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# HPLC-MS and HPTLC Finger printing of fractions of *Asteracantha longifolia* Leaf extract

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**Abstract**
Chromatographic techniques can be used to investigate the molecular fingerprinting of the herbal compounds. In the present study, we explore the HPLC-MS and HPTLC finger printing pattern of three fractions (methanol soluble, water soluble, and residual fraction) of hot water leaf extract of *Astercantha longifolia* (AL) Linn. Nees. Methanolic and residual fractions were fractionated into two peaks each by HPLC with retention times of 1.07, 4.72 min and 0.95, 1.15 min, respectively. On the contrary, only one peak was observed in the aqueous fraction with a retention time of 0.91 min using a Nova- Pak ® C-18 column. Considering the m/z values it could be attributed that methanolic and aqueous fraction may contain a relatively high percentage of a compound having m/z value of 118. Methanolic and residual fraction has an unique m/z value of 114 which was absent in the aqueous fraction. Methanolic fraction has distinct m/z values of 132, 142 and 261 which makes it separate from the other two fractions. Thus, methanolic fraction contains the more number of compounds in comparison to other two fractions. Scanning of HPTLC developed plates of different fractions at 254 nm showed the presence of nine, six and five peaks in methanolic, aqueous and residual fractions, respectively. However, scanning at 366 nm showed the presence of only three, four and two peaks respectively in these fractions. Thus, 254 nm was found to be optimal wavelength for identification of more compounds compared to 366 nm.

**Keywords:** *Asteracantha longifolia,* HPLC-MS, HPTLC, m/z, TIC

## Introduction
Some aquatic weeds and wetland herbs are being observed to have immense medicinal values and *Astercantha longifolia* (AL) Linn. Nees. [Synonym(s) *Hygrophila spinosa* T. Anders] is one of them [1]. Traditional medicinal applications and pharmacological studies of the plant revealed by ancient literature and modern researches showed that AL is a plant of immense medicinal and ethnobotanical importance [1]. AL Nees (Common Bengali name "Kullakhara"; Hindi name "Tal-Makhana", Gokulakanta; Local name "Mokhla"; Sanskrit name "Kokilaksha"; German name "Langblattriger Steindorn") is a commonly found herb in India and being used as a leafy vegetable in some states like Odisha, Chhattisgarh and West Bengal [2, 3]. It has also been observed that boiled aerial parts of succulent plant of pre-flowering and flowering stages are randomly used as haematonic by the rural people of these states [1]. Many reports suggest that AL contains various groups of phytoconstituents which includes carbohydrates, aminoacids, fatty acids, enzymes, alkaloids, polyphenols, flavonoids, phytosterols, different essential minerals, vitamins etc and is found to be useful in the treatment of different diseases like anasarca, diseases involving urinogenital tract, dropsy of chronic Bright's disease, dysentry, asthma, different blood diseases, menorrhagia and different diseases involving genital tract of both male and females etc [4, 5, 6]. In the era of organic farming, AL is to be given particular attention in the treatment of anaemia [1]. Ethanolic extract of the aerial part was reported to increase hemopoietic parameters and also can act as a hepatoprotective agent [7, 8]. Recently, it has been reported that oral administration of AL increases haemoglobin and lymphocytes and reduces neutrophils in the blood of humans and this maintains upto 6-8 months [9]. The application of AL for treatment of different diseases in animals are absent in the literature but different study conducted in different diseased animal models suggests different solvent extracts of AL can be used for the treatment of different liver diseases and also act as haematinics. There is also reports showing its use as an anti-tuberculosis drug. There are many reports suggesting probable use of AL as a therapeutic agent for male and female genital tract diseases [1, 10]. The literature about the molecular fingerprinting of leaf extracts of AL is scanty. Hence, the present study was designed to

investigate the HPLC-MS and HPTLC fingerprinting pattern of different fractions of leaf hot water extract of AL.

**Materials and Methods**

Fresh leaves and aerial parts of AL at its pre-flowering stage were collected aseptically in bulk from water-logged areas of Anjora, Durg, Chhatisgarh during the month of August-September and were cleaned off any extraneous dust or other adhered particles. The cleaned leaves and aerial parts were then dried under shade and grounded into a fine powder form using a domestic mixer grinder. Fine powder thus obtained was stored in airtight containers inside a desiccator for further processing. The plant was identified and authenticated by Professor and Head, Department of Botany and Microbiology, Govt. VYT PG Autonomous College, Durg (CG) which has a specimen no. 04/EVM/Durg and a voucher specimen was preserved for further reference.

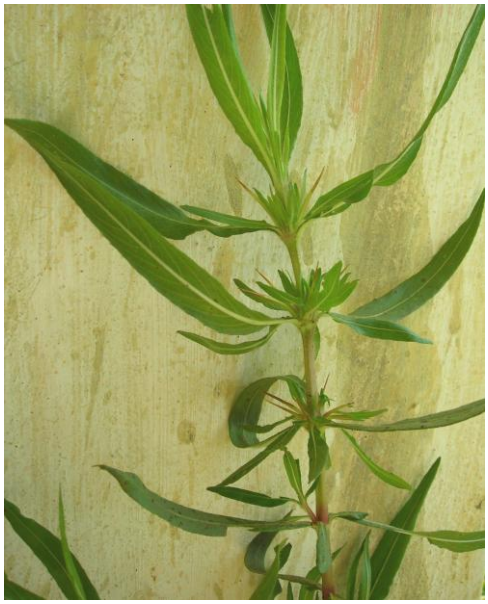


**Fig 1:** *Astercantha longifolia* (AL) Linn. Nees. (pre-flowering stage)

The powdered leaves and aerial parts of AL were processed to obtain concentrate hot water extracts. Briefly describing, dried powdered leaves and aerial parts of the plant (80 g) was dispersed in 1 litre of boiled distilled water (100 °C) with continuous stirring and cooled at room temperature in a closed glass container. The content was filtered with the help of double-layered muslin cloth, and the water infusion was collected. The solution was centrifuged at 5,000 rpm for 10 min, and the supernatant was collected and filtered using Whatman No. 40 filter paper. The extract obtained after filtration was semi dried in the rotary vacuum evaporator (Model-MSW-191, MAC, and New Delhi) at 60 $^{o}$C [11]. The semidried plant hotwater extract was removed from rotary vacuum evaporator and was air dried at room temperature to get complete dried extract. The dried extract was weighed, and the percent (%) recovery of extract was recorded on the dry weight basis. The extract was kept in air tight container and preserved at 4 $^{o}$C for further use [10].

Hot water extract thus obtained was subjected to sequential solvent fractionation using absolute methanol followed by doubled distilled water at room temperature. For methanol soluble fraction, saturated volume of methanol was added to the dried hot water extract and was subjected to semidrying in the rotary vacuum evaporator (MAC Rotary Vacuum Evaporator, BUCHI Type; MSW-191) at 60 $^{0}$C temperature and low pressure, then was completely dried at room temperature. After complete evaporation of the methanol, the weight of the fraction was noted. The leftover fraction of the hot water extract (primary extract) after methanol treatment was subjected to saturated volume of double distilled water treatment in the same manner as the methanol treatment and the final dried product was termed as water soluble fraction. After removal of these two fractions from the primary hot water extract, the residual fraction (third fraction) was dried at room temperature. Then weight of dry residual fraction was recorded. Percent (%) recovery of all the above three fractions were recorded on the dry weight basis. The fractions were kept in air tight containers and preserved at 4 °C for further use. At each step of separation, the change in pH was also noted by using digital pH/mV meter (MSW- 552, MAC, India) [10]. The methanolic extraction was tried to retrieve remnant of methanol soluble phytochemicals, if any from the hot aqueous extract.

**Preparation of samples for HPLC-MS and HPTLC**

Samples for analysis were prepared by dissolving 0.125g of lipholized methanolic, aqueous and residual fraction in 10 ml of HPLC grade distilled water (Merck, Germany) and filtered through a syringe filter with a pore size of 0.22 µm (Nylon acrodisc, Pall Life Sciences ).

Chromatographic and mass spectrometric analysis was performed using a high-performance liquid chromatograph–mass spectrometer (Waters, Milford, Mass, USA). The HPLC-MS system consisted of a pump (Model Delta 600, Waters, USA), on-line degasser, UV detector (Model 2487, Waters) and was coupled online to a mass spectrometric analyzer (Micromass Quattro microTM API). Compounds were separated on a HPLC column Nova- Pak ® C-18, 4µm, 3.9 x 150mm i.d. (Waters, Milford, Mass) using gradient mobile phase of acetonitrile (HPLC grade, J.T. Baker) and 0.1% aqueous formic acid (v/v) (HPLC grade formic acid, Spectrochem Pvt. Ltd., Mumbai, India and HPLC grade Water, Merck, Germany**)** with a flow rate of 1ml / min, source cell temperature of 100 $^{o}$C and desolvation temperature of 350 $^{o}$C. Sample (5µL) was injected, and chromatograms were recorded at 254 and 360 nm using UV-VIS detector (Model 2487, Waters, USA). The MS was set for total ion monitoring between 100 and 1000 Dalton using a positive ion mode [ESI (+)], and column eluent was adjusted at 0.5ml/min for mass spectrometry. Before applying the samples, the base line for HPLC was optimized.

HPTLC finger printing of the fractions were performed using HPTLC assembly of CAMMAG, Switzerland with all its accessories and aluminium plates of 10x10 cm (silica gel $60F_{254}$ of 200 µm thickness, E. Merck, Germany).

Each sample (10 µl) was applied 10 mm above the base of the plate using a semiautomatic sample application device 'LINOMAT - 5' fitted with a syringe of 100 µl capacity using nitrogen gas at 6 psi and then TLC plates were air dried at room temperature.

The HPTLC plates were developed in a twin-trough chamber using the mobile phase toluene: ethyl acetate: methanol (15: 3: 2) v/v. The plates were developed in a pre-equilibrated closed chamber till the solvent reached to 90% height of the plates. The plates were removed solvent fronts were marked and dried on a plate dryer. The developed plates were visualized at 254 nm and 366 nm under a visualizer and photographed by the documentation system. The chromatographic conditions had been previously optimized to achieve the best resolution and peak shape.

## Results and Discussions

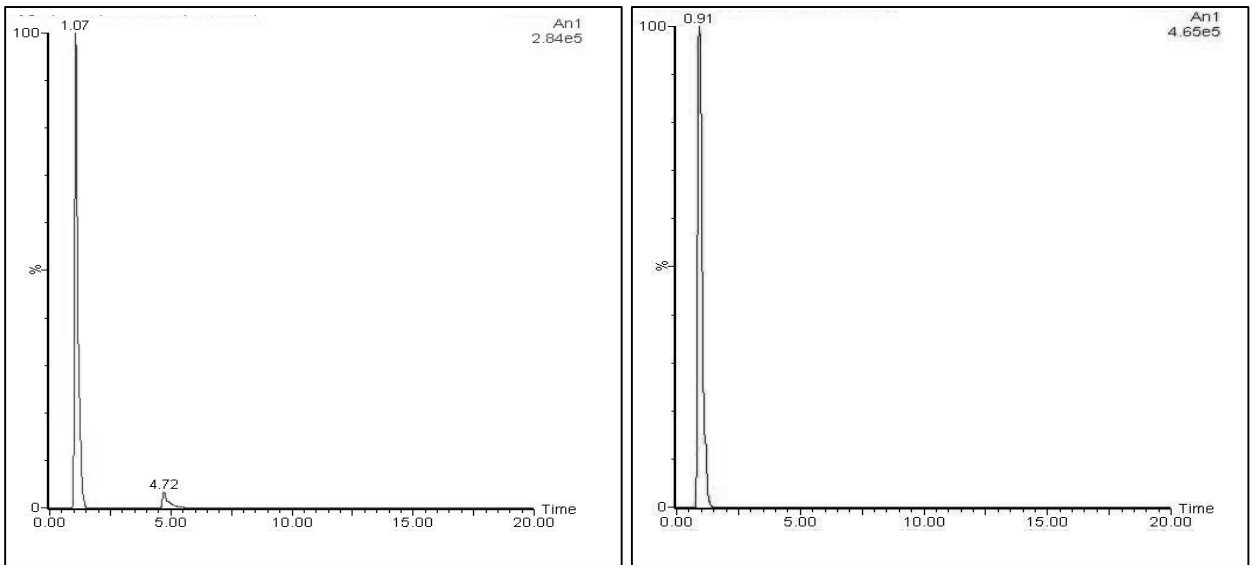
HPLC chromatogram showed different peaks in all the three fractions with different retention times (rt) and the chromatogram patterns found at 254 and 360 nm were similar (Table 1 and Fig. 2). Methanolic and residual fraction showed two peaks with rt of 1.07 and 4.72 min and 0.95 and 1.15 min, respectively. On the contrary, only one peak was observed in aqueous fraction with a rt of 0.91 min.

**Table 1:** HPLC pattern of different fractions in acetonitrile and 0.1% aqueous formic acid gradient

| Name of fraction | Retention time (min) | | | | |
|---|---|---|---|---|---|
| | **0.91** | **0.95** | **1.07** | **1.15** | **4.72** |
| Methanolic fraction | - | - | √ | - | √ |
| Aqueous fraction | √ | - | - | - | - |
| Residual fraction | - | √ | - | √ | - |

' √ ' represents the presence of a peak at the mentioned retention time.

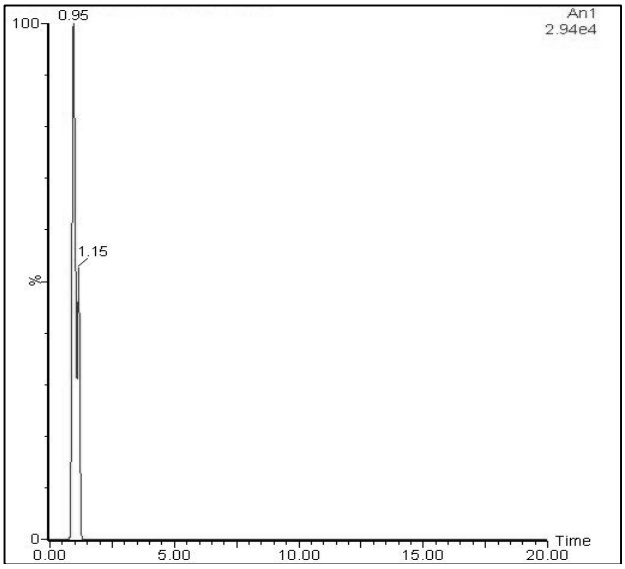
' – ' represents the absence of a peak at the mentioned retention time.




**(a)**




**(b)**




**(c)**

**Fig 2:** HPLC chromatogram of fractions at 254 nm. (a) methanolic fraction at 254 nm, (b) aqueous fraction at 254 nm, (c) residual fraction at 254 nm.

Different peaks were also found in various fractions on analysis using positive total ion current chromatogram (TIC) (Fig. 3, 4, 5). However, methanolic and aqueous fraction showed one peak with almost similar rts 1.22 and 1.23 min, respectively, with a high relative percentage and with a same m/z value of 118. The same m/z value was also found in the methanolic fraction for other two peaks on TIC scan data with rt of 1.12 and 4.43 min whereas one peak in residual fraction with rt of 1.28 min. All these peaks shared a higher relative percentage in both the fractions. Methanolic and residual fraction showed four and one peak with rt of 4.55, 4.84, 6.67 and 7.48 and 2.33 min, respectively, having 114 m/z value on TIC scan. The fraction with similar m/z value (114) was not noticed on TIC scan of the aqueous fraction. Three peaks with 132 m/z was only recorded in TIC scan of methanolic fraction (rt, 1.12, 1.22 and 4.43 min). Peaks with 142 m/z was also present only in TIC scan of methanolic fraction (rt, 1.22, 4.55, 4.84, 6.67, 7.48 min). A major and a minor peak in methanolic fraction (rt, 4.43, 4.55 min) and a minor peak in aqueous fraction (rt, 2.16 min) have m/z value of 277.The peak with 261 m/z was only found in methanolic fraction (rt, 4.43 min).

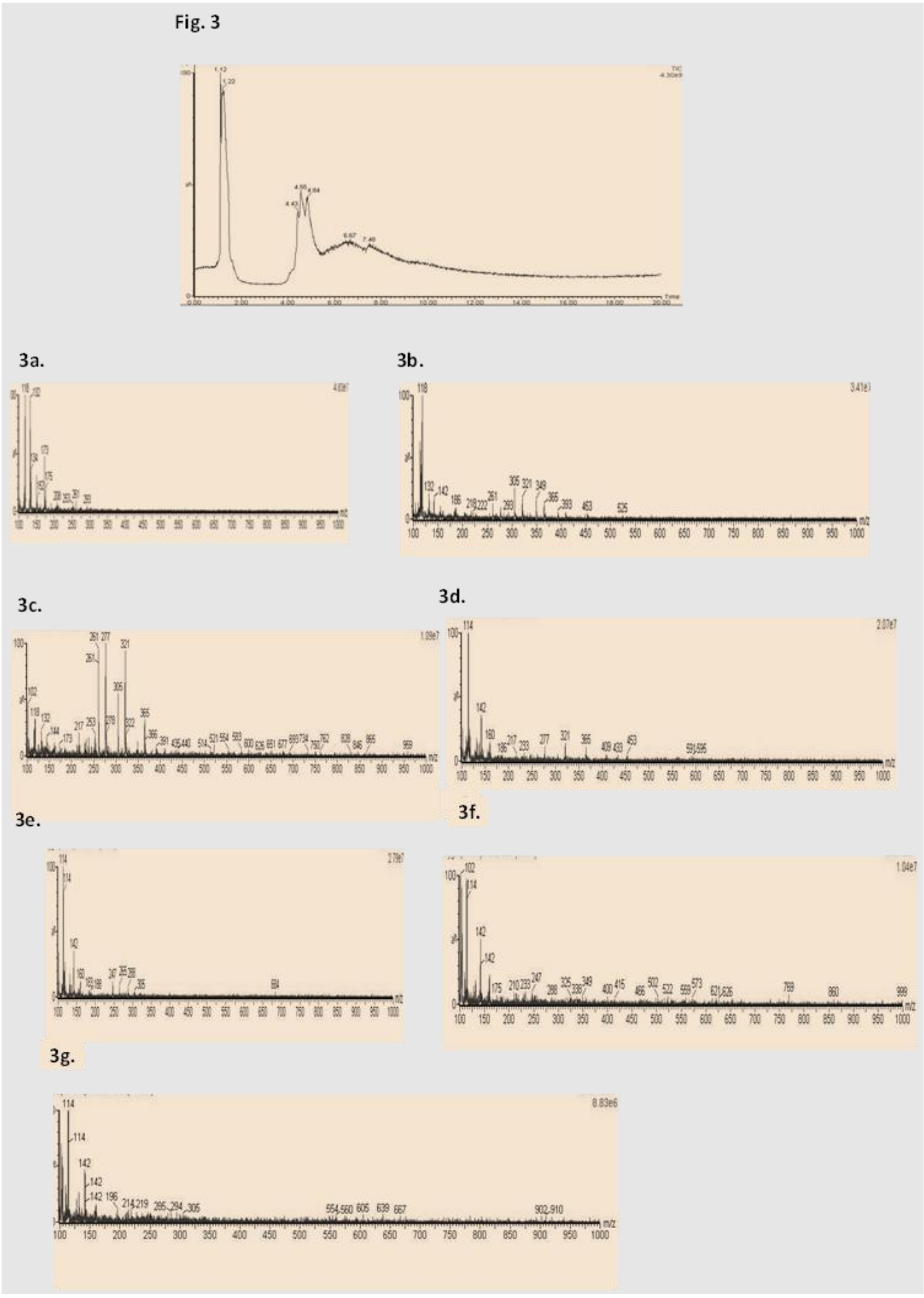



**Fig 3:** Total ion chromatogram (TIC) and Scanned m/z values of peaks of TIC found in methanolic fraction of hot water extract of *A. longifolia* leaf and areal parts. (a) TIC scan of peak found at retention time 1.12, (b) TIC scan of peak found at retention time 1.22 min, (c) TIC scan of peak found at retention time 4.43 min, (d) TIC scan of peak found at retention time 4.55 min, (e) TIC scan of peak found at retention time 4.84 min, (f) TIC scan of peak at retention time 6.67 min, (g) TIC scan of peak found at retention time 7.48 min.

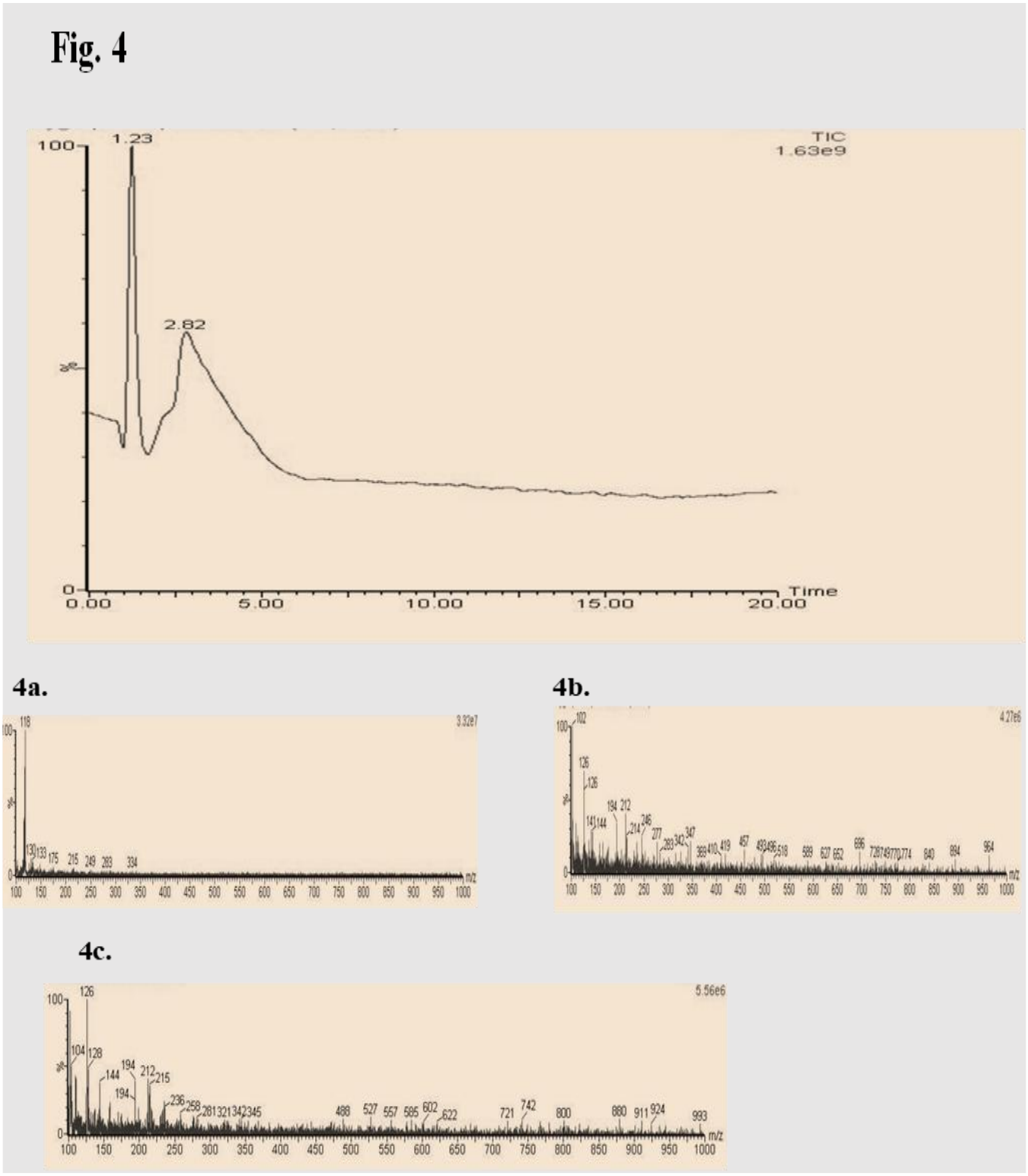



**Fig 4:** Total ion chromatogram (TIC) and scanned m/z values of peaks of TIC of aqueous fraction of *A. longifolia* leaf and areal parts. (a) TIC scan of peak found at retention time 1.23 min, (b) TIC scan of peak found at retention time 2.16 min, (c) TIC scan of peak found at retention time 2.82 min.

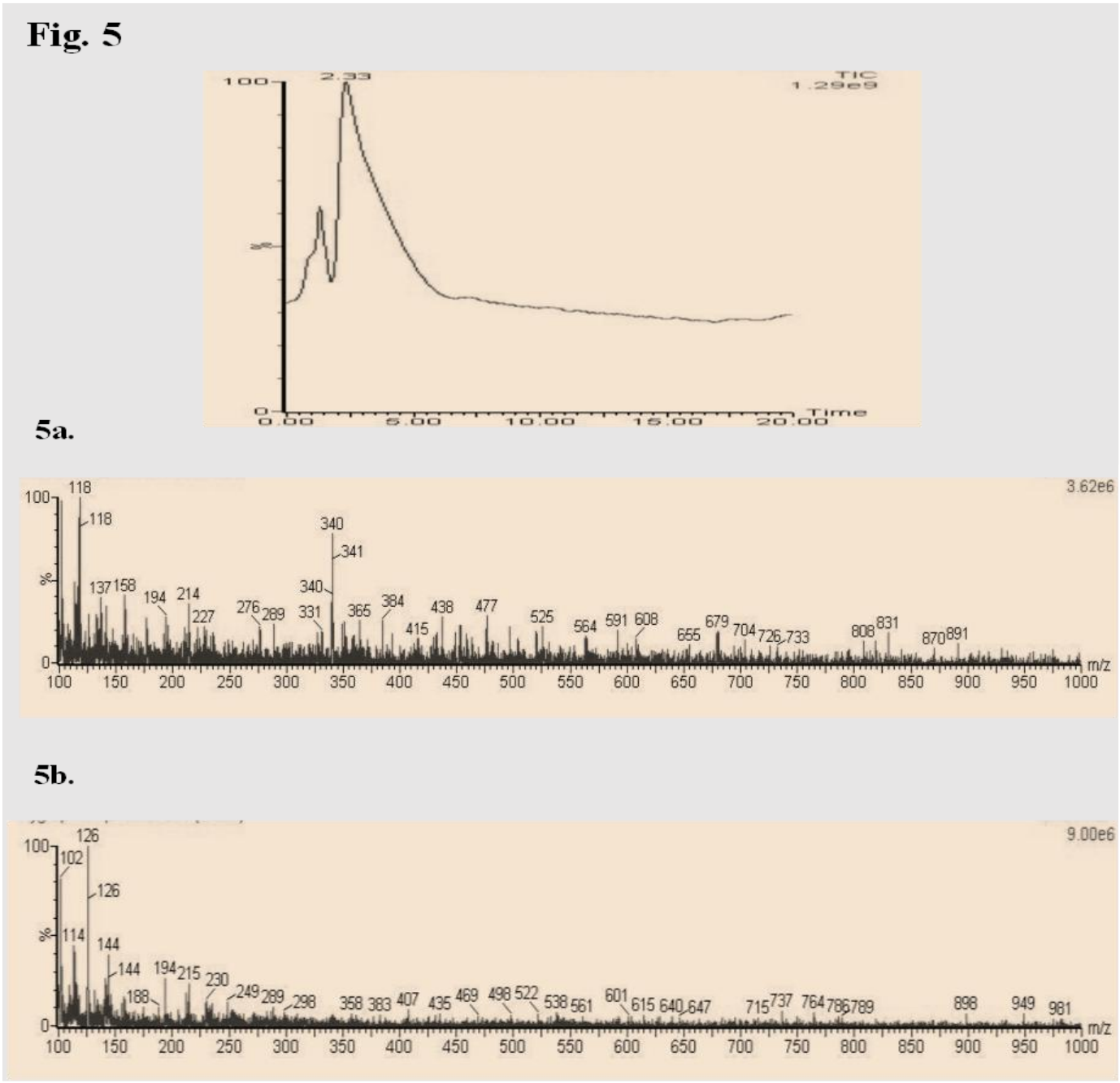



**Fig 5:** Total ion chromatogram (TIC) and scanned m/z values of peaks of TIC of residual fraction of hot water extract of *A. longifolia* leaf and areal parts. (a) TIC scan of peak found at retention time 1.28 min. (b) TIC scan of peak found at retention time 2.33 min.

HPTLC fingerprinting of the fractions were analyzed using mobile phase toluene: ethyl acetate: methanol (15: 3: 2) v/v and visualized at 254 and 366 nm. The results obtained by visualizing and scanning the plates at 254 and 366 nm were presented in Table 2 and Fig. 6. Scanning data of HPTLC plates of different fractions at 254 nm attributed to the presence of nine peaks in methanolic fraction followed by six peaks in aqueous fraction and 5 peaks in residual fraction of hot water extract of AL (Table 2 and Fig. 6). However, scanning data at 366 nm showed the presence of only three peaks in the methanolic fraction, four in aqueous fraction and two peaks in the residual fraction. A higher percentage of area was observed in scan data at 366 nm for the peaks with Rf value of 0.36 and 0.95 in methanolic fraction, peaks with Rf value 0.01, 0.03, 0.4 in aqueous fraction and peaks with Rf value of 0.01, 0.06 in residual fraction. One peak of methanolic fraction and one peak of aqueous fraction with Rf value of 0.03 and 0.69, respectively, revealed a higher area percentage on scanning at 254 nm compared to that of 366 nm. Considering the result of HPTLC scan data it was obvious that 366 nm was not an optimal wavelength for some of the compounds and 254 nm was also not optimal wavelength for some other compounds. However, 254 nm was found to be optimal wavelength for more compounds compared to 366 nm. So, further study is needed to obtain an optimum wavelength suitable for all the compounds present in each of the fractions. The analysis of Rf values calculated on the basis of scanning data showed some similar values in different fractions which are suggestive of the presence of similar compounds as revealed by HPLC-MS data on TIC scan.

Our findings were in partial corroboration with the findings of other workers [12] who reported four spots having Rf values 0.13, 0.54, 0.63 and 0.89 in petroleum ether extract of AL leaf in mobile phase containing toluene and ethyl acetate in the ratio of 8:1; five spots having Rf values 0.40, 0.58, 0.66, 0.74

and 0.90 in chloroform extract in mobile phase containing toluene, ether and cyclohexane in the ratio of 5:2:1; five spots having Rf values 0.08, 0.44, 0.53, 0.75 and 0.89 in mobile phase containing toluene, ethyl acetate and pyridine in the ratio of 17:2:1 in alcoholic fraction and aqueous fraction gives two spots having Rf values 0.37 and 0.67 in mobile phase containing methanol, chloroform and pyridine in the ratio of 2:2:1. The HPTLC estimation of lupeol and sitosterol in various part like root, leaves, seeds and stems of AL was reported in solvent system toluene: ethyl acetate: methanol 15:3:1.5 (% v/v) [13]. Steroidal alkaloids in the methanolic extract of AL leaf were reported using solvent system butanone/xylene/methanol/diethylamine (20:10:5: 1) [14]. The presence of terpenoids, flavonoid and phenolic compounds was confirmed by the TLC pattern developed using n-hexane: ethyl acetate (7:3) as a solvent system [15].

**Table 2:** Rf values and their area % obtained from visualization and scanning of HPTLC plates of three fractions

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Methanolic fraction | 254 nm | Rf value | - | 0.03 | 0.06 | 0.35 | - | 0.40 | 0.43 | 0.58 | - | 0.80 | 0.88 | 0.95 |
| | | Area % | - | 7.59 | 15.78 | 52.84 | - | 2.29 | 13.90 | 2.82 | - | 1.89 | 1.32 | 1.57 |
| | 366 nm | Rf value | - | 0.03 | - | 0.36 | - | - | - | - | - | - | - | 0.95 |
| | | Area % | - | 7.01 | - | 76.58 | - | - | - | - | - | - | - | 16.41 |
| Aqueous fraction | 254 nm | Rf value | 0.01 | 0.03 | - | 0.35 | 0.38 | 0.40 | - | - | 0.69 | - | - | - |
| | | Area % | 55.16 | 13.62 | - | 12.62 | 4.12 | 11.30 | - | - | 3.18 | - | - | - |
| | 366 nm | Rf value | 0.01 | 0.03 | - | - | - | 0.40 | - | - | 0.69 | - | - | - |
| | | Area % | 60.41 | 25.59 | - | - | - | 11.80 | - | - | 2.21 | - | - | - |
| Residual fraction | 254 nm | Rf value | 0.01 | - | 0.06 | - | 0.38 | - | 0.43 | - | - | - | 0.88 | - |
| | | Area % | 6.50 | - | 9.63 | - | 14.41 | - | 26.94 | - | - | - | 42.52 | - |
| | 366 nm | Rf value | 0.01 | - | 0.06 | - | - | - | - | - | - | - | - | - |
| | | Area % | 17.54 | - | 82.46 | - | - | - | - | - | - | - | - | - |

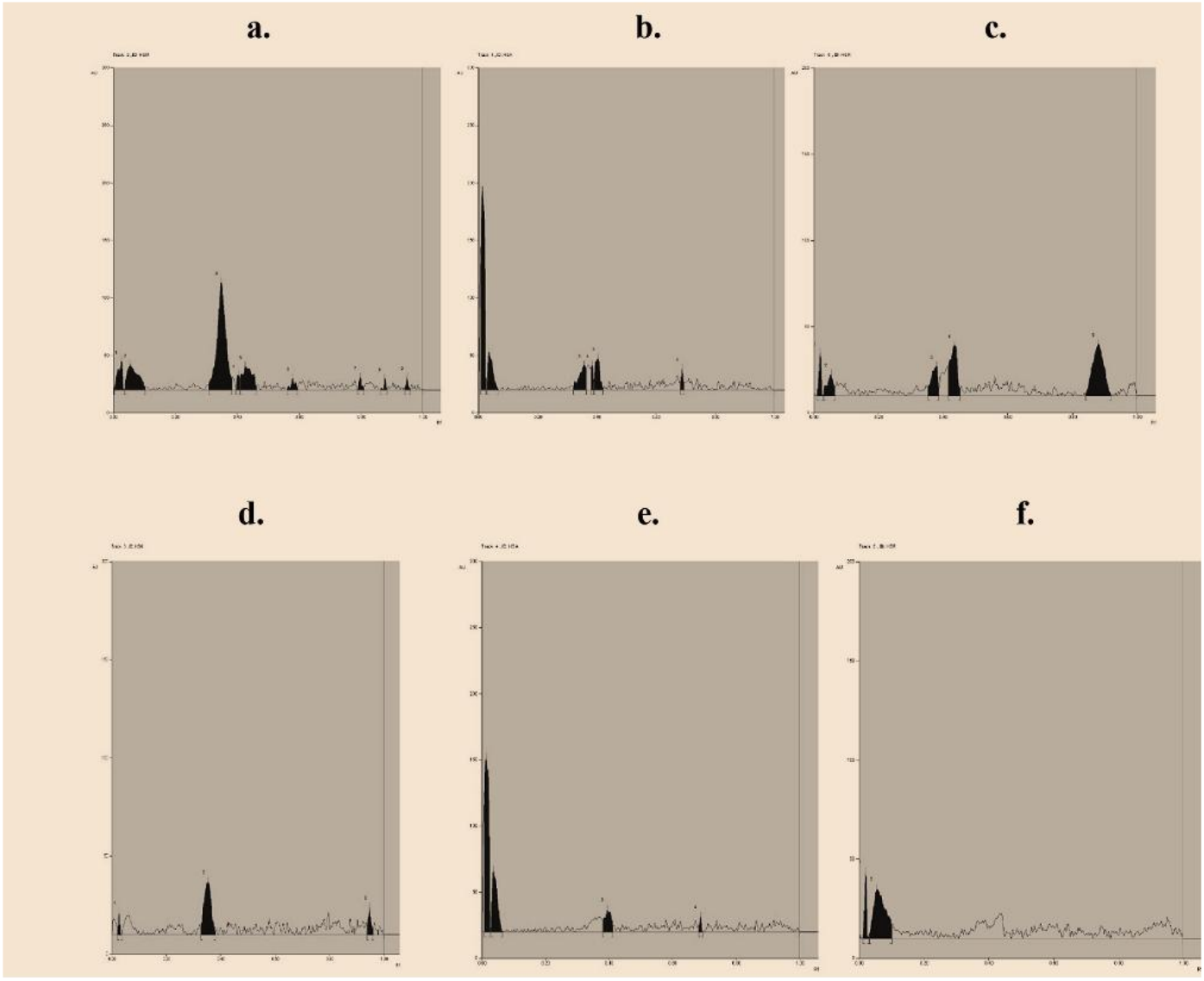



**Fig 6:** HPTLC scan showing peaks of different fractions at 254 and 366 nm. (a) methanolic fraction scanned at 254 nm. (b) aqueous fraction scanned at 254 nm. (c) residual fraction scanned at 254 nm. (d) methanolic fraction scanned at 366 nm. (e) aqueous fraction scanned at 366 nm. (f) residual fraction scanned at 366 nm.

## Conclusions

From the HPLC fingerprinting of AL, it can be concluded that methanolic and residual fraction may contain two different categories of compounds each (rt of 1.07 and 4.72 min and 0.95 and 1.15 min, respectively) and only one category of compound was observed to be present in aqueous fraction with a rt of 0.91 min. From MS data it can be concluded that methanolic and aqueous fraction might have almost similar compounds (rts 1.22 and 1.23 min, respectively) and have a high relative percentage of same m/z value of 118. The m/z value of 118 was also detected in the methanolic fraction with rt of 1.12 and 4.43 min whereas one peak in residual fraction with rt of 1.28 min. Methanolic and residual fraction has an unique m/z value of 114 which was found on four and one peak with rt of 4.55, 4.84, 6.67 and 7.48 and 2.33 min, respectively and was absent in the aqueous fraction. Methanolic fraction has one distinct m/z value of 132, 142 and 261 [(rt, 1.12, 1.22 and 4.43 min), (rt, 1.22, 4.55, 4.84, 6.67, 7.48 min) and rt, 4.43 min which makes it separate from the other two fractions. Thus, methanolic fraction contains the more number of compounds in comparison to other two fractions. The scanning data of HPTLC resolution pattern showed that 254 nm is more optimum wavelength for the compound present in those fractions. From this study, it can be concluded that AL leaf extract fractions although have some similar compounds, the overall composition differs from each other. This fingerprinting can act as a stepping stone in identification of the compound (s) present in AL leaf. This study is completely an *in-vitro* work and the ultimate efficacy of these fractions need to be further studied in animal models which are not the scope of the present study.

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