Advances in Pharmacology and Pharmacy 13(3): 305-322, 2025 http://www.hrpub.org
DOI: 10.13189/app.2025.130303

Evaluation of Anticancer Activity of *Eupatorium adenophorum* Leaves Extracts via MTT Assay and Further Characterization of Chemical Constituents Using Liquid Chromatography Mass Spectroscopy (LCMS)

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Received April 10, 2024; Revised September 26, 2024; Accepted March 23, 2025

Cite This Paper in the Following Citation Styles

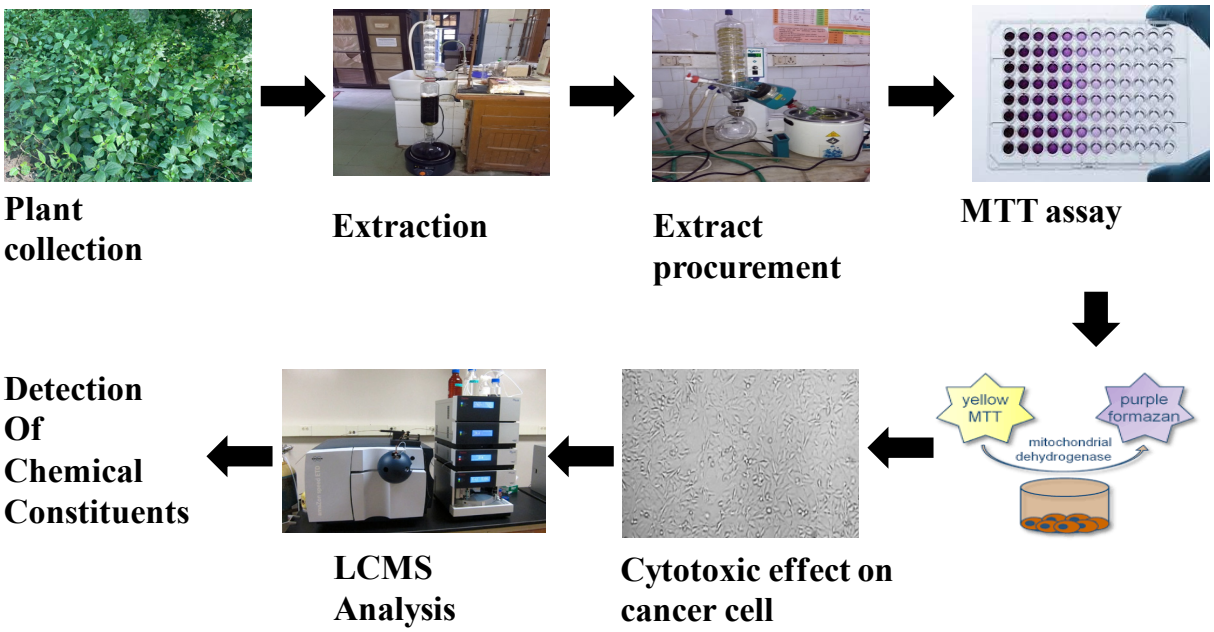
(a): *[1] Shilpa Rana, Uddipak Rai, Vineet Kumar, Mansi Butola , "Evaluation of Anticancer Activity of Eupatorium adenophorum Leaves Extracts via MTT Assay and Further Characterization of chemical Constituents Using Liquid Chromatography Mass Spectroscopy (LCMS)," Advances in Pharmacology and Pharmacy, Vol. 13, No. 3, pp. 305 - 322, 2025. DOI: 10.13189/app.2025.130303.*

(b): *Shilpa Rana, Uddipak Rai, Vineet Kumar, Mansi Butola (2025). Evaluation of Anticancer Activity of Eupatorium adenophorum Leaves Extracts via MTT Assay and Further Characterization of chemical Constituents Using Liquid Chromatography Mass Spectroscopy (LCMS). Advances in Pharmacology and Pharmacy, 13(3), 305 - 322. DOI: 10.13189/app.2025.130303.*

Abstract **Background:** Globally, breast cancer is the most common malignancy in women and the second most common cause of cancer-related death among women. There is therefore a need to identify more efficacious therapies for this neoplasm. Therapeutic treatment of liver cancer has been also a great challenge. Use of herbal drugs in treatment of cancer has been a great choice for the researcher. *Eupatorium adenophorum* also known as *Ageratina adenophora* belongs to the family Asteraceae which is a new plant found with anti-cancer properties. **Methodology:** The successive extraction of the leaves was carried out using Petroleum ether (PET), Chloroform ($CHCl_3$) and Methanol (MeOH). The three extracts obtained were carried out for anti-cancer activity via 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay on breast cancer cell lines (MDA-MB-231) and liver cancer cell lines (HepG2). **Result:** Best anti-cancer activity was found in PET and MeOH extract in HepG2 and MDA-MB-231 cell lines respectively. Further characterization was carried out via Liquid Chromatography-Mass Spectroscopy (LCMS) in order to identify the chemical constituents responsible for anti-cancer activity. **Conclusion:** The LCMS data confirmed the presence of chemicals from polyphenol and flavonoid and fattyacid category in MeOH and PET extracts which could be responsible for inhibiting the viable cancer cells. This study indicates that MeOH and PET have better anti-cancer activity. So *Eupatorium adenophorum* can be the greater choice for the researcher in the treatment of cancer.

Keywords *Eupatorium adenophorum*, Extraction, Cancer, MTT, LCMS, MDA MB-231, HepG2

Graphical Abstract



1. Introduction

Cancer is a pathological condition characterized by abnormal proliferation of cells that has the potential to metastasize to different parts of the body. It is caused by genetic or epigenetic alterations in somatic cells. They constitute a cluster of neoplasms. A "tumor" is a collection of cells that proliferate uncontrollably, forming a lump or mass that can also spread extensively [1]. In 91 of the 172 countries on the planet, cancer is either the top or second-leading cause of death before the age of 70[2]. Globally, 9.6 million people died from cancer in 2018, according to GLOBOCAN. By 2040, it is projected that there will be around 29.5 million new cases of cancer and 16.3 million deaths caused by cancer [3]. Fast urbanization, increase in pollution and inactive lifestyle are some of the factors, contributing to the cancer burden and this affects the low to middle income countries like India [5]. According to the Global Burden of Disease (GBD) 2016 report, cancer was the only disease responsible for 5% of disability-adjusted life years (DALYs) and 8.3% of fatalities [4]. More than 0.7 million cancer deaths and 1.1 million new cases of cancer were both reported by GLOBOCAN 2018. More than 1 million people will die from cancer and over 2 million additional cases are anticipated in 2040 [6].

Worldwide, breast cancer is one of the most commonly diagnosed form of cancer and the primary cause of cancer-related mortality among women [7]. Finding efficient treatments with little adverse effects is urgently needed to lessen this load. A growing body of research indicates that chemicals produced from plants may be very effective in treating a variety of malignancies. Interestingly, several phytochemicals have started to show promise in treating breast cancer in clinical trials. For instance, the herbal extract from *Scutellaria barbata* (NCT00028977) has successfully completed phase II clinical trials for metastatic breast cancer, and the herbal preparation from *Blue Citrus* (NCT00702858) is presently undergoing clinical trials for estrogen receptor positive (ER+) post-menopausal breast cancer [8]. One of the deadliest types of cancer, liver cancer has a high mortality rate globally. The most frequent form of primary liver cancer is hepatocellular carcinoma (HCC), with cholangiocarcinoma making up the remainder. A bad prognosis is still established after regular treatment, with or without surgical resection [9]. Despite the wide range of available cancer treatments, each one is associated with specific detrimental effects. Chemotherapy is the prevailing and highly effective treatment for the majority of malignancies. [10]. Formerly, it was believed that chemotherapy treatments solely worked to kill cancer cells, but it is now well known that they also harm healthy cells, causing side effects that depend on the dosage of the drug, such as exhaustion, nausea, hair loss, vomiting, and even death in extreme situations. Chemotherapy medications primarily employ the strategy of specifically targeting tumor cells, primarily through genotoxicity, which is partially done by the production of reactive oxygen species [11], and which does not only harm the cancer cells but also healthy cells [12]. The US Food and Drug Administration has approved a total of 132 cancer chemotherapeutic medicines, 56 of which have been linked to oxidative stress [13]. Several chemo protectants, including glutathione, amifostine, mesna, and dexrazoxane, have been shown to protect specific tissue types in a drug-dependent manner. Nevertheless, overall

survival has not been shown to increase with the use of these medications, in part because they may also protect cancer cells [14]. Consequently, despite research into medications that are especially targeted for cancer cells, there should be a fundamentally unique strategy that kills cancer cells selectively while sparing healthy cells. According to one of the studies, it was found that around 70% of the cancer patients experienced the most frequent side effect of chemotherapy like weakness, fatigue, nausea, hair loss and vomiting in 95%, 90%, 77%, 76% and 75% patients respectively. Traditional plant extract-based medicinal regimens are being researched as an alternative therapeutic approach to addressing the shortcomings of existing therapies [9]. Researchers have studied the use of naturally derived products in drug development in great detail, and they frequently use these compounds in cancer research. Plants are essential to the medical system because they provide herbal chemicals that can be used alone or in combination to treat disease [15,16]. Plant-derived medicines have long been employed in the traditional therapeutic systems of India and the rest of the world to treat a wide range of illnesses [16,17]. Scientists are constantly working to extract compounds from various plant parts and characterize them for their functional components and antioxidant, antibacterial, and anticancer properties. An important issue that requires major attention is the establishment of current scientific data supporting the conventional therapy system [18,19]. *Eupatorium adenophorum* Spreng (Ageratina adenophora-Spreng) belongs to the Family: Asteraceae. *E. adenophorum* is a "forest killer" plant which is often known as crofton weed, banmara, sticky snakeroot, etc. In countries like China, India, Nepal, Pakistan etc., the Asteraceae family is extensively spread [20]. It is frequently applied as traditional Chinese medicine in China. *E. adenophorum* has reportedly been used historically to treat cuts and bruises, hyperglycemia, inflammation, fever, jaundice and dysentery. The chemical and pharmacological characteristics of *E. adenophorum* have been studied in various ways throughout the last few years. Sesquiterpenoids, triterpenes, flavonoids, phenolics, coumarins, steroids, and phenylpropanols are just a few of the many phytochemicals that have been isolated and identified from *E. adenophorum* [21,22]. The extract of *E. adenophorum* was found to have a variety of biological properties, including antioxidant, antibacterial, anticancer, wound healing, analgesic, antipyretic, and anti-inflammatory action [23]. Since no anti-cancer activity has been conducted on *E. adenophorum* extracts so this plant has become a matter of research for anti-cancer activity as it possesses chemical constituents like terpenoids, flavonoids, steroids and phenolic compound, which could make this plant a greater choice for anti-cancer activity. The objective of the study is to take the plant for successive extraction, then to make the extract be subjected to MTT assay for studying apoptosis on breast cancer and liver cancer cell lines and finally to analyze those chemical constituents responsible for anti- cancer activity via LCMS.

2. Materials and Methods

2.1. Plant Procurement and Authentication

Plant leaves were collected in the month of July from Forest Research Institute (FRI), Dehradun, Uttarakhand. The specimen of the plant was submitted to Botanical Survey of India, Dehradun, Uttarakhand for authentication. The accession No. of the specimen is 1302.

2.2. Extract Preparation

For extraction, chemicals were procured from FRI and all the chemicals were of analytical grade. Leaves were cleaned with water before being bought to the laboratory in sterile bags. Leaves were air dried to remove moisture. Total sample size was 350 gm and further size reduction was done. Then sample was carried for successive extraction i,e solvent was added in ascending order of polarity [24,25]. The method used for extraction was hot percolation (Soxhlet extraction). Assembly was set up on heating mantle and the temperature was maintained at 60^0C. Firstly 5 liters of Petroleum ether were added to the sample and extraction was carried out till the yellow color of solvent became colorless in siphon tube. Sample was removed and solvent was evaporated in rotary evaporator till the solid mass was extracted. To the same sample chloroform and methanol were added respectively following the same procedure as aforementioned above [26,27]. Three extracts were obtained, petroleum ether extract (PET), chloroform extract ($CHCl_3$) and methanol extract (MeOH). Further samples were subjected for cytotoxicity study on breast cancer cell line (MDAMB231) and liver cancer cell line (HepG2).

2.3. *In vitro* Cytotoxicity and Apoptosis (MTT Assay)

The MTT assay was used to assess mitochondrial activity, relying on the conversion of MTT into formazan crystals by viable cells. This experiment is mostly conducted to evaluate the in vitro cytotoxic effects of medications on cell lines, as the overall mitochondrial activity is generally associated with the percentage of viable cells in most cell populations [28,29].

Cytotoxicity study of the obtained samples (PET, $CHCl_3$ and MeOH extracts) was performed on MDAMB231 and HepG2 cell line via MTT Assay. A total of 10,000 cells were cultured in each well of a 96-well plate for 24 hours at a temperature of 37°C with 5% CO_2. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic solution. The cells were exposed to formulations at concentrations ranging from 1 to 1000 µg/ml the following day. Various concentrations were made in a full

medium. The MTT solution was introduced to the cell culture and then kept in an incubator for 24 hours at a concentration of 250 µg/ml. Following the experiment, the cell layer matrix was diluted in 100µl of dimethyl sulfoxide (DMSO) and analyzed at wavelengths of 540 nm and 660 nm using an Elisa plate reader (iMark, Biorad, USA) [29-31].

2.4. LCMS of the Extracts

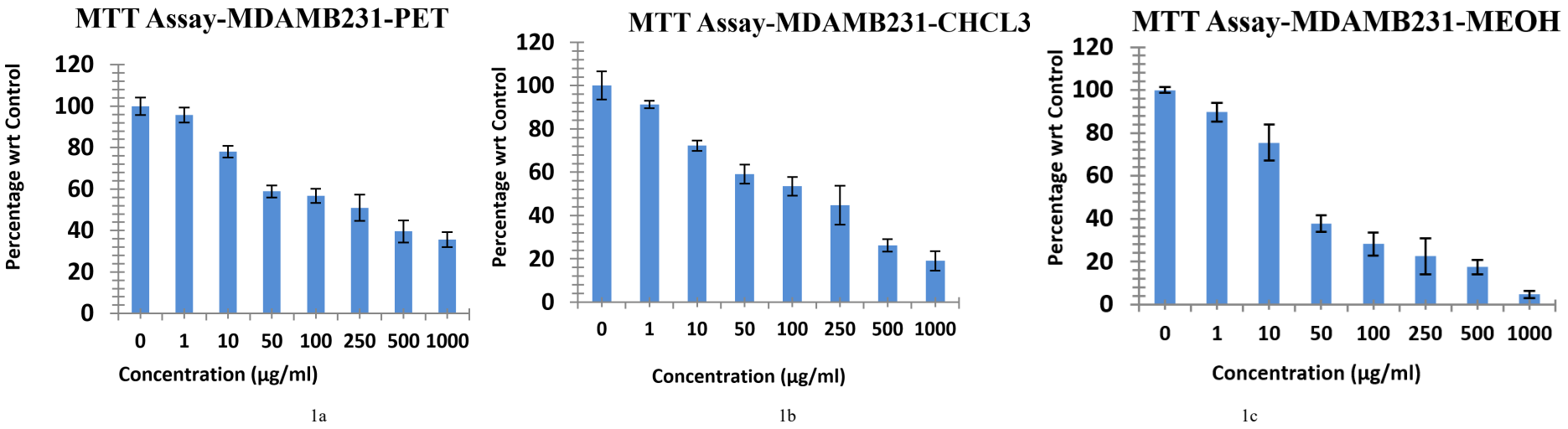
Liquid chromatography-mass spectrometry (LC-MS/MS) is a method that combines mass spectrometry and liquid chromatography (or HPLC). It is an analytical technique that combines the mass analysis skills of mass spectrometry with the physical separation capabilities of liquid chromatography (or HPLC). For the qualitative and quantitative examination of drug compounds, drug products, and biological samples, laboratories frequently use LC-MS/MS [32]. LC-MS is an analytical method that combines sensitive and accurate mass spectral detection with high resolution chromatographic separation. In LC-MS, mass spectroscopy aids in sample structure elucidation and elemental composition analysis [33]. The fractions of PET extract and MeOH extract, which exhibited anti-cancer activity, were further processed for identification of bioactive compounds by LC MS analysis at Aakar Biotechnologies Private Limited, Lucknow, using LC InstrumentXEVO-TQD#QCA1232; Column**:** SUNFIRE C18, 250 X 2.1, 2.6um. The detection was performed through direct injection mode with Electron Spray Ionization (ESI) probe. The both methanolic extract and petroleum ether extract were centrifuged at 12,000 rpm for 15 min before analysis. The HPLC system consisted of two pumps and an automated injector. Separation was achieved on a C-18 column. Two mobile phases were used i,e Acetonitrile in ammonium buffer and formic acid in water. The sample flow rate was set at 0.2 ml/minute. The mass range was selected from negative and positive ionization modes between m/z 150 and 2000 [27,34].

3. Results

In the MTT assay, as the concentration of extract was increased the viability of the cell was decreased with respect to control in PET, $CHCl_3$ and MeOH extracts. In MDAMB 231 cell line the maximal inhibitory concentration (IC50) was found to be 37.5 µg/ml for MeOH extract, 123.8µg/ml for $CHCl_3$ extract and 201.4 for PET extract (Table 1; Figures 1a-1c). This indicates MeOH extract is more potent than $CHCl_3$ and PET extracts. In case of HepG2 cell line IC50 of PET was 138.2 µg/ml, $CHCl_3$ was 504 µg/ml and MeOH was 664.2 µg/ml. This indicates that PET extract is more active than MEOH and $CHCl_3$ extracts (Table 1; Figures 2a-2c). The finding of MTT assay of *Eupatorium adenophorum* extract indicates it as a potent anti-cancer drug. Studies on ethno medical properties of *Eupatorium adenophorum* have indicated the presence of many chemical constituents that might show anti-cancer properties [35]. To identify those chemicals responsible for anti-cancer activity of PET and MeOH extracts LCMS was carried out. List of chemical compounds obtained from LCMS of MeOH extract has been given below (Tables 2a and 2b; Figures 3a and 3b) while chemicals found in PET extract responsible for anti-cancer activity have been enlisted in (Tables 3a and 3b; Figures 4a and 4b). The compounds identified in methanolic extracts possessing anticancer activity are chalcone, acacetin and luteolin (flavonoids category), linoleic acid (fatty acid), malvidin chloride (polyphenol category) and compounds identified in petroleum ether extracts are 1-Isothiocyanato-9-(methylsulfinyl)-nonane, 1-Isothiocyanato-7-(methylsulfinyl)-heptane (antioxidants), Chalcone, Rhamnetin (flavonoid), Scoulerin, Sinapic acid (alkaloid), and Resveratrol (polyphenols) that are responsible for anti-cancer effects.

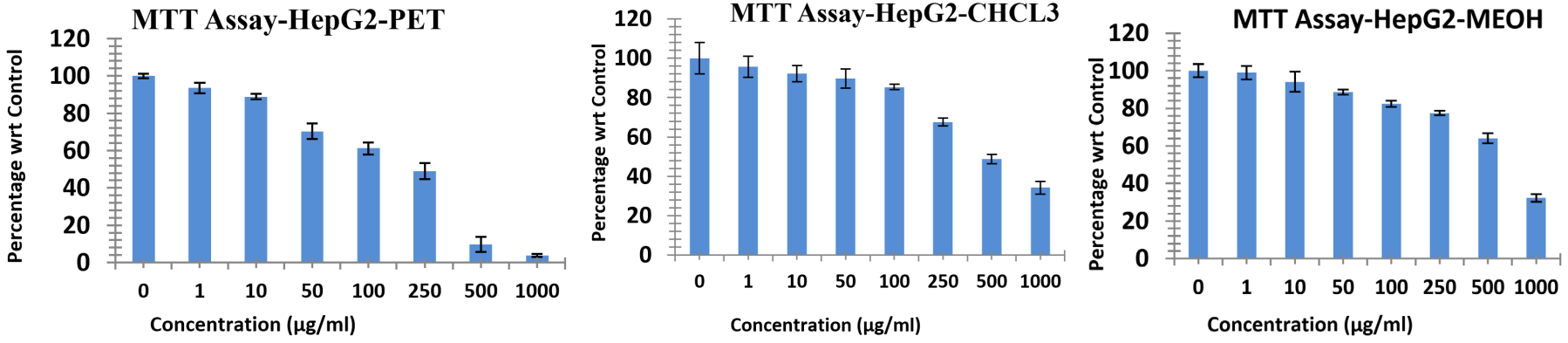
Table 1. IC_{50} of MDA MB 231 (Breast cancer) and HepG2 (Liver cancer) cell lines. PET: Petroleum Ether Extract, $CHCl_3$: Chloroform Extract, MeOH: Methanol Extract, IC_{50}: Inhibitory Concentration of viable cells

MDA MB 231 cell line		**HepG2 cell line**	
Sample	IC_{50} Value (µg/ml)	Sample	IC_{50} Value (µg/ml)
PET	201.4	PET	138.2
$CHCl_3$	123.8	$CHCl_3$	504
MeOH	37.5	MeOH	664.2



1a 1b 1c

Figure 1. 1a: MTT assay for breast cancer cell line (MDAMB231) against various concentration of Petroleum ether extract. All values are represented as mean± SEM, n=4; 1b: MTT assay for breast cancer cell line (MDAMB231) against various concentration of Chloroform extract. All values are represented as mean± SEM, n=4; 1c: MTT assay for breast cancer cell line (MDAMB231) against various concentration of Methanolic extract. All values are represented as mean± SEM, n=4



2a 2b 2c

Figure 2. 2a: MTT assay for liver cancer cell line (HepG2) against various concentration of Petroleum ether extract. All values are represented as mean ± SEM, n=4; 2b: MTT assay for liver cancer cell line (HepG2) against various concentration of Chloroform extract. All values are represented as mean ± SEM, n=4; MTT assay for liver cancer cell line (HepG2) against various concentration of Methanolic extract. All values are represented as mean ± SEM, n=4

Table 2a. Ions in positive mode in methanol

Retention time	Score	Compound Name	Category	Ion	Formula	Exact mass	Observed mass	Mass Difference
1.55	0.949	Amantadine hydrochloride	Adamantanes	positive	C10H17N	151.136	154.1747	-3.04
7.72	0.905	3,4-Dihydroxy-L-phenylalanine	Dopamine	[M+H]+	C9H11NO4	197.19	197.2303	-0.04
11.68	0.809	1-Isothiocyanato-7-(methylsulfinyl)-heptane	Antioxidants	positive	C9H17NOS2	219.075	219.2309	-0.16
13.96	0.901	1,10-Phenanthroline monohydrate	Hetro-cyclic organic compound	positive	C12H8N2	180.068	181.2362	-1.17
14.24	0.811	Linoleic acid	Fatty acids	positive	C18H32O2	280.24	281.2183	-0.98
14.99	0.817	Cytidine-5'-diphosphate sodium salt hydrate,from yeast	Carbohydrate	[M+H]+	C9H15N3O11P2	403.18	403.2747	-0.09
16.69	0.745	Malvidin Chloride	Polyphenols	positive	C17H15O7	331.081	331.2617	-0.18
16.76	0.841	Caffeine, Anhydrous	Alkaloid	[M+H]	C8H10N4O2	194.19	194.2609	-0.07
16.93	0.63	Sinapine	Alkaloid	positive	C16H24NO5	310.165	313.3769	-3.21
17.65	0.972	Chalcone	Flavonoids	positive	C15H12O	208.088	209.2091	-1.12
18.47	0.726	trans-Zeatin-riboside	Carbohydrates	positive	C15H21N5O5	351.154	253.3985	97.76
19.80	0.87	luteolin-3',7-di-O-glucoside	Flavonoid	positive	C27H30O16	610.153	609.4714	0.68
21.06	0.827	1-Isothiocyanato-8-(methylsulfinyl)-octane	Glycosides	positive	C10H19NOS2	233.09	237.2836	-4.19
21.53	0.381	Kaempferol-3-O-beta-D-glucoside-7-O-alpha-L-rhamnoside	Flavonoid	positive	C27H30O15	594.158	634.6143	-40.46
21.67	0.381	Pelargonin chloride	Anthocyanidin	positive	C27H31O15	595.166	593.4072	1.76
21.53	0.381	Pelargonin chloride	Anthocyanidin	positive	C27H31O15	595.166	634.6143	-39.45
21.67	0.801	Kaempferol-3-O-beta-D-glucoside-7-O-alpha-L-rhamnoside	Flavonoids	positive	C27H30O15	594.158	593.4072	0.75
22.28	0.755	Peonidin-3,5-O-di-beta-glucopyranoside	Carbohydrates	positive	C28H33O16	625.176	625.5021	-0.33
22.73	0.688	Kaempferol-3-O-beta-D-glucoside-7-O-alpha-L-rhamnoside	Flavonoids	positive	C27H30O15	594.158	593.4409	0.72
22.86	0.787	Delphinidin-3-O-(2"-O-beta-xylopyranosyl-beta-glucopyranoside)	Flavonoids	positive	C26H29O16	597.145	600.5956	-3.45
23.00	0.73	1,2-Dipalmitoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt	Lipid	positive	C38H75O10P	722.509	556.5203	165.99
23.17	0.637	syringetin-3-O-galactoside	Flavonoids	positive	C23H24O13	508.121	512.6144	-4.49
23.31	0.66	Sissotrin	Flavonoid	positive	C22H22O10	446.121	451.4309	-5.31
23.44	0.94	2'-Deoxyadenosine-5'-diphosphate sodium salt	Purine	positive	C10H15N5O9P2	411.034	407.4930	3.54

Table 2a continued

23.58	0.41	Guanosine-5'-monophosphate disodium salt from Yeast	Purine	[M+H]+	C10H14N5O8P	363.22	363.3872	-0.17
2378	0.706	Adenosine-3',5'-cyclicmonophosphate	Purine	[M+H]+	C10H12N5O6P	329.21	329.2032	0.01
26.34	0.915	6-(gamma,gamma-Dimethylallylamino)purine riboside	Glycoside	positive	C15H21N5O4	335.159	338.4494	-3.29
26.44	0.929	5-Aminoimidazole-4-carboxamide-1-beta-D-ribofuranosyl 5'-monophosphate	Glycoside	positive	C9H15N4O8P	338.062	338.4494	-0.39
27.84	0.623	1-O-b-D-glucopyranosyl sinapate	Glycoside	positive	C17H22O10	386.121	391.3961	-5.28
29.17	0.467	Cytidine-5'-diphosphate sodium salt hydrate, from yeast	Pyrimidine	[M+H]+:	C9H15N3O11P2	403.18	391.3624	11.82
30.13	0.615	isorhamnetin-3-O-rutinoside	Flavonoid	positive	C28H32O16	624.169	623.4435	0.73

Table 2b. Ions in negative mode in Methanol Extract

R. Time	Score	Compound Name	Category	Ion	Formula	Exact Mass	Observed Mass	Mass Diff
1.16	0.989	D-(-)-Quinic acid	Monocarboxylic acid	negative	C7H12O6	192.063	191.0891	0.97
15.31	0.936	Pentachlorophenol	Organochlorine compound	[M-H]-	C6HCl5O	266.34	265.3923	0.95
18.45	0.703	Piperacillin sodium salt	Ureidopenicillin	negative	C23H27N5O7S	517.163	513.5256	3.64
2073	0.972	6-Phosphogluconic acid Barium salt hydrate	Carbohydrate	negative	C6H13O10P	276.024	277.3714	-1.35
21.14	0.938	2'-Deoxycytidine	Pyrimidine	negative	C9H13N3O4	227.09	227.2280	-0.14
22.30	0.873	D-Glucosamine-6-phosphate sodium salt	Amino sugar	negative	C6H14NO8P	259.045	253.2782	5.77
23.05	0.974	gamma-Linolenic acid	Fatty acid	[M-H]-	C18H30O2	278.43	279.3624	-0.93
25.85	0.977	2'-Deoxyinosine	Purine	negative	C10H12N4O4	252.085	255.3703	-3.29
26.39	0.95	Luteolin	Flavonoids	negative	C15H10O6	286.047	281.3870	4.66
28.78	0.799	Formononetin	Phytoestrogen	negative	C16H12O4	268.073	269.3066	-1.23
32.50	0.946	Acacetin	Flavonoids	negative	C16H12O5	284.068	283.4454	0.62
32.57	0.979	Acacetin	Flavonoids	negative	C16H12O5	284.068	283.4117	0.66
32.70	0.931	Xanthosine	Purine	negative	C10H12N4O6	284.075	283.4454	0.63

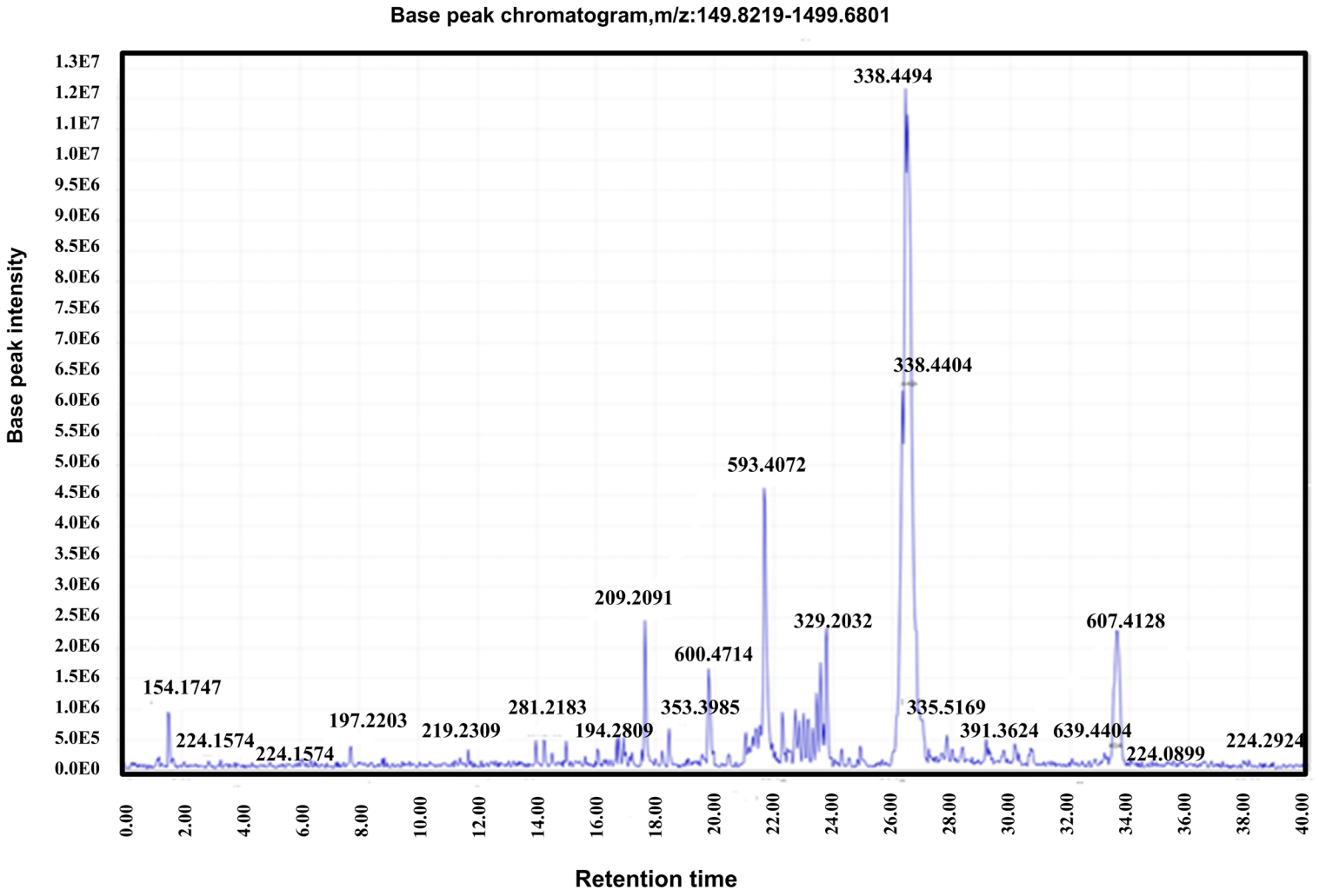


Figure 3a. Total Ion Chromatogram (Positive Mode) for Methanolic Extract. The peak intensity represents different components at different retention time. 181.2362 peak represents 1,10-Phenanthroline monohydrate and 600.5956 peak represents Delphinidin-3-O-(2"-O-beta-xylopyranosyl-beta-glucopyranoside)

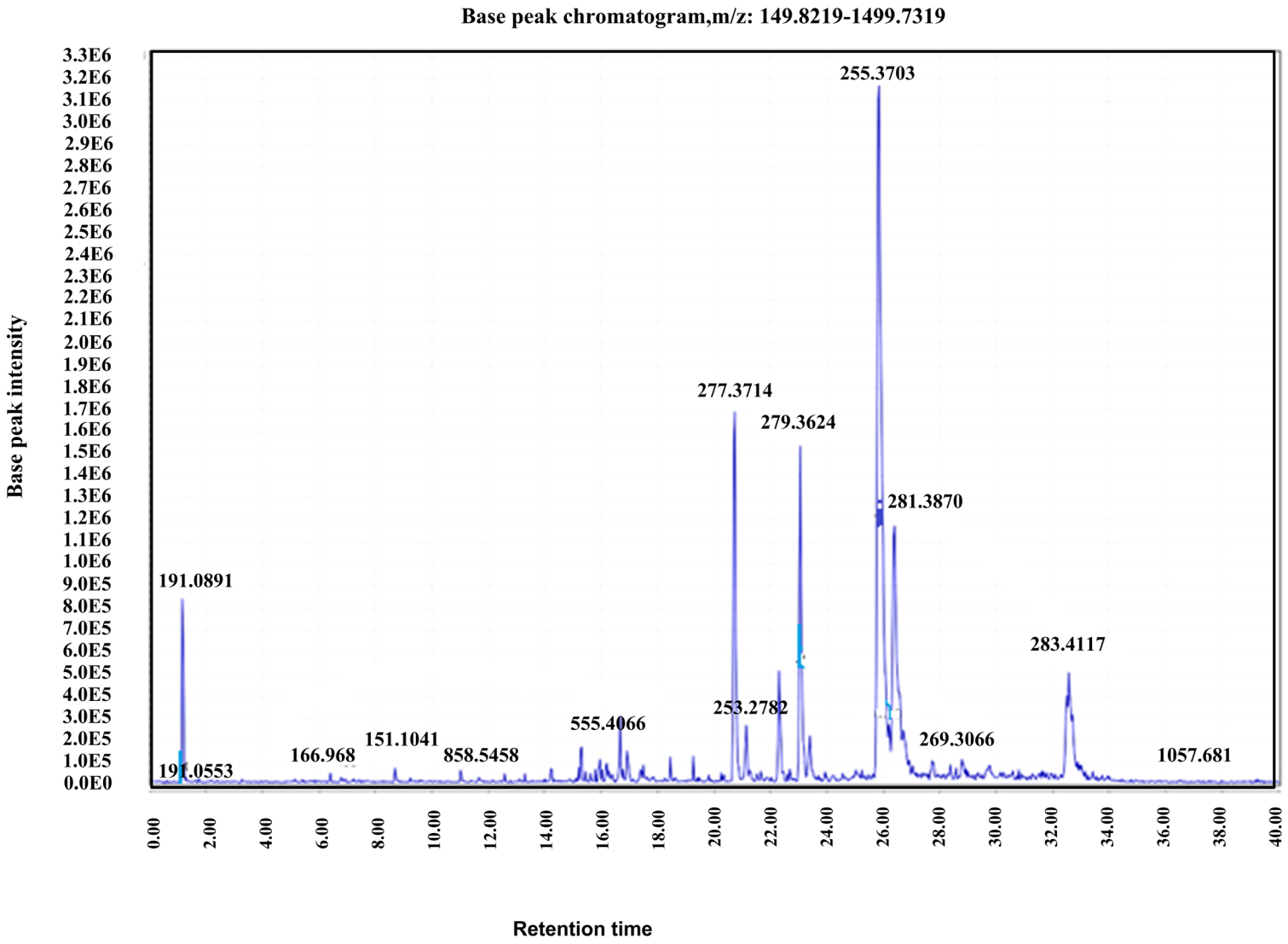


Figure 3b. Total Ion Chromatogram (Negative Mode) for Methanolic Extract. The peak intensity represents different components at different retention time. 191.0891 peak represents D-(-)Quinic acid, 277.37 peak represents 6-Phosphogluconic acid Barium salt hydrate and 279 peak represents gamma-Linolenic acid

Table 3a. Ions in positive mode in Petroleum Ether Extract

R.Time	Score	Compound Name	Category	Ion	Formula	Exact Mass	Observed Mass	Mass Diff
7.76	0.985	3,4-Dihydroxy-L-phenylalanine	Dopamine	Positive	C9H11NO4	197.068	197.4199	-0.3519
8.81	0.788	Luteolin	Flavonoid	[M+H]+	C15H10O6	286.25	291.4836	-5.2336
9.70	0.853	Pyridoxal-5'-phosphate monohydrate	Vitamin	Positive	C8H10NO6P	247.024	249.4637	-2.4397
11.03	0.853	1-Isothiocyanato-9-(methylsulfinyl)-nonane	Antioxidant	Positive	C11H21NOS2	247.106	247.4387	-0.3327
12.33	0.683	1-Isothiocyanato-7-(methylsulfinyl)-heptane	Antioxidant	Positive	C9H17NOS2	219.075	219.4255	-0.3505
12.77	0.78	Chalcone	Flavonoid	Positive	C15H12O	208.088	207.3764	0.7116
14.00	0.952	1,10-Phenanthroline monohydrate	Hetro-cyclic organic compound	Positive	C12H8N2	180.068	181.3883	-1.3203
14.20	0.688	1-Isothiocyanato-8-(methylsulfinyl)-octane	Glycosides	Positive	C10H19NOS2	233.09	231.4408	1.6492
14.48	0.96	gamma-Glu-Cys	Dipeptide	Positive	C8H14N2O5S	250.062	251.4888	-1.4268
14.99	0.893	cysteinylglycine	Dipeptide	Positive	C5H10N2O3S	178.041	177.4057	0.6353
15.43	0.782	Calcium (+)- pantothenate	Vitamin	[M+H]+	C9H17NO5	219.23	219.4592	-0.2292
15.50	0.828	Sodium pantothenate	Vitamin	Positive	C9H17NO5	219.11	219.4592	-0.3492
15.60	0.926	1-Isothiocyanato-7-(methylsulfinyl)-heptane	Antioxidants	Positive	C9H17NOS2	219.075	219.4255	-0.3505
16.86	0.611	Cystathionine	Aminoacid	[M+H]+	C7H14N2O4S	222.26	220.4718	1.7882
17.48	0.832	4-Nitrophenyl phosphate disodium salt hexahydrate	Weak base	Positive	C6H6NO6P	218.993	219.4592	-0.4662
17.54	0.688	1-Isothiocyanato-7-(methylsulfinyl)-heptane	Antioxidants	Positive	C9H17NOS2	219.075	219.4592	-0.3842
17.95	0.983	Calcium (+)-pantothenate	Vitamin	[M+H]+	C9H17NO5	219.23	217.4342	1.7958
18.06	0.983	Harmaline hydrochloride dihydrate	Alkaloid	[M+H]+	C13H14N2O	214.28	217.4342	-3.1542
19.18	0.769	Scoulerin	Alkaloids	Positive	C19H21NO4	327.147	327.4282	-0.2812
19.63	0.521	1-Isothiocyanato-8- (methylsulfinyl)-octane	Antioxidants	Positive	C10H19NOS2	233.09	237.4484	-4.3584
21.40	0.692	L-Tryptophane	Amino acid	[M+H]+	C11H12N2O2	204.23	205.4189	-1.1889
21.47	0.707	L-Tryptophane	Amino acid	[M+H]+	C11H12N2O2	204.23	205.3514	-1.1214
23.68	0.619	Leupeptin hemisulfate salt	Peptide	[M+H]+	C20H38N6O4	426.58	425.5419	1.0381
23.79	0.588	Scoulerin	Alkaloids	Positive	C19H21NO4	327.147	329.4532	-2.3062

Table 3b. Ions in negative mode in Petroleum Ether Extract

R.Time	Score	Compound Name	Category	Ion	Formula	Exact Mass	Observed Mass	Mass Diff
7.20	0.673	trans-3,5-Dimethoxy-4-hydroxycinnamaldehyde	Aldehyde	Negative	C11H12O4	208.073	207.4439	0.6291
8.76	0.712	Sinapic acid	Alkaloid	Negative	C11H12O5	224.068	223.4418	0.6262
9.51	0.823	D-Mannose-6-phosphate barium salt hydrate	Carbohydrate	Negative	C6H13O9P	260.029	261.5465	-1.5175
9.62	0.703	alpha-D-glucose-1-phosphate dipotassium salt dihydate	Carbohydrate	Negative	C6H13O9P	260.029	261.4790	-1.45
10.16	0.793	trans-3,5-Dimethoxy-4-hydroxycinnamaldehyde	Aldehyde	Negative	C11H12O4	208.073	207.3427	0.7303
10.81	0.753	alpha-L-(-)-Fucose 1-phosphate bis(cyclohexylammonium) salt	Carbohydrate	Negative	C6H13O8P	244.034	245.4136	-1.3796
11.90	0.75	Rhamnetin	Flavonoid	Negative	C16H12O7	316.058	311.4978	4.5602
11.94	0.651	2'-Deoxyuridine-5'-monophosphate disodium salt	Pyrimidine	Negative	C9H13N2O8P	308.04	311.5315	-3.4915
12.69	0.724	Pentachlorophenol	Organo chlorine compound	[M-H]-	C6HCl5O	266.34	267.4867	-1.1467
13.47	0.912	Resveratrol	Polyphenol	Negative	C14H12O3	228.078	229.4157	-1.3377
13.54	0.81	D-Arabinose-5-phosphate disodium salt	Carbohydrate	Negative	C5H11O8P	230.019	229.4832	0.5358
14.87	0.699	Pyridoxal-5'-phosphate hydrate	Vitamin	Negative	C8H10NO6P	247.024	247.5399	-0.5159
15.89	0.664	Kaempferide	Flavonoid	Negative	C16H12O6	300.063	297.4574	2.6056
16.03	0.866	Pentachlorophenol	Organo chlorine compound	[M-H]-	C6HCl5O	266.34	265.5629	0.7771
16.10	0.753	2'-Deoxyguanosine monohydrate	Puine	Negative	C10H13N5O4	267.096	265.5291	1.5669
16.40	0.696	N-acetylneuraminic acid	Carbohydrate	Negative	C11H19NO9	309.105	309.5740	-0.469
16.74	0.952	N-acetylneuraminic acid	Carbohydrate	Negative	C11H19NO9	309.105	311.5315	-2.4265
16.81	0.9	N-acetylneuraminic acid	Carbohydrate	Negative	C11H19NO9	309.105	311.5315	-2.4265
17.08	0.78	2'-Deoxyuridine-5'-monophosphate disodium salt	Pyrimidine	Negative	C9H13N2O8P	308.04	311.5315	-3.4915
17.56	0.73	Uridine-5'-monophosphate	Pyrimidine	Negative	C9H13N2O9P	324.035	325.4706	-1.4356
17.63	0.749	Cytidine-5'-monophosphate monohydrate	Pyrimidine	Negative	C9H14N3O8P	323.051	325.5719	-2.5209
17.94	0.69	(+)-Epicatechin	Flavonoid	Negative	C15H14O6	290.079	293.5761	-3.4971
18.11	0.815	(+)-Epicatechin	Flavonoid	Negative	C15H14O6	290.079	293.5423	-3.4633
18.31	0.972	(+)-Epicatechin	Flavonoid	Negative	C15H14O6	290.079	293.6098	-3.5308
18.48	0.862	(+)-Catechin hydrate	Polyphenols	Negative	C15H14O6	290.079	291.5173	-1.4383
18.59	0.929	Eriodictyol	Flavonoids	Negative	C15H12O6	288.063	291.5510	-3.488

Table 3b continued

18.65	0.909	(+)-Catechin hydrate	Polyphenols	Negative	C15H14O6	290.079	291.5173	-1.4383
19.34	0.823	Kaempferide	Flavonoids	Negative	C16H12O6	300.063	295.6011	4.4619
19.78	0.858	(+)-Catechin hydrate	Polyphenols	Negative	C15H14O6	290.079	293.5086	-3.4296
19.88	0.886	(+)-Catechin hydrate	Polyphenols	Negative	C15H14O6	290.079	293.6436	-3.5646
21.69	0.814	Kaempferide	Flavonoids	Negative	C16H12O6	300.063	295.5336	4.5294
24.59	0.94	gamma-Linolenic acid	Fatty acid	[M-H]-	C18H30O2	278.43	277.6119	0.8181
24.76	0.992	6-Phosphogluconic acid Barium salt hydrate	Carbohydrate	Negative	C6H13O10P	276.024	277.5444	-1.5204
26.74	0.924	gamma-Linolenic acid	Fatty acid	[M-H]-	C18H30O2	278.43	279.6032	-1.1732
26.84	0.917	L-saccharopine		Negative	C11H20N2O6	276.132	279.6370	-3.505
26.91	0.948	6-Phosphogluconic acid Barium salt hydrate	Carbohydrate	Negative	C6H13O10P	276.024	279.6370	-3.613
28.82	0.671	Uridine-5'-monophosphate	Pyrimidine	Negative	C9H13N2O9P	324.035	327.6982	-3.6632
30.21	0.93	D-Glucosamine-6-phosphate sodium salt	Amino sugar	Negative	C6H14NO8P	259.045	255.6401	3.4049
30.32	0.938	2'-Deoxyinosine	Purine	Negative	C10H12N4O4	252.085	255.5726	-3.4876
30.42	0.89	D-Mannose-6-phosphate barium salt hydrate	Carbohydrate	Negative	C6H13O9P	260.029	255.5051	4.5239
30.69	0.748	2'-Deoxyinosine	Purine	Negative	C10H12N4O4	252.085	255.5726	-3.4876

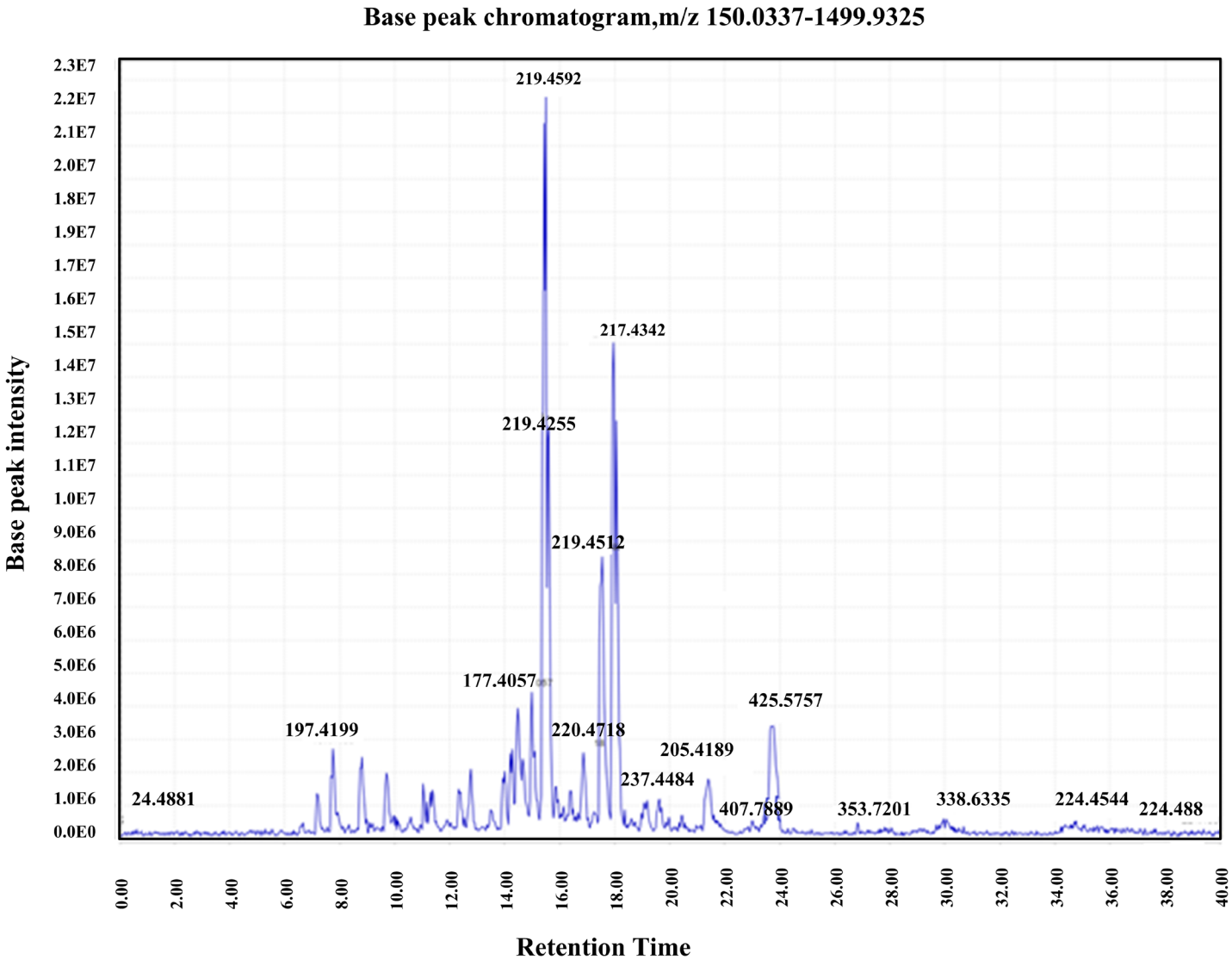


Figure 4a. Total Ion Chromatogram (Positive Mode) for Petroleum Ether extract. The peak intensity represents different components at different retention time.197.4199 peak represents 3,4-Dihydroxy-L-phenylalanine, 247.4387 represents peak for 1-Isothiocyanato-7-(methylsulfinyl)-heptane and 327.4282 peak represents Scoulerin

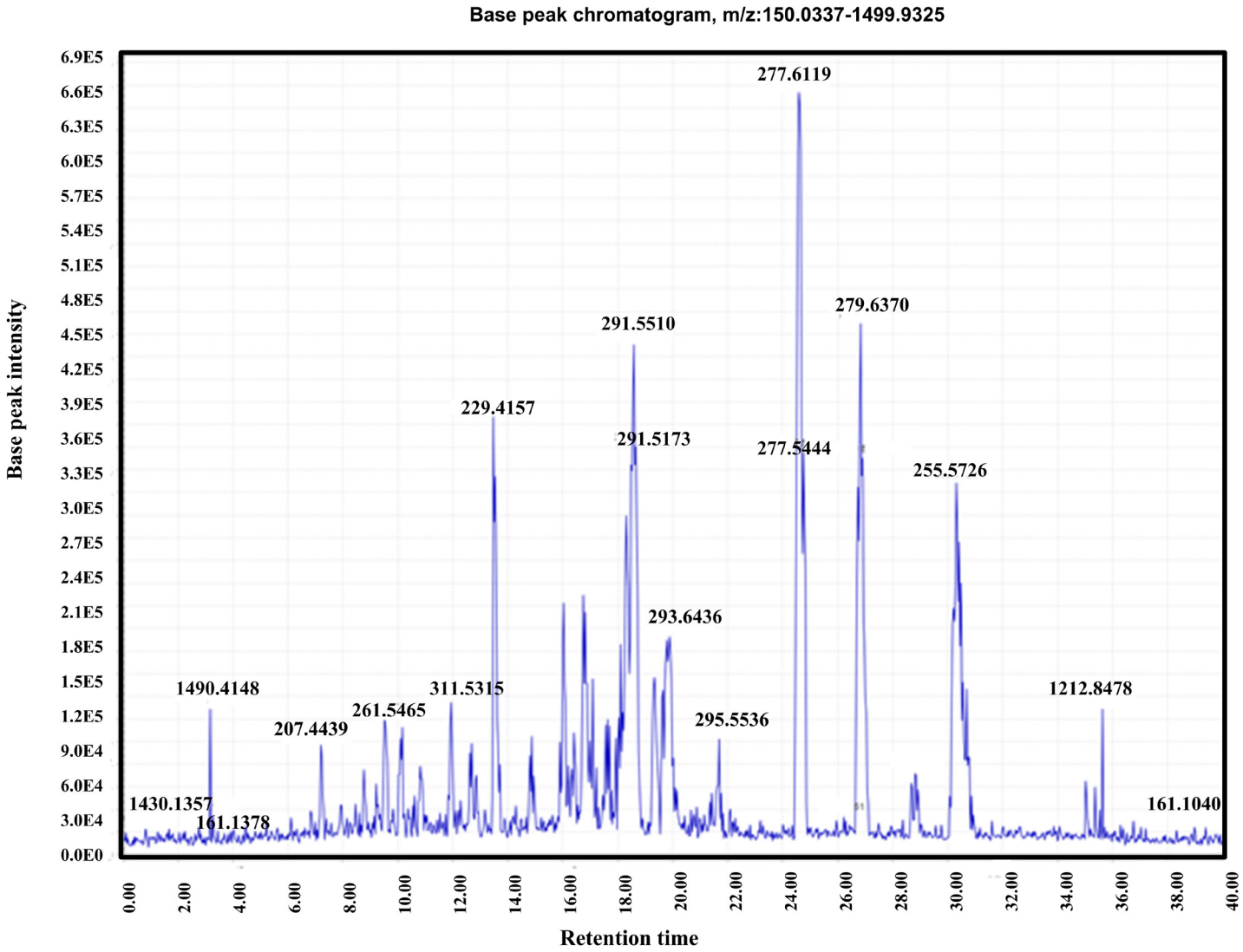


Figure 4b. Total Ion Chromatogram (Negative Mode) for Petroleum Ether extract. The peak intensity represents different components at different retention time. 207.4439 peak represents trans-3,5-Dimethoxy-4-hydroxycinnamaldehyde, 327.6982 peak represents Uridine-5'-monophosphate and 311.531 peak represents N-acetylneuraminic acid

In a mixture of compounds the individual component is separated by ionization and separation of ions on the basis of mass/charge ratio. The chemicals are obtained in positive and negative modes. The chemicals obtained in MeOH extract in positive as well as negative mode are polyphenols and flavonoids and most of the chemicals obtained in PET extract are of flavonoids and alkaloids category responsible for anti-cancer activity.

4. Discussion

The LCMS data confirms the presence of different chemicals from flavonoids and polyphenols category in MeOH extract and chemical compounds like glucosamine, uridine, catechin, kaempferol, linoleic acid, resveratroletc in PET extract making it suitable drug for anti-cancer activity.

It is believed that polyphenols have useful apoptosis-inducing characteristics that exhibit anti-cancer capabilities. By controlling the mobilization of copper ions that are linked to chromatin and cause DNA fragmentation, polyphenols are considered to trigger the apoptotic process. Resveratrol was found to be able to degrade DNA when Cu (II) was present [36]. Plant polyphenols also has the ability to prevent the growth of cancer cells by interfering with the proteins found in cancer cells. With the direct bonding of the polyphenol, which controls acetylation, methylation, or phosphorylation, cancer agents may be modified [37]. Flavonoids were discovered to exhibit cytotoxicity on cancer cells and to have high free radical scavenging activity by Cao *et al*. in 2013 [38]. Via intrinsic and extrinsic signalling pathways, purified flavonoids have also demonstrated anticancer effects against additional human malignancies, such as hepatoma (Hep-G2), cervical carcinoma (Hela), and breast cancer (MCF-7) [39]. Apoptotic proteins are induced, which drastically lowers the mitochondrial membrane potential. Cancer cells are unable to survive when their mitochondria are damaged [40,41]. A revolutionary strategy known as "membrane lipid treatment" recently came into existence. It is predicated on the idea that using particular lipids may change the content and structure of cancer membranes. By altering downstream pathways that are essential for the proliferation of tumor cells, this can dismantle the lipid raft architecture and change the localization and function of membrane-associated proteins [42]. Lineolic acid may therefore function along this route. Likewise certain other chemical constituents follow specific pathways in killing the cancer cells. Glycosides inhibit the proliferation of cancer cell and block the tumor growth, by inducing immunogenic cell death [43]. The newly found plant has confirmed its role as a major anti-cancer drug in *in vitro* studies and the presence of chemical constituents like polyphenols, flavonoids, fatty acids and glycosides might play a major role in its anticancer activity. Cell viability was studied at different concentration for PET, $CHCl_3$ and MeOH extracts in MDA MB 231 cell line and at the concentration of 1000µg/ml maximum viable cells were inhibited by MeOH extract (Figure 5). In case of HepG2 cell line at the concentration of 1000µg/ml maximum viable cells were inhibited by PET extract (Figure 6). The *E. adenophora* leaves extract has confirmed its role as an anti-cancer drug.

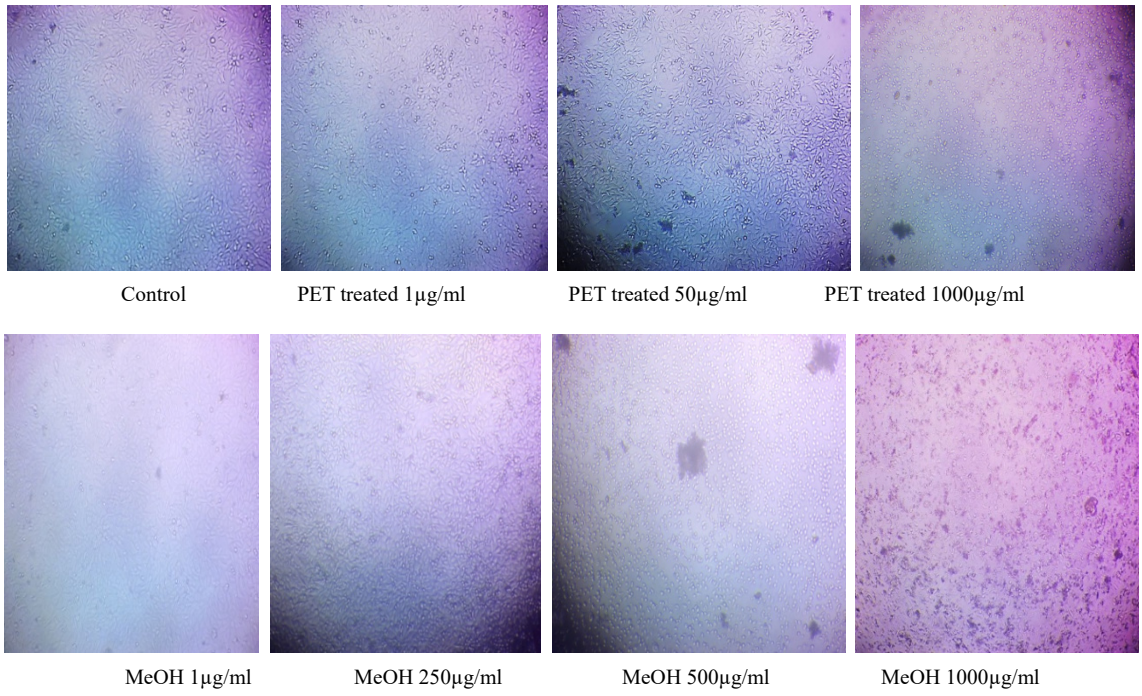


Figure 5. These images indicate that Methanolic extract has less IC50 value when compared to other extracts i,e Chloroform and Petroleum Ether. At the concentration of 1000µg/ml maximum cell viability was inhibited by Methanol extract in MDA MB -231 cell line

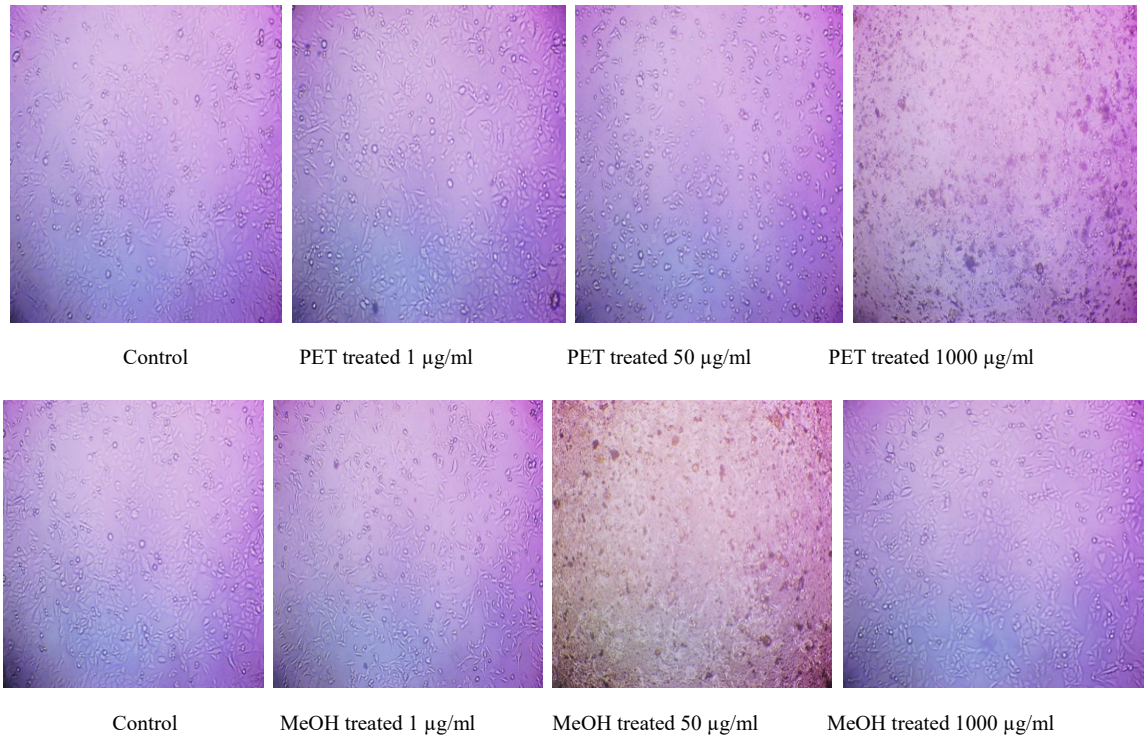


Figure 6. These images indicate that Petroleum Ether extract has less IC50 value when compared to other extracts i,e Chloroform and Methanol. At the concentration of 1000µg/ml maximum viable cells were inhibited by Petroleum ether extract in Hep2 cell line

5. Conclusions

The results from the MTT assay are showing that the extracts from the leaves of *E. adenophorum* are having good anti-cancer potential. This result is also validated through the LCMS reports which indicates the presence of various chemical constituents such as flavonoids, polyphenols, glucosamine and uridine with high anti-cancer efficacy. Therefore, *E. adenophorum* leaves extract may be a better therapeutic alternative over conventional therapeutic regime in the amelioration of breast and liver cancer.

Acknowledgements

The author would like to thank Forest Research Institute and DIT University for their support in processing of practical work.

Conflicts of Interest

None.

List of Abbreviations

PET: Petroleum Ether, **CHCl3:** Chloroform, **MeOH:** Methanol, **MTT:** (3-(4,5-Dimethylthiazo2-yl)-2,5-diphenyltetrazolium bromide, **LCMS:** Liquid Chromatography-Mass Spectroscopy, **GBD:** Global Burden of Disease, **ER+:** Estrogen Receptor positive, **E. Adenophorum:** Eupatorium adenophorum, **DMEM:** Dulbecco's Modified Eagle Medium, **FBS:** Fetal Bovine Serum, **DMSO:** Dimethyl sulfoxide, **IC50:** Inhibitory concentration.

Funding Source

None.

Summary

Eupatorium adenophorum contains many chemical constituents that can be used for cancer treatment. But the plant has not been explored much and no studies has been conducted on its anti cancer property. This study reveals the chemical constituents present in the plants and in vitro study confirms its anti cancer activity. Further in vivo study will be conducted on its extract to confirm its use in clinical study.

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