	395742
340.1	1	85713
341	1	248876
377		71782
683.2	1	105745

m/z	z	Abund
785.6	1	203807
786.6	1	133413
787.6	1	63475


+ESI TIC Scan Frag=135.0V FC2012060195.d Smooth (3)
x10 7
* 2.736
291590952
* 5.163
71524516
* 4.273
153745347
* 6.283
48387939
Counts vs. Acquisition Time (min)


+ESI Scan (2.460-3.161 min, 90 scans) Frag=135.0V FC2012060195.d
x10 1
127.0
202.1
641.4
764.5
Counts (%) vs. Mass-to-Charge (m/z)





IV. Conclusion:

The insilico studies have shown that the chalcones have a binding energy similar to that of established drugs. The compounds such as 4'-hydroxyderricin, isofissistin and fissistin can be isolated from the natural sources (*Toshihiro et.al, 2003*) (*Alias Y et.al, 1995*). The methanolic extracts *Bridelia* and *Polyalthia* showed good antimitotic activity and can be considered for further further studies.

Chalcones are present in various parts of plants and can be extracted in its pure form, from these plants. Chemically synthesized commercially available drugs come with a heavy price tag which can be checked by

these naturally occurring compounds. Since chalcones are natural compounds possibility of side effects, which can occur as result of chemically synthesized drugs, could be reduced. Thus we can conclude that chalcones can be harnessed as natural potent alternate therapeutic drugs against cancer.

5. Declarations of interest: none

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