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RESEARCH ARTICLE

CHROMATOGRAPHIC FINGERPRINT ANALYSIS OF FLAVONOIDS OF *ACHYRANTHES ASPERA* LINN. BY HPTLC

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ABSTRACT

The study was carried out with an aim to determine the chemical profile and flavanoid composition of the medicinally important plant *Achyranthes aspera* Linn. from methanolic extract of root, stem and leaf. Preliminary phytochemical screening was done followed by HPTLC studies. Ethyl acetate: Formic acid: Glacial acetic acid : water 10:0.5:0.5:1.3 was used as mobile phase for the separation of flavanoids. The flavanoid fingerprint of leaf exhibited 9 peaks each in methanolic neutral, acidic and basic fractions, while that of root exhibited 8 in neutral, 11 in acidic and 7 in basic methanolic extracts. The fingerprint of stem revealed the presence of 8 peaks in neutral, 9 in acidic and 7 in basic methanolic fractions. The study revealed diverse forms of flavonoids in large number in the root, stem and leaf of *Achyranthes aspera* Linn. It can be concluded that HPTLC fingerprint analysis of root, stem and leaf extract of *Achyranthes aspera* Linn. can be used as a diagnostic tool for the correct identification of the plant and it is useful as a phytochemical marker.

Key words: *Achyranthes aspera* Linn. Root, stem, Leaf, Phytochemical Screening, HPTLC Fingerprinting, flavonoids.

1. INTRODUCTION:

Flavonoids comprise a large group of plant secondary metabolites characterized by a Diphenyl propane structure (C6-C3-C6). Numerous pre-clinical and some clinical studies suggest that flavonoids have potential for the prevention and treatment of several diseases. Some epidemiological studies support a protective role of diets rich in foods with flavonoids and a reduced risk of developing cancer and cardiovascular diseases [1-3]. Pre-clinical in vitro and in vivo investigations have shown plausible mechanisms by which flavonoids may confer cancer and cardiovascular protection [4]. In addition to their preventive potential, certain flavonoids may be useful in the treatment of several diseases. Some evidence supporting the therapeutic potential of flavonoids comes from the study of plants used in traditional medicine to treat a wide range of diseases, which has shown that flavonoids are common bioactive constituents of these plants [5]. Flavonoids have been reported to possess many useful properties, including anti-inflammatory activity, oestrogenic activity, enzyme inhibition, antimicrobial activity, anti-cancer, anti-allergic activity, antioxidant activity, vascular activity and cytotoxic anti-tumour activity [6]. Epidemiological studies have illustrated that heart diseases are inversely related to flavonoid intake. Studies have shown that flavonoids prevent the oxidation of low-density lipoprotein thereby reducing the risk for the development of atherosclerosis.

Achyranthes aspera Linn. is an indigenous medicinal plant of Asia, South America and Africa. It is found throughout tropical India as a common weed in fields and wasteland [7] belonging to the family Amaranthaceae. The plant is known for various medicinal properties and used widely for the treatment of different diseases in human. In the recent time, *A. aspera* Linn. is reported to have array of medicinal compounds and medicinal properties. The plant is astringent, digestive, diuretic, laxative, purgative and stomachic. The juice of the plant is used in the treatment of boil, diarrhoea, dysentery, haemorrhoids, rheumatic pains, itches and skin eruptions. The ash from the burnt plant, often mixed with mustard oil and a pinch of salt, and is used as a tooth powder for cleaning teeth. It is believed to relieve pyorrhoea and tooth ache. The leaf is emetic and a decoction is used in the treatment of diarrhoea and dysentery. A paste of the leaves is applied in the treatment of rabies, nervous disorders, hysteria, insect and snake bite [8]. *Achyranthes aspera* Linn. possesses wound healing activity, immune stimulatory properties, larvicidal activity, antibacterial activity and

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antifungal activity. Roots of *A. aspera* Linn. has antioxidant activity and anti-inflammatory properties [9-15]. The main limitation in the use of traditional remedies is the lack of standardization of raw material, manufacturing process and the final product. A biomarker on the other hand is a group of chemical compounds which are in addition to being unique for that plant material also correlates with biological efficacy. So the need arises to lay standards by which the right material could be selected and incorporated into the formulation. HPTLC is a valuable tool for reliable identification because it can provide chromatographic fingerprints that can be visualized and stored as electronic images [16-19]. The present study was intended to resolve the chemical profile and flavonoids constituents present in the stem, leaves, root of *Achyranthes aspera* Linn., which will be useful for the proper identification of commercial samples.

2. MATERIALS AND METHODS:

A. Collection of plant material:

Whole plant parts of *Achyranthus aspera* Linn. were collected in the month of August- September 2013 from natural habitats in Vasai region of Thane district. The plants were authenticated at Blatter's herbarium; St. Xavier's College, Mumbai and the specimens voucher were deposited in the St. Xavier's College Herbarium for further reference. The accession number for *Achyranthes aspera* L. is 62490.

B. Preparation and Extraction of Plant Material:

After confirmation of its botanical identity the leaf, stem and roots were subjected for preliminary phytochemical studies and HPTLC finger print studies. The leaf, stem and roots of *Achyranthus aspera* Linn. were separated, washed thoroughly in distilled water and cut into small pieces. They were shade dried at room temperature. Dried pieces were then uniformly grinded separately using mechanical grinder to make fine powder. The powdered form of plant leaves, roots and stems were stored for future use. The powdered material is then used for preliminary phytochemical studies and HPTLC fingerprinting.

C. Phytochemical Screening:

The preliminary phytochemical investigation of the leaf, stem and roots of *Achyranthus aspera* Linn. was carried out with standard protocol [20]. The extracts were subjected to preliminary phytochemical investigation for detection of flavonoids. The results are presented in Table 1.

D. HPTLC Profile (High Performance Thin Layer Chromatography)

HPTLC studies were carried out following the method of Harborne [21] and Wagner [22] *et al.*

i. Sample Preparation:

Methanolic acidic, basic and neutral extracts obtained by sonication were used for sample application. All the solvents used for HPTLC analysis was obtained from MERCK.

ii. Developing Solvent System:

Different compositions of the mobile phase for HPTLC analysis were tested in order to obtain high resolution and reproducible peaks. The desired aim was achieved using Ethyl Acetate: Formic acid: Glacial Acetic acid: water (10:0.5:0.5:1.3) as the mobile phase

iii. Sample Application:

Chromatograph was performed on 20x10 cm aluminium packed TLC plate coated with 0.2 mm layer of silica gel 60F254 ((E. Merck Ltd, Darmstadt, Germany) stored in a dessicator. 15 µl aliquots of each of the extracts was applied on 8 mm wide band by Hamilton microsyringe (Switzerland), with the nitrogen flow providing a delivery speed of 150 nl/s. The syringe was mounted on a Linomat V applicator attached to CAMAG HPTLC system and was programmed through WIN CATS software. Spotting was performed at 25±2^{o}C ascending development of the plate with elution distance of 80 mm (distance to the lower edge was 10 mm).

iv. Development of Chromatogram:

After the application of sample, the chromatogram was developed in Twin trough glass chamber 20 x 10 cm saturated with solvent vapours of Ethyl Acetate: Formic acid: Glacial Acetic acid: water (10:0.5:0.5:1.3) for 20 minutes. The linear ascending development was carried out and 20 mL of mobile phase was used per chromatography run.

v. Detection of spots:

The developed plate was dried by hot air to evaporate solvents from the plate. The developed plate was sprayed with anisaldehyde sulphuric acid reagent as spray reagent and dried at 100^{o}C on CAMAG plate heater for 3 min.

vi. Photodocumentation:

The plate was kept in photodocumentation chamber (CAMAG REPROSTAR 3) and captured the images under UV light at 366 nm and visible light. The Rf values and finger print data were recorded by WIN CATS software

vii. Densitometric scanning:

Finally, the plate was fixed in scanner stage and scanning was done at 366 nm. Densitometric scanning was performed on Camag TLC scanner III and operated by CATS software (V 3.15, Camag).

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3. RESULTS AND DISCUSSION:

A. Phytochemical Screening:

The phytochemical test on methanolic extracts of *Achyranthus aspera* Linn. leaf, stem and roots showed the presence of flavonoid. (Table 1).

Table1: Flavonoid screening of methanolic extracts of different parts of *Achyranthus aspera* Linn.

Sr. No.	Secondary metabolites	Methanolic Extracts		
		Root	Stem	Leaf
1	Flavonoids	+	+	+

B. HPTLC finger printing of Flavanoids of *Achyranthus aspera* Linn.:

Flavonoids belong to a group of natural substances with variable phenolic structures and are found in fruit, vegetables, grains, bark, roots, stems, flowers, tea, and wine [23]. These natural products were known for their beneficial effects on health long before flavonoids were isolated as the effective compounds. More than 4000 varieties of flavonoids have been identified, many of which are responsible for the attractive colors of flowers, fruit, and leaves. [24] Flavonoid intakes protect against coronary heart disease. [25, 26] Hertog et al [25] stated that the flavonoids in regularly consumed foods might reduce the risk of death from coronary heart disease in elderly men. flavonoids, as antioxidants, can inhibit carcinogenesis. [27] The antiviral activity of flavonoids was shown in a study by Wang et al [28]. Some of the viruses reported to be affected by flavonoids are herpes simplex virus, respiratory syncytial virus, parainfluenza virus, and adenovirus.

Our studies revealed an array of Flavanoids present in the different parts of the plant. The methanolic extract of stem, leaves and root of *Achyranthes aspera* Linn. showed the presence of different types of flavonoids with Rf values ranging from 0.01 to 0.91 (Table 2 - 10). In general more degree of flavonoids diversity has been observed in leaf when compared to the stem and root. Maximum number of flavonoids has been observed in leaves followed by root and stem. Among the 23 different flavonoids of leaf, 12 flavonoids with Rf values 0.05, 0.09, 0.29, 0.32, 0.34, 0.43, 0.52, 0.54,0.57, 0.83, 0.87 and 0.91 are unique to leaf only (Table 2). Sixteen different types of flavonoids have been observed in stem of *Achyranthes aspera* Linn. Among these different flavonoids of leaves, the five flavonoids with Rf values 0.16, 0.68, 0.72, 0.77 and 0.82 are unique to the stem and they are not present in leaves and roots of *Achyranthes aspera* Linn. The flavonoids with Rf values 0.03, 0.15, 0.20, 0.25, 0.39, 0.45, 0.55, 0.69, 0.84 and 0.90 are showed their unique presence only in the root of *Achyranthes aspera* Linn. These are among the twenty two different flavonoids found in the root. The flavonoids with the Rf value 0.19, 0.44 are present in leaf, stem and root of the plant. The flavonoids with the Rf value is found in leaves and stem of the plant. The flavonoids with the Rf values 0.35 is expressed jointly in root and stem of *Achyranthes aspera* Linn.

4. CONCLUSION:

Standardisation of plant materials is the need of the day. An HPTLC fingerprint is suitable for rapid and simple authentication. The HPTLC fingerprint developed may serve as a supplement chromatographic data and the information thus generated may be explored further as a tool for standardization. HPTLC analysis revealed a better separation of array of flavanoids. The plant can be used to discover bioactive products that may serves leads for the development of the new pharmaceuticals that address hither to unmet therapeutic needs. These plant derived bioactive compounds in addition of being developed directly as drugs can also served as prototype drug molecules known as " Lead Compounds" and as pharmacological probes to help better understand biochemical and physiological mechanisms. [29] Bioactivity guided fractionation can lead to the isolation of active principle of this plant and some of the chemical entities with acceptable pharmaceutical qualities can be developed as drugs in their original form directly. In addition to their medicinal use some secondary metabolites from these plants can also serve as powerful "pharmacological tool" to help explain the mechanism underlying human diseases. [30,31]

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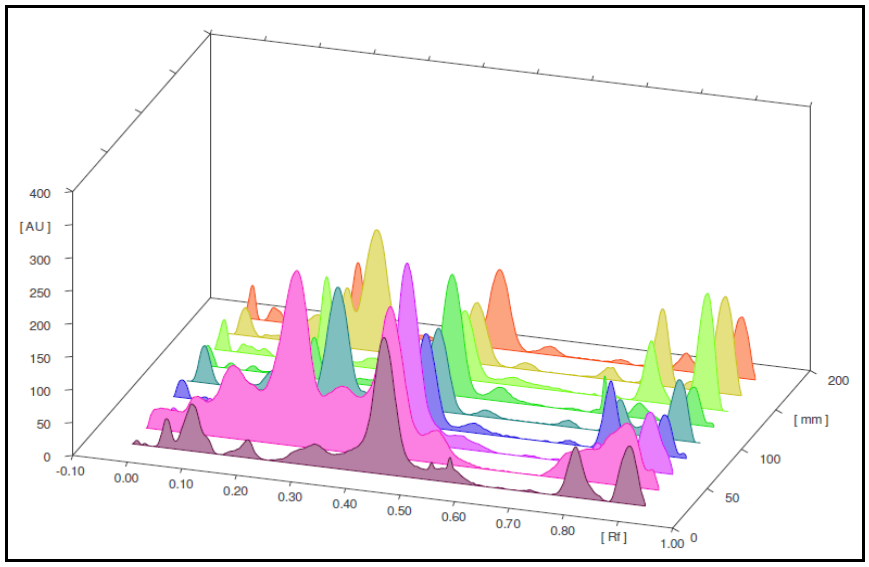

Figure 1: Flavonoids all tracks chromatogram of the methanolic extracts of *Achyranthes aspera* Linn Leaf, stem and root.

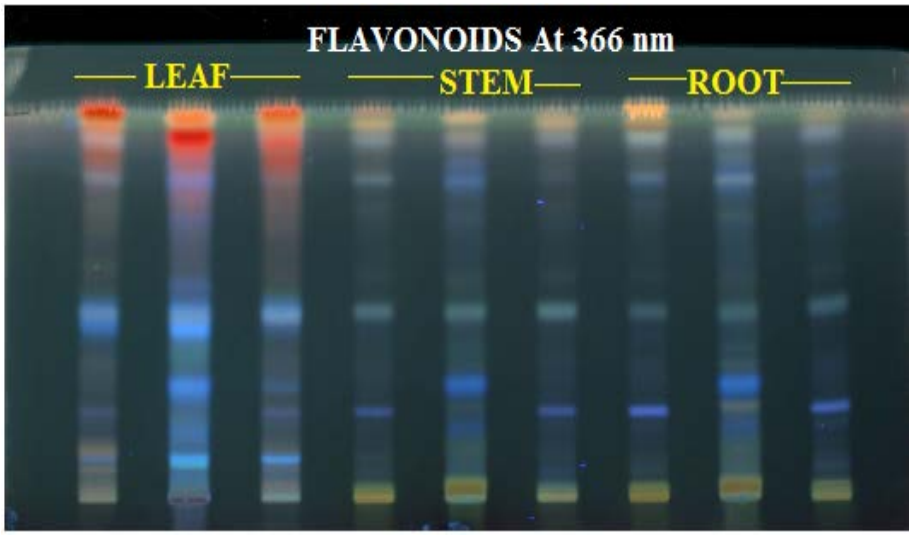

Plate 1: HPTLC Profile of Flavonoids in methanolic extracts of leaf, stem and root of *Achyranthes aspera* Linn. at 366 nm after derivatization.

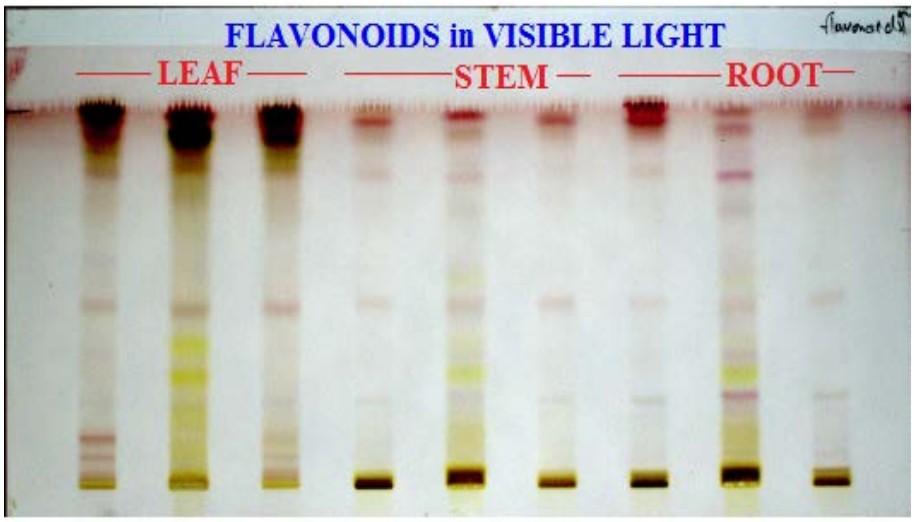

Plate 2: HPTLC Profile of Flavonoids in methanolic extracts of leaf, stem and root of *Achyranthes aspera* Linn. in visible light after derivatization.

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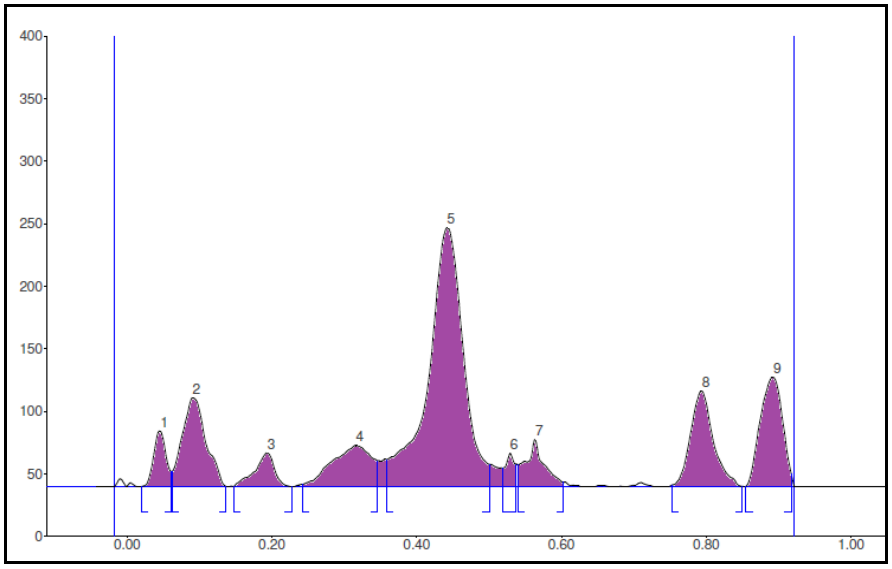

Figure 2: Flavonoids chromatogram of the methanolic neutral extracts of *Achyranthes aspera* Linn Leaf

Table 2: Flavonoids profile of the methanolic neutral extracts of *Achyranthes aspera* Linn. Leaf

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.02	0.1	0.05	44.7	7.28	0.06	11.9	637.8	3.46
2	0.06	12.1	0.09	71.6	11.67	0.14	0.2	1958.0	10.61
3	0.15	0.2	0.19	27.3	4.45	0.23	0.1	614.0	3.33
4	0.24	2.0	0.32	33.3	5.42	0.35	20.8	1519.7	8.23
5	0.36	21.8	0.44	207.2	33.78	0.50	17.8	8382.1	45.41
6	0.52	14.9	0.53	27.0	4.39	0.54	18.0	280.0	1.52
7	0.54	18.0	0.57	37.9	6.18	0.60	3.5	796.7	4.32
8	0.75	1.4	0.80	76.7	12.51	0.85	0.5	2017.8	10.93
9	0.86	0.4	0.89	87.8	14.31	0.92	10.7	2252.5	12.20

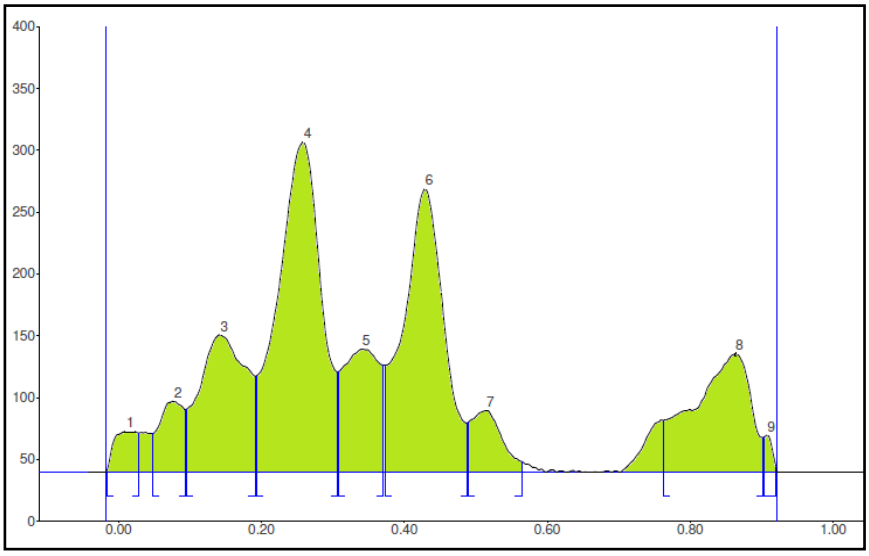

Figure 3: Flavonoids chromatogram of the methanolic acidic extracts of *Achyranthes aspera* Linn Leaf

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Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	-0.02	0.3	0.01	33.1	3.40	0.03	31.7	890.3	1.95
2	0.05	31.2	0.08	57.5	5.92	0.09	50.5	1661.1	3.63
3	0.10	50.8	0.14	111.1	11.42	0.19	77.5	6055.0	13.25
4	0.19	77.6	0.26	266.8	27.43	0.31	80.9	13527.6	29.60
5	0.31	81.2	0.34	99.5	10.23	0.37	86.6	4239.6	9.28
6	0.37	86.3	0.43	229.0	23.54	0.49	39.7	10860.9	23.76
7	0.49	40.1	0.52	49.9	5.13	0.57	8.5	1807.5	3.96
8	0.76	41.9	0.87	95.6	9.83	0.90	27.8	6381.3	13.96
9	0.91	28.1	0.91	30.1	3.09	0.92	3.8	279.3	0.61

Table 3: Flavonoids profile of the methanolic acidic extracts of *Achyranthes aspera* Linn Leaf

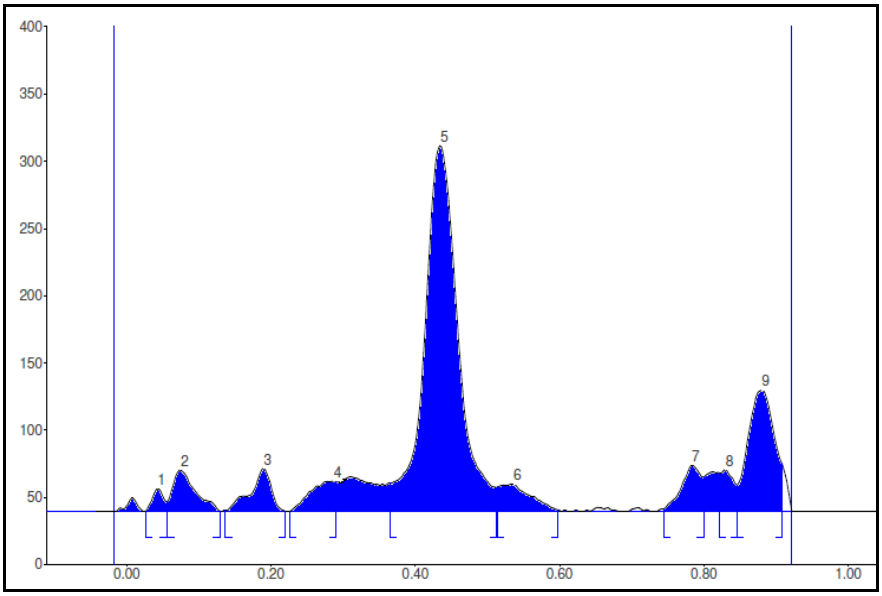

Figure 4: Flavonoids chromatogram of the methanolic basic extracts of *Achyranthes aspera* Linn Leaf

Table 4: Flavonoids profile of the methanolic basic extracts of *Achyranthes aspera* Linn Leaf

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.03	0.0	0.04	16.8	3.06	0.06	6.9	213.2	1.20
2	0.06	7.1	0.08	30.6	5.58	0.13	0.1	792.7	4.47
3	0.14	0.2	0.19	31.7	5.78	0.22	0.0	751.6	4.24
4	0.23	0.0	0.29	22.4	4.09	0.29	22.1	649.5	3.67
5	0.37	20.7	0.44	271.9	49.60	0.51	17.6	10561.8	59.62
6	0.52	18.0	0.54	20.1	3.66	0.60	1.0	737.3	4.16
7	0.75	1.5	0.79	34.0	6.19	0.80	25.3	791.1	4.47
8	0.82	28.4	0.83	30.8	5.61	0.85	19.4	478.2	2.70
9	0.85	19.5	0.88	90.1	16.43	0.91	35.8	2740.6	15.47

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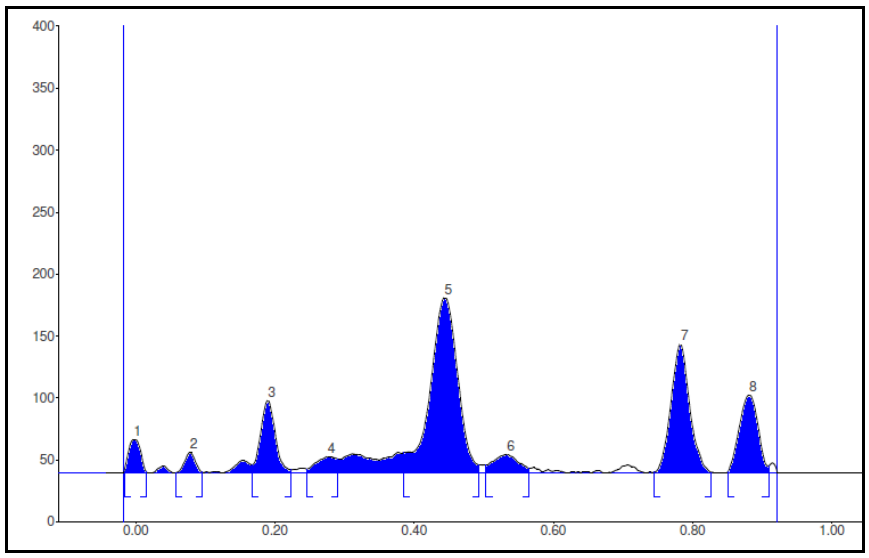

Figure 5: Flavonoids chromatogram of the methanolic neutral extracts of *Achyranthes aspera* Linn Stem

Table 5: Flavonoids profile of the methanolic neutral extracts of *Achyranthes aspera* Linn Stem

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	-0.02	1.2	-0.00	26.9	6.15	0.02	0.5	388.0	3.60
2	0.06	0.1	0.08	16.4	3.75	0.10	0.4	205.1	1.90
3	0.17	6.3	0.19	58.2	13.31	0.22	2.2	1024.0	9.49
4	0.25	3.0	0.28	13.1	3.00	0.29	11.1	311.1	2.88
5	0.39	16.3	0.44	141.4	32.33	0.49	6.1	4664.8	43.24
6	0.50	6.2	0.53	14.9	3.41	0.57	3.5	450.1	4.17
7	0.75	0.3	0.78	104.0	23.76	0.83	0.1	2334.2	21.64
8	0.85	0.9	0.88	62.5	14.29	0.91	5.1	1411.0	13.08

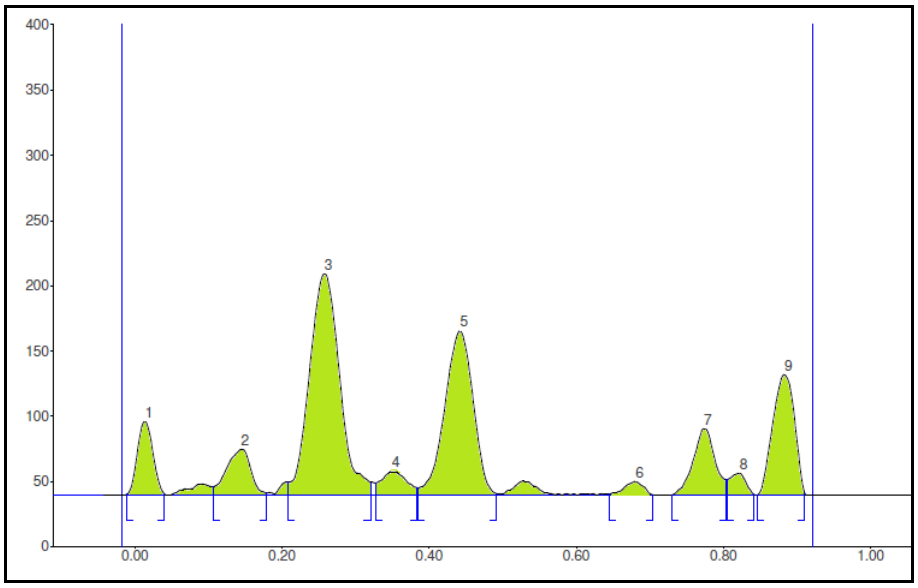

Figure 6: Flavonoids chromatogram of the methanolic acidic extracts of *Achyranthes aspera* Linn Stem

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Table 6: Flavonoids profile of the methanolic acidic extracts of *Achyranthes aspera* Linn Stem

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	-0.01	0.3	0.01	56.6	9.83	0.04	0.6	989.3	6.05
2	0.11	6.4	0.14	35.0	6.08	0.18	1.6	952.3	5.82
3	0.21	9.6	0.26	169.5	29.46	0.32	9.4	5805.8	35.50
4	0.33	9.0	0.35	18.2	3.17	0.38	5.6	520.7	3.18
5	0.39	5.3	0.44	125.9	21.88	0.49	1.0	4041.5	24.71
6	0.65	0.4	0.68	10.4	1.80	0.71	0.1	217.6	1.33
7	0.73	0.1	0.77	50.9	8.85	0.81	11.5	1254.8	7.67
8	0.81	11.9	0.82	16.8	2.91	0.84	0.2	301.3	1.84
9	0.85	0.1	0.88	92.2	16.02	0.91	1.3	2271.6	13.89

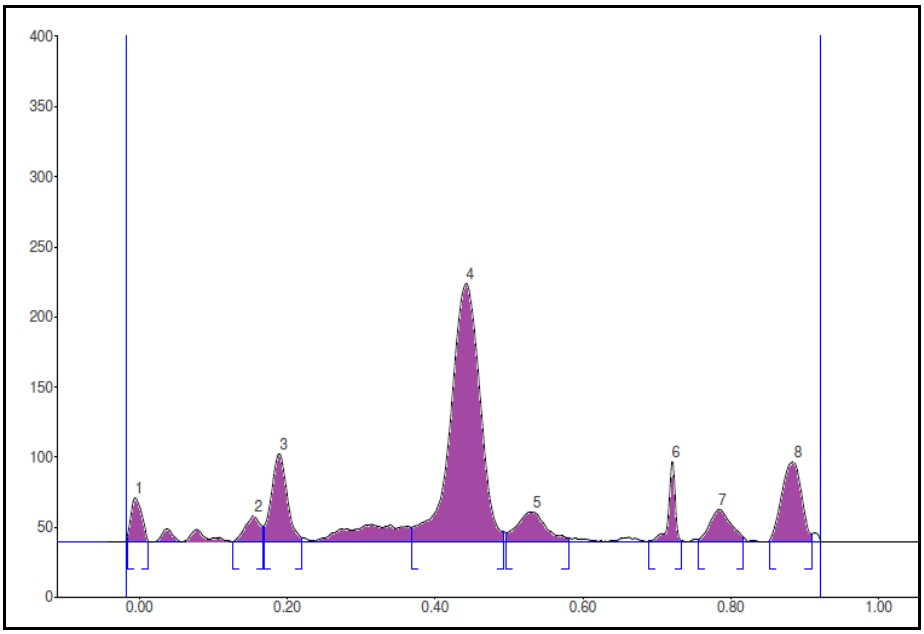

Figure 7: Flavonoids chromatogram of the methanolic basic extracts of *Achyranthes aspera* Linn Stem

Table 7: Flavonoids profile of the methanolic basic extracts of *Achyranthes aspera* Linn Stem

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	-0.02	2.5	-0.01	31.2	6.85	0.01	0.1	385.8	3.53
2	0.13	0.0	0.16	18.3	4.02	0.17	10.7	311.8	2.85
3	0.17	11.1	0.19	62.6	13.77	0.22	2.6	1114.3	10.19
4	0.37	10.3	0.44	184.0	40.45	0.49	7.0	6165.9	56.36
5	0.50	6.3	0.53	21.2	4.65	0.58	2.8	695.4	6.36
6	0.69	0.3	0.72	57.6	12.67	0.73	0.7	437.8	4.00
7	0.76	1.3	0.79	23.1	5.09	0.82	3.6	548.9	5.02
8	0.85	0.6	0.89	56.9	12.50	0.91	5.5	1280.7	11.71

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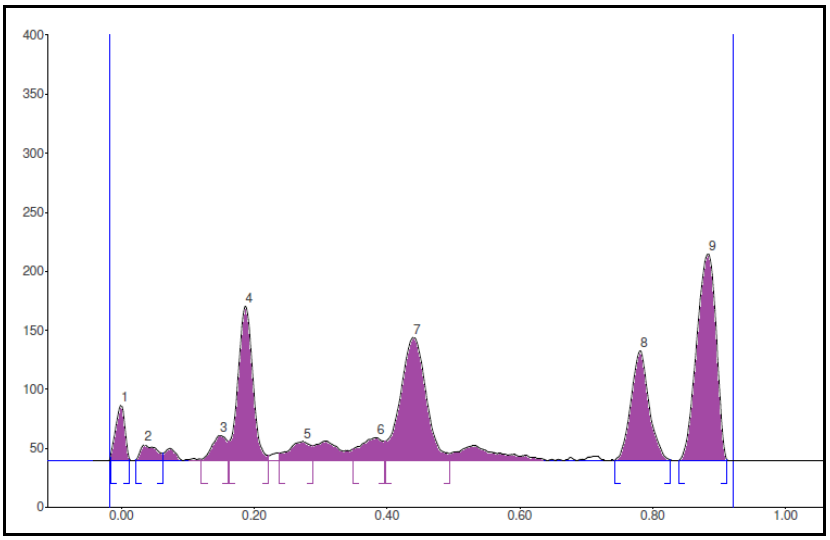

Figure 8: Flavonoids chromatogram of the methanolic neutral extracts of *Achyranthes aspera* Linn Root

Table 8: Flavonoids profile of the methanolic neutral extracts of *Achyranthes aspera* Linn Root

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	-0.02	3.0	0.00	46.8	7.54	0.01	1.2	550.2	3.81
2	0.02	0.5	0.03	13.5	2.17	0.06	5.9	269.0	1.86
3	0.12	0.9	0.15	21.3	3.43	0.16	16.0	389.5	2.70
4	0.16	16.1	0.19	130.8	21.09	0.22	4.2	2433.1	16.85
5	0.24	6.4	0.28	16.1	2.59	0.29	12.4	439.1	3.04
6	0.35	9.9	0.39	19.2	3.10	0.40	16.2	544.1	3.77
7	0.40	16.3	0.44	104.4	16.84	0.50	5.7	3386.7	23.45
8	0.74	0.0	0.78	93.0	15.00	0.83	0.4	2120.8	14.69
9	0.84	0.2	0.89	175.0	28.23	0.91	0.6	4307.8	29.83

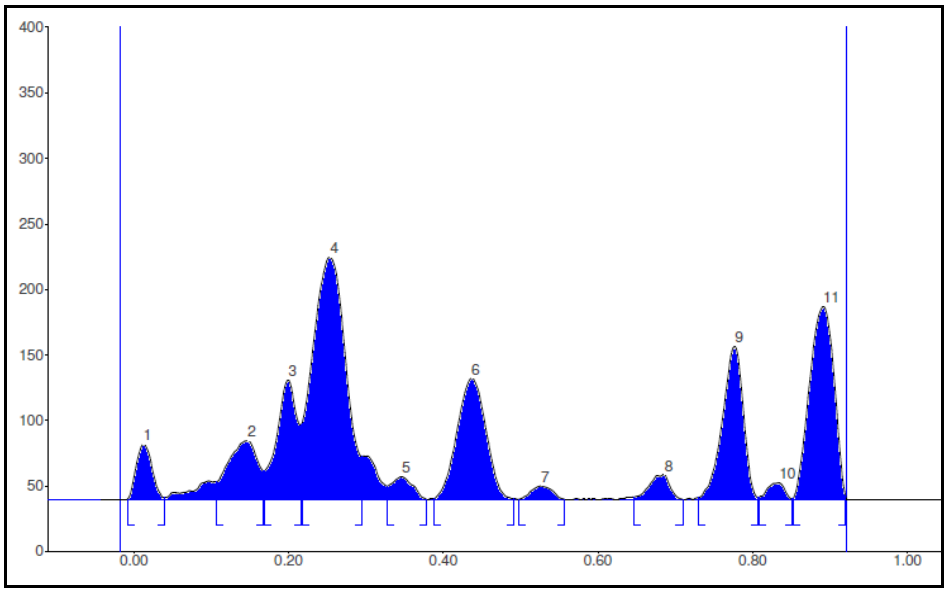

Figure 9: Flavonoids chromatogram of the methanolic acidic extracts of *Achyranthes aspera* Linn Root

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Table 9: Flavonoids profile of the methanolic acidic extracts of *Achyranthes aspera* Linn Root

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	-0.01	0.1	0.01	42.2	5.42	0.04	1.3	721.3	3.35
2	0.11	13.3	0.15	45.0	5.77	0.17	21.6	1438.8	6.68
3	0.17	21.9	0.20	91.2	11.70	0.22	56.8	2039.6	9.47
4	0.22	58.0	0.25	184.8	23.72	0.30	32.9	6602.2	30.65
5	0.33	10.2	0.35	17.4	2.24	0.38	0.2	405.7	1.88
6	0.39	0.6	0.44	92.5	11.87	0.49	0.4	2890.6	13.42
7	0.50	0.1	0.53	10.3	1.33	0.56	0.1	235.0	1.09
8	0.65	2.0	0.69	18.9	2.43	0.71	0.0	421.3	1.96
9	0.73	1.7	0.78	116.8	14.99	0.81	1.4	2645.4	12.28
10	0.81	1.5	0.84	12.8	1.64	0.85	0.3	233.2	1.08
11	0.85	0.6	0.89	147.3	18.90	0.92	2.4	3907.8	18.14

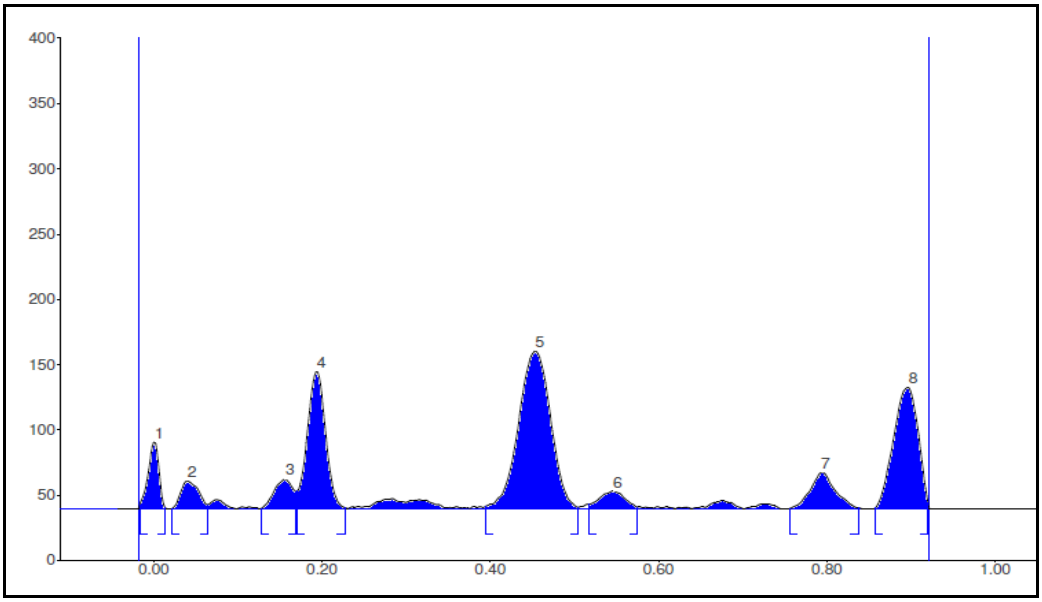

Figure 10: Flavonoids chromatogram of the methanolic basic extracts of *Achyranthes aspera* Linn Root

Table 10: Flavonoids profile of the methanolic basic extracts of *Achyranthes aspera* Linn Root

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	-0.02	2.7	0.00	51.1	11.21	0.01	0.4	547.5	5.28
2	0.02	0.2	0.04	21.4	4.70	0.06	3.0	371.9	3.58
3	0.13	0.0	0.16	22.7	4.98	0.17	13.1	413.3	3.98
4	0.17	13.2	0.19	105.3	23.11	0.23	0.8	1907.2	18.38
5	0.40	1.9	0.45	120.7	26.50	0.51	0.9	3840.1	37.02
6	0.52	3.3	0.55	13.4	2.94	0.58	1.2	338.3	3.26
7	0.76	0.2	0.80	28.0	6.15	0.84	0.7	681.1	6.57
8	0.86	0.5	0.90	93.0	20.41	0.92	4.3	2274.4	21.92

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