CHAPTER - 3

EXTRACTION, PURIFICATION, AND CHARACTERIZATION OF GEDUNIN FROM *AZADIRACHTA INDICA*

3.1. Introduction

Millennium medicines have been the primary agents from natural sources (Mother Nature), particularly plants. Neem, also known as *Azadirachta indica*, is a plant renowned for its absence from sickness and has been used on the Indian and African continents for thousands of years. Many plant components, including flowers, leaves, grains, and cords, utilized as insecticides, antimicrobials, larvicidal, antimalarial, antibacterial, antiviral, and spermicidal agents, have been employed for the treatment of both acute and chronic human diseases. In traditional medicine, neem plant components have been used for generations to treat several illnesses, including cancer. Seeds, leaves, blossoms, and neem-fruit extracts have consistently demonstrated chemical and antitumor benefits in various cancers. The few bioactive components of neem that have been investigated extensively are azadirachtin, gedunin, and nimbolide, although further studies are needed into several more bioactive ingredients **(Chibber *et al.*, 2014).** Neem significant anticancer actions of neem components on malignant cells include inhibition of cell growth, induction of cellular death, suppression of angiogenesis from cancer, and restoration of cell reduction/oxidation (redox). Although the actual mechanisms of these actions are largely unknown, the removal of the NF-B signalling pathway is partly implicated in neem-component anticancer activity.

Neem is a rare plant that exhibits therapeutic qualities in its leaves, flowers, seeds, fruits, roots, and bark (**Gupta *et al.,* 2017).**In industrialized and impoverished countries, neem is a potentially underused tree. Antimicrobial bioactivities (antibacterial, anti-inflammatory, antipyretic, contraception, antiulcorrient, and anti-constituent of neem) have always been used for antimicrobial purposes (antibacterial, antiviral, and anti-fungal). Neem leaves to feed on dryland ruminants and effectively cure snakes and bug bites. Its twigs are rich in alkaloids, resins, fluoride, sulfur, tannins, oils, saponins, and calcium. Its seeds are rich in protein and fatty acids but remain primarily unused because of bitter, toxic compounds. Its fruits have potential anti-venom and anticancer activities (**Alzohairy *et al.,* 2016).**

Gedunin is a tetranortriterpenoid derived from the Indian neem tree that is used in traditional Indian medicine to treat malaria and other infectious diseases. Furthermore, gedunin has been demonstrated to inhibit cell proliferation in a variety of cell lines, including the prostate, colon, and ovary. The heat shocks reaction, like celastrol and gedunin, produces a strong thiol-reactive electrophile. It is comparable to the triterpenoid structure, which is well renowned for its involvement in anti-venom **(Kamath *et al*., 2009).**

The earliest analytical method for liquid chromatography was the separation of colored components in the early twentieth century. Here, chrome denotes color; the graph implies writing and is derived from the term chroma. A primitive type of chromatographic separation was employed by Mikhail S. Tswett, a Russian botanist, to clear blends of plant colors into pure components, which were separated based on their connection to the stationary phase necessary for chromatographic separation. Powdered chalk and aluminum were the stationary

phases used as the moving solvents. After packing in one column with a solid fixed phase (usually a long, hollow, and glass tube), the plant and the solvent were mixed with the column. He dumped an additional solvent into the column until the samples eluted at the bottom of the column. As plant pigments pass through the static stage, they are separated into bands of pure components, which is the most important conclusion of this study. The original chromatography method, contemporary high-performance chromatography (HPLC), is derived from this separation. The chromatographic process has developed significantly over the last 100 years **(Vogeser *et al.,* 2008).**

Here in extraction, purification, and characterization of gedunin from *Azadirachta indica*, extraction of gedunin from neem using a soxhlet-based method was performed, which was further purified and characterized using column chromatography, HPLC, HR-LCMS, FTIR respectively.

3.2. Experimental

3.2.1. Collection of plant material

Azadirachta indica (neem) ripened fruits were collected from ICAR DELHI, INDIA (accession numbers IC-268588 and IC-268579, respectively).

3.2.2. Extraction and characterization

50 g of *Azadirachta indica* ripened fruits were collected, after which 70% and 80% acetone, 300 ml of chloroform, distilled water, methane (50%), chloroform (30%), acetonitrile (15%) were used to perform the extraction. 50 g of *Azadirachta indica* seeds were used and washed with tap water, distilled water, and 70% acetone, respectively. After the seeds had been converted into powder form, a Soxhlet apparatus was set up with powdered seeds and 300 ml of

chloroform and operated at 50° C for 3 hours. The supernatant liquid was collected, and 80% acetone was added; then, the flask was kept on a shaker for 24 h at 400°C and 150 rpm. After filtering, the supernatant was collected and performed column chromatography with a running buffer composed of methane (50%), chloroform (30%), acetonitrile (15%), and water (5%) at a speed of 7 cm/minute **(Sahai*et al.*, 2020).**

HPLC (High-performance liquid chromatography) was performed on a Shimadzu LC using acetonitrile as the mobile phase and DMSO as the solvent for the lyophilized extracted and purified samples (using column chromatography) acting as the stationary phase **(Bhattacharjee *et al.*, 2020).** The injection volume was 20 µl, 45 minutes run time, and PDA Ch1 254 nm 4 nm.

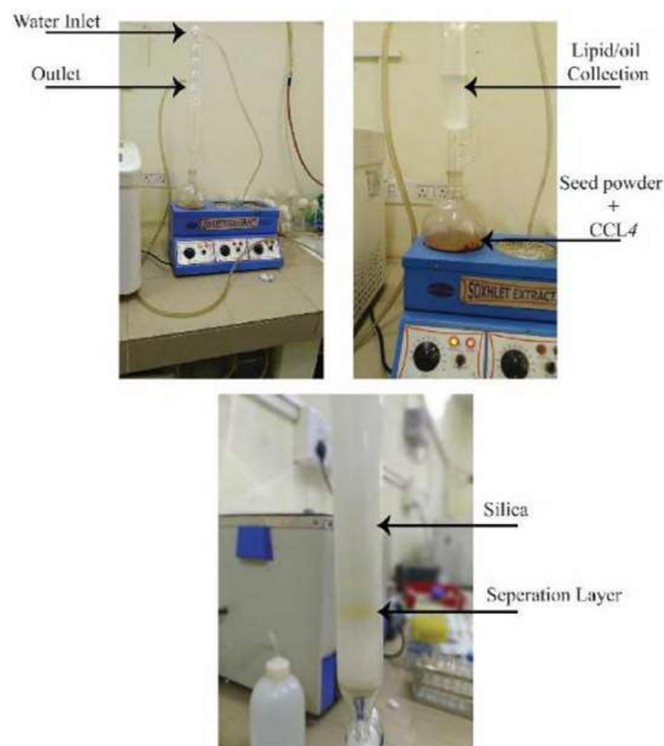


Figure.3.1. Depicting the experimental setup of the Soxhlet-based method and Column chromatography

HRLC- MS –Q-TOF (High-resolution liquid chromatograph mass spectrometer quadruple time of flight) (G6550A model) was used for qualitative and quantitative prediction of fraction three components. Dual AJS-ESI was the ion source, and 100 ul/Min eject and draw speed. The flow rate was 0.300 ml/min, and acetonitrile was run for 30 min at a temperature of 40° C**(Adepiti*et al.,* 2020).**

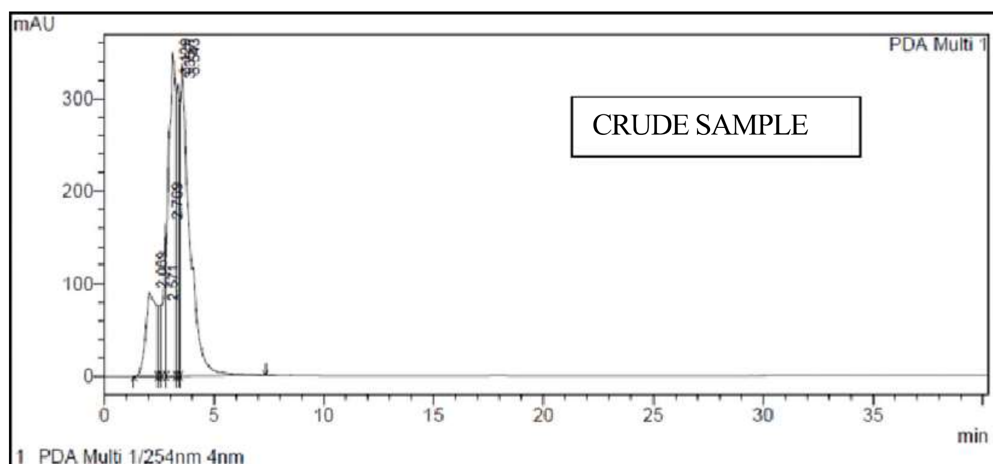
3.3. Results and Discussion

Solvent-based extraction of analytes can be used to analyze large samples **(Figure 3.1.)**. The Soxhlet-based method, which works on the principle of polarity, shows the extraction of gedunin from a neem sample using non–polar to polar solvent for extraction. The gedunin is the non-polar compound that elutes faster in a non-polar solvent; the ratio of solvent is methane(50%): CCL_4(30%): acetonitrile(15%): water(5%). The percent yield of gedunin in fraction 3 was 90 % with 18 gm actual yield. The extracted supernatant was further subjected to column chromatography based on the principle of adsorption. The solutes of the solution with acetonitrile and chloroform of the mobile phase passed through the silica of the stationary phase to separate the components of a sample from the components in the layers.

HPLC was performed using Shimadzu LC software equipped with a pump and a sample injector (manual). The samples were filtered using a filter of 0.22 meters. Separation was carried out using a 5m Agilent and C18 Eclipse column. The mobile phase of acetonitrile was isocratic elution with a flow rate of 1.6 ml/min at 32°C in the test sample **(Toscano *et al.,* 1996). Figure 3.2.** shows the analytical results showing the presence of gedunin in the raw and purified

samples and other components in the range of 240-245 nm wavelength and 2-3 min retention time.

HRLC-MSQ-TOF predicted the presence of gedunin (molecular weight 482.6) at a retention time of 17.559 min. A base peak at m/z 134.8938 was the $C_9H_{14}O$ group of gedunin with M-4 difference in mass units, a base peak at m/z 153 was $C_{10}H_{10}O_2$ with M-9 difference in mass units, and a base peak at m/z 116 was C_8H_{12} with M-8 difference in mass units and the height of the peak was 160804, as shown in **(Figure 3.4.)**

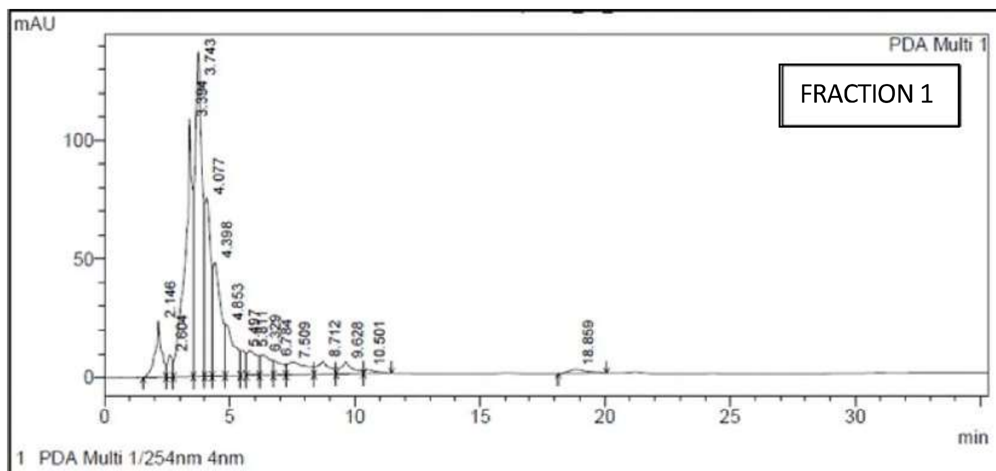


PeakTable

PDA Ch1 254nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.069	2987776	90858	11.570	6.811
2	2.571	782271	76754	3.029	5.754
3	2.769	1415062	164582	5.480	12.338
4	3.129	7812408	349499	30.252	26.200
5	3.357	2947017	315823	11.412	23.676
6	3.543	9879766	336426	38.258	25.220
Total		25824300	1333942	100.000	100.000

Figure 3.2. HPLC characterization of a crude sample of Azadirachta *indica.*

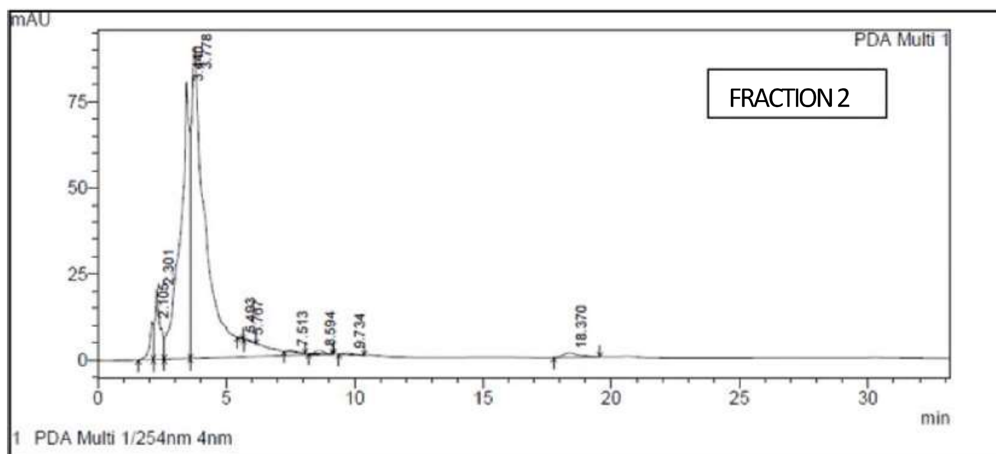


PeakTable

PDA Ch1 254nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.146	403026	23714	4.070	4.958
2	2.604	137976	9138	1.393	1.910
3	3.394	2238346	108656	22.605	22.717
4	3.743	2693484	136723	27.201	28.584
5	4.077	1185999	75146	11.977	15.711
6	4.398	1086636	48076	10.974	10.051
7	4.853	549538	21677	5.550	4.532
8	5.497	144999	10756	1.464	2.249
9	5.811	294136	10551	2.970	2.206
10	6.329	253681	8438	2.562	1.764
11	6.784	149747	6266	1.512	1.310
12	7.509	283863	5324	2.867	1.113
13	8.712	188036	5510	1.899	1.152
14	9.628	164707	4953	1.663	1.036
15	10.501	48605	1720	0.491	0.360
16	18.859	79294	1666	0.801	0.348
Total		9902075	478312	100.000	100.000

Figure. 3.2.1. HPLC characterization fraction 1 of column chromatography.

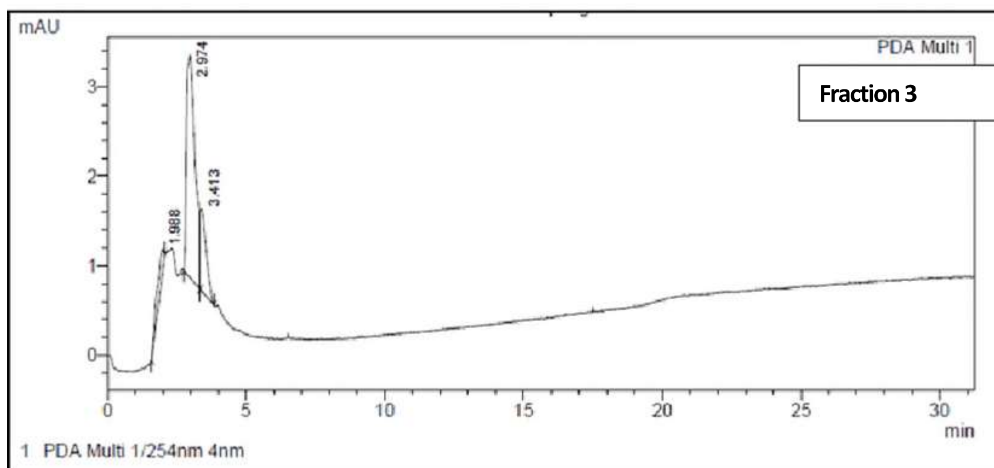


PeakTable

PDA Ch1 254nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.105	107462	10968	1.715	5.335
2	2.301	283410	20575	4.524	10.008
3	3.440	2058180	80130	32.852	38.975
4	3.778	3708960	90314	59.201	43.929
5	5.493	1133	163	0.018	0.079
6	5.767	6711	398	0.107	0.194
7	7.513	10128	470	0.162	0.229
8	8.594	21545	902	0.344	0.439
9	9.734	12372	420	0.197	0.204
10	18.370	55092	1254	0.879	0.610
Total		6264994	205593	100.000	100.000

Figure 3.2.2 HPLC characterization of fraction 2 of column chromatography.



PeakTable

PDA Ch1 254nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	1.988	6265	261	8.505	7.125
2	2.974	54057	2491	73.382	68.026
3	3.413	13343	910	18.114	24.849
Total		73666	3662	100.000	100.000

Figure 3.2.3 HPL Ccharacterization of fraction 3 of column chromatography.

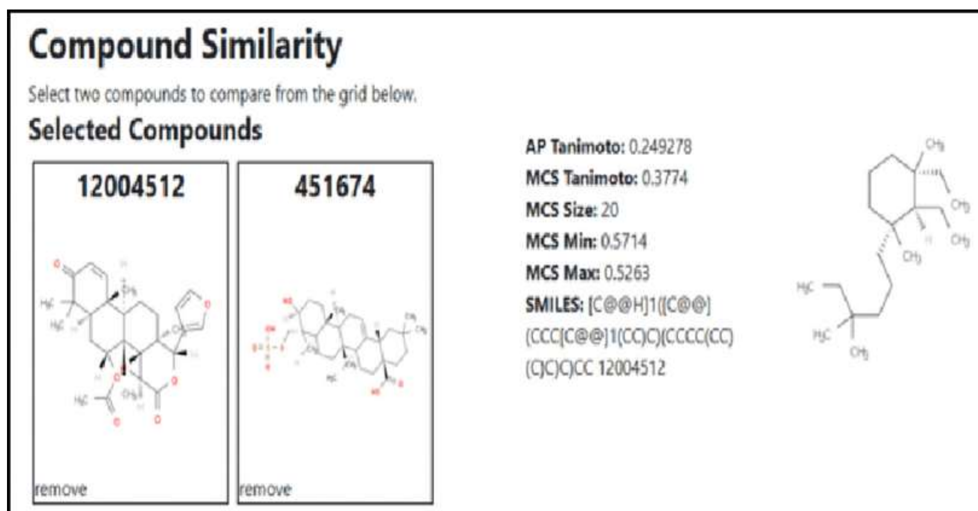


Figure 3.3the similarity between gedunin and triterpenoid

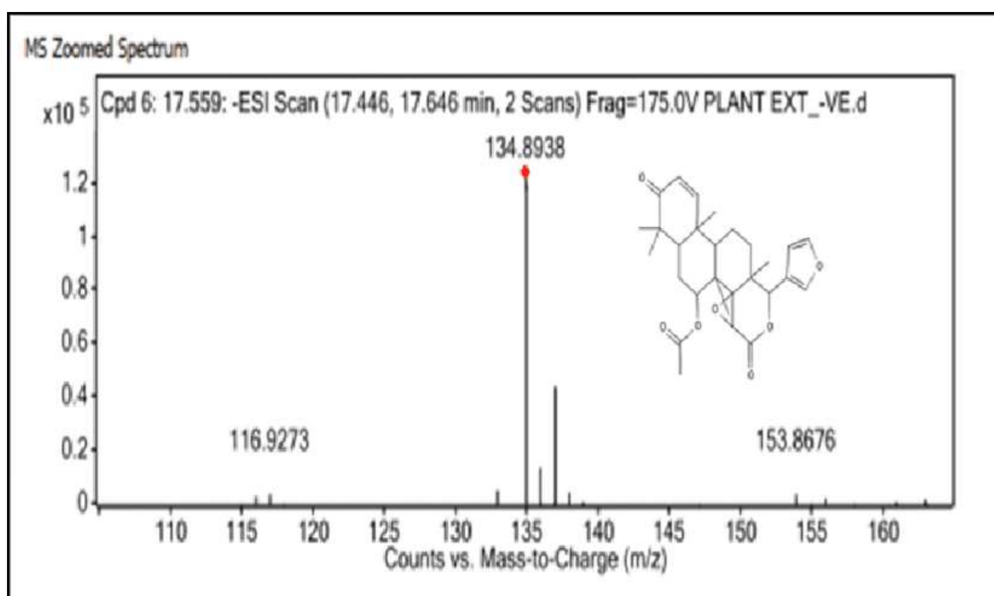


Figure 3.4 HRLCMSQ-TOF analysis of fraction 3 shows the presence of gedunin in a fragmentation pattern with the highest peak at 134.8938.

3.4. Conclusion

The nature and type of solvents employed for extraction and purification of components from A. indica vary; gedunin was extracted using Soxhlet and non-Soxhlet polarity-based methods using non-polar to polar solvents. Gedunin is a

non-polar chemical that elutes quicker in non-polar solvents; the solvent ratio is methane (50%): CCL4 (30%): acetonitrile (15%): and water (5%). The material was purified using column chromatography and analysed using HPLC and HRLC-MSQ-TOF. HPLC analysis indicated the presence of gedunin in fraction 3 of the seed extract with a retention time of 2.974. HRLC-MSQ-TOF proved the presence of gedunin in seed extract and gave extensive data such as a base peak at m/z 134.8938 was C9H14O group of gedunin with M-4 difference in mass units, a base peak at m/z 153 was C10H10O2 with M-9 difference in mass units and a base peak at m/z 116 was C8H12 with M-8 difference in mass units.