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Research Article

ISOLATION AND HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY ANALYSIS OF CONDITIONAL AMINO ACIDS FROM THE FRESH LEAVES OF *ALTERNANTHERA SESSILIS*

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ABSTRACT

Objective: The objective of the study was to isolate the conditional amino acids present in the fresh leaves extracts of *Alternanthera sessilis* and compare the isolated amino acids with amino acid standards.

Method: The fresh plant material was used for extraction. For the extracted sample solution, paper chromatography and high-performance thin-layer chromatography (HPTLC) fingerprinting analysis were then carried out.

Results: Since *A. sessilis* is rich in protein, various amino acids have been isolated in aggregates and paper chromatography has been performed. Amino acids were separated and identified using trial and error method. Cysteine, glycine, aspartic acid, and proline are the standard amino acids used for comparing with the isolated amino acids by HPTLC fingerprint analysis, which gives an idea for the authentication of the plant, its constituents and also provides a parameter for quality control.

Conclusion: The results scientifically validate the use of *A. sessilis* in the traditional medicine and also as a food.

Keywords: Fresh leaves extract, *Alternanthera sessilis,* Amino acids, High-performance thin-layer chromatography, Traditional medicine.

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INTRODUCTION

Metabolites are intermediate metabolic product and are of two types - primary and secondary. Primary metabolites usually perform intrinsic function and so present in all plants. Some common examples are carbohydrates, ethanol, lipids, nucleic acid, and proteins. Secondary metabolites usually not directly involved growth, development or reproduction but involved in defense function. Some common examples are alkaloids, terpenoids, glycosides, antibiotics, pigments, and plant growth regulators [1].

Primary metabolites

Primary metabolites are regarded as essential due to their function. They are components or products of fundamental metabolic pathways or cycles such as glycolysis, the Krebs cycle, and the Calvin cycle. Apart from its basic function, they act as a precursor for secondary metabolites and also an end product. Some antibiotics use primary metabolite as precursors, such as actinomycin created from primary metabolite, tryptophan [2].

Amino acid

Amino acids are primary metabolite, occurs in plant both in the free state and as the basic units of proteins and other metabolites. Some 20 different amino acids are isolated from proteins while other occurs in a free state, isolated from plants and microorganisms. Plant and bacteria can synthesize all 20 amino acids whereas mammals can able to synthesize only about half of them. Amino acid those synthesized in mammals are considered to be non-essential amino acid and not needed in the diet, whereas the remainder amino acids are considered an essential amino acid must be obtained from food [3]. As per the WHO guidelines, essential amino acid recommended for adult (70 kg) as follows, Tryptophan - 7 mg/g, Threonine - 27 mg/g, Isoleucine - 25 mg/g, Leucine - 55 mg/g, Lysine - 51 mg/g, methionine and cysteine - 25 mg/g, phenylalanine and tyrosine - 47 mg/g, valine - 32 mg/g, and histidine - 18 mg/g [4].

Six other amino acids are considered conditionally essential in the human diet, meaning their synthesis can be limited under special pathophysiological conditions, such as prematurity in the infant or individuals in severe catabolic distress. They are arginine, cysteine, gylcine, glutamine, proline, and tyrosine. The recommended daily intakes for children aged 3 years and older are 10–20% higher than adult levels, and those for infants can be as much as 150% higher in the 1st year of life. Cysteine (or sulfur-containing amino acids), tyrosine (or aromatic amino acids), and arginine are always required by infants and growing children [3].

Glycine is not essential to the human diet, as it is biosynthesized in the body from the amino acid serine, which is, in turn, derived from 3-phosphoglycerate, but the metabolic capacity for glycine biosynthesis does not satisfy the need for collagen synthesis. Pharmaceutical grade glycine is produced for some pharmaceutical applications, such as intravenous injections, where the customer's purity requirements often exceed the minimum required under the USP grade designation [3].

Aspartic acid deficiency leads to a decrease in cellular energy. Reduces stamina and chronic fatigue are the issues associated with this deficiency. It may also lead to depression. Amino acids help in the removal of excess ammonia from the body so aspartic acid deficiency may result in the elevated ammonia levels in the blood. This condition is responsible for the damage to liver, brain, and nervous system. D-aspartic acid is the supplement, responsible to increase the levels of testosterone in men. It helps the men to become muscular faster. However, the excess use of D-aspartic acid results in various side effects on health [4].

Many secondary metabolites are biosynthetically derived from an amino acid (primary metabolites). Some examples are proline, hydroxyl proline, ornithine, and arginine act as a precursor in the production of secondary metabolites like alkaloids. Amino acids have lots of applications in bodybuilding functions in plants, mammals and also precursors for secondary metabolites (biomolecules) [2].

Herbs are usually rich in amino acid, one such plant is *A. sessilis,* and its phytochemical analysis reveals the presence of amino acid [5] and also possesses wound healing activity [6]. *A. sessilis* commonly known as sessile Joyweed is a perennial herb, often found in and near ponds, canals, and reservoirs reported 5% of protein. In traditional medicine, the whole plant is used internally and externally and used to treat diarrhea, skin diseases, night blindness, indigestion, and fever. *A. sessilis* is being widely used for cooking by people of Tamil Nadu and other Southern states of India.

METHODS

Collection of plant material

A. sessilis leaves were collected in and around in Coimbatore District, Tamil Nadu, India, and authenticated by Dr. C. Murugan, Scientist "D" and Head of office, T.N.A.U, Coimbatore, Tamil Nadu, India. Authentication number BSI/SRC/5/23/2017/Tech-3527.

Isolation of free amino acid

Fresh leaves (150 mg) of *A. sessilis* were collected and kept in a frozen condition along with the liquid nitrogen to prevent degradation. Frozen leaves were grinded in a dry mortar along with liquid nitrogen and 600 µl of a mixture of solutions containing water, chloroform, and methanol in a ratio (3:5:12 v/v). Addition of methanol makes the mixture homogenized. This homogenate is completely transferred to 2 ml centrifuge tube and remaining in the mortar was also rinsed with mixture of solution and transferred to the centrifuge. Immediately the mixture was centrifuged at full speed for 2 min and supernatant solution was collected separately.

Re-extract the residue with another 600 µl of mixture of solutions containing water, chloroform, and methanol for about 2 min and only the supernatant solution were transferred to fresh tube. Centrifugation continued till clear solution is obtained. Samples were stored at −80°C and the extracted amino acids were quantified using ninhydrin assay. Usually, amino acid will be degraded within 1 week, so it is being quantified immediately [7].

This method is more reliable and less costly. Hence, free amino acid can be isolated from the fresh leaves of *A. sessilis* and can be used for further work.

Paper chromatography

Paper chromatography is an analytical method used to separate colored chemicals or substances. In paper chromatography, the sample mixture is applied to a piece of Whatman filter paper using the capillary tube and the edge of the paper is immersed in a solvent (mobile phase) and the solvent moves up the paper by capillary action. The filter paper is then dried and sprayed with ninhydrin reagent. Once again stationary phase dried in a hot air oven for 110°C.

Amino acids isolated from fresh leaves are separated using chromatogram and compared with various standard amino acids using trial and error method [8].
Paper development: Ascending Techniques
Stationary phase: Whatman filter paper Grade 1
Mobile phase: n-Butanol:acetic acid:water (4:1:5)
Detecting agent: Ninhydrin reagent.

High-performance thin-layer chromatography (HPTLC) of Amino acid

HPTLC based methods are considered as a good alternative, as they are being explored as an important tool in routine drug analysis. Major advantage of HPTLC is its ability to analyze several samples simultaneously using a small quantity of mobile phase. This reduces time and cost of analysis. In addition, it minimizes exposure risks and significantly reduces disposal problems of toxic organic effluents, thereby reducing the possibilities of environment pollution. HPTLC also facilitates repeated detection of chromatogram with same or different parameters.

Cysteine, glycine, aspartic acid, and proline are four amino acids used as a standard, and the solution containing free amino acids isolated as per the method described above was used as a test solution for HPTLC analysis. n-butanol - 40%+ glacial acetic acid - 10%+water - 50% was used as a solvent system to develop HPTLC fingerprint profile for amino acids containing the sample. 1 µl of the standard solution and 2 µl of test solution were loaded as 5 mm band length separately on precoated silica gel 60F 254 aluminum sheets (3×10 cm) using a Hamilton syringe with the help of Linomat 5 applicator attached to a Camag HPTLC system, which was programmed through WIN CATS software. After the application of spots, the chromatogram was developed in twin trough glass chamber (20×10 cm) pre-saturated with respective mobile phase. The air-dried plates were kept in a CAMAG Visualizer and images were captured at visible light, UV 366 nm and UV 254 nm. The chromatograms were scanned by a CAMAG TLC Scanner after spraying with respective spray reagents and dried at 100° in a hot air oven. The peak number with its height, area and R f values of fingerprint data were recorded by WIN CATS (1.3.4 version) software [9-14].

Stationary phase:
Plate size (x × y) : 10.0 × 10.0 cm
Material: HPTLC plate silica gel 60 F 254
Manufacturer : E. MERCK KGaA
Sample application- CAMAG Linomat 5:
Instrument: CAMAG Linomat 5 "Linomat5_170147"
Linomat 5 Application Parameters
Spray gas : Inert gas
Sample solvent type: Methanol
Dosage speed : 150 nl/s
Predosage volume: 0.2ul
Sequence
Syringe size : 100 microliter
Number of tracks: 5
Application position Y : 8.0 mm
Band length : 8 mm
Development-glass tank:
Chamber type : Twin Trough Chamber 10×10 cm
Executed by : AAS
Pre Conditioning
Mobile phase : n-Butanol 40%: Glacial acetic acid 10%: Water 50%
Solvent front position: 70.0 mm
Volume : 10.0 ml
Drying device : oven
Temperature: 60°C
Time : 5 min
Detection
Instrument : CAMAG TLC Scanner

RESULTS AND DISCUSSION

Isolation of free amino acid

From fresh leaves juice of *A. sessilis,* as per the procedure discussed earlier various free amino acids are isolated in the form of aggregates. Hence, to separate the amino acids aggregates and also to assume the amino acid, paper chromatography method is applied.

Paper chromatography

Since paper chromatography is one of the reliable methods for determination of amino acid, isolated amino acids are identified using trial and error method. n-Butanol:glacial acetic acid:water in a ratio 4:5:1 is used as a mobile phase since polar side chain amino acids (aspartic acid, cysteine, lysine, etc.) and shorter non-polar amino acids (proline, alanine, and glycine) migrates faster in this solvent. The sample is separately developed and viewed at 362 nm reported in Fig. 1 and also viewed after spraying detecting agent (ninhydrin reagent) is reported in Fig. 2.

Then using trial and error method, the sample containing amino acid is being compared with various standard amino acids and four amino acids have been assumed based on the coinciding R_f values.

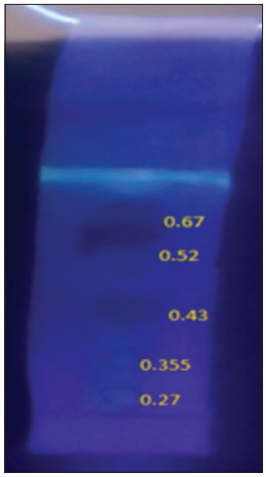

Fig. 1: Paper chromatography of isolated amino acid detected at UV 362nm

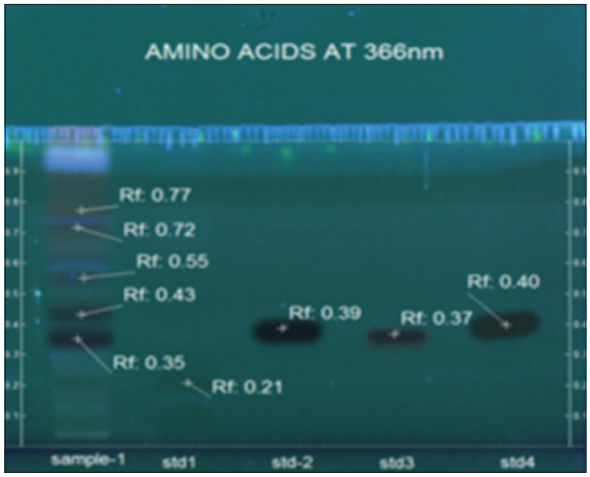

Fig. 4: High-performance thin-layer chromatography chromatogram of the amino acid at 366 nm

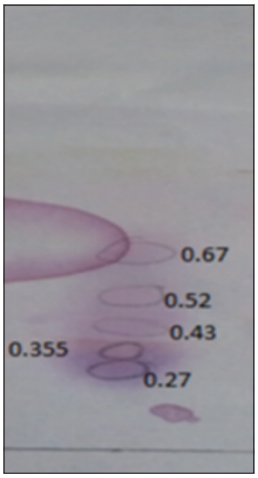

Fig. 2: Paper chromatography of isolated amino acid detected using ninhydrin

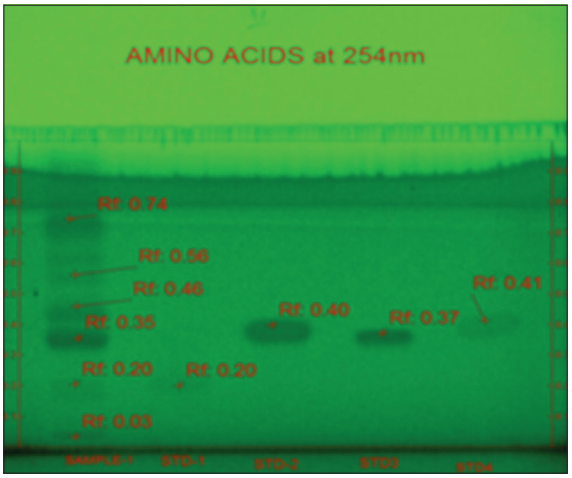

Fig. 5: High-performance thin-layer chromatography chromatogram of the amino acid at 254 nm

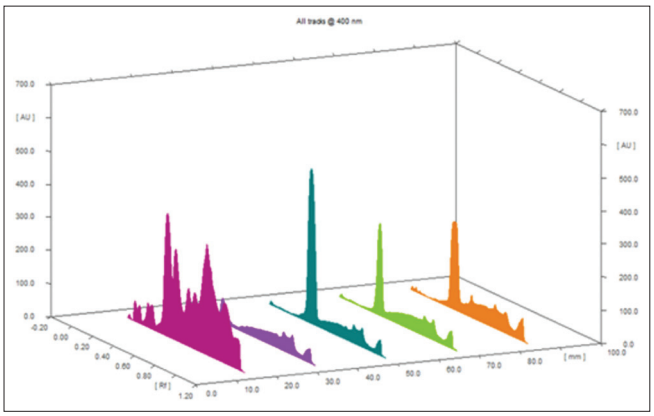

Fig. 3: 3- Dimensional view of chromatogram of sample with four standard amino acids

The amino acids assumed by paper chromatography are cysteine, glycine, aspartic acid, and proline.

HPTLC analysis of amino acid

Seven different types of amino acids were observed with peak value from the isolated sample of *A. sessilis.* The suitable solvent system selected for evaluation was n-Butanol:glacial acetic acid:water (4:1:5).

The chromatogram of the sample was developed along with the four standard amino acids. HPTLC fingerprint of a plant species provides basic information regarding isolation, purification, and characterization. This present study provides sufficient information about amino acids present in fresh leaves of *A. sessilis* and also identification, standardization and quality control of the medicinal plant. HPTLC chromatogram of the sample was reported in Table 1. indicates the presence of seven compounds.

3- D dimension of sample with standard amino acid represented in Fig. 3. The results from HPTLC scanned at 366 nm and reported in Fig. 4, for the amino acid isolated from fresh leaves of *A. sessilis*, reveals the presence of 5 amino acids with the R_f values: 0.35, 0.43, 0.55, 0.72, and 0.77. The R_f values for standard amino acids cysteine, glycine, aspartic acid, and proline are 0.21, 0.39, 0.37, and 0.40, respectively. Comparing with the four standard amino acids, the R_f value of standard (I) 0.37 coincide with R_f value of sample 0.35 and the R_f value of standard (II) 0.40 coincide with R_f value of sample 0.43.

The results from HPTLC scanned at 254 nm and reported in Fig. 5, for an isolated sample containing amino acid reveals the presence of 6 amino acids with the R_f values: 0.03, 0.20, 0.35, 0.46, 0.56, and 0.74. The R_f values for standard amino acids cysteine, glycine, aspartic acid, and proline are 0.20, 0.40, 0.37, and 0.41, respectively. When comparing with the four standard amino acids, the R_f value of standard (I) 0.20 coincide with R_f value of sample 0.20, the R_f value of standard (II) 0.40 coincide with R_f value of sample 0.35, and the R_f value of standard (III) 0.41 coincide with R_f value of sample 0.46.

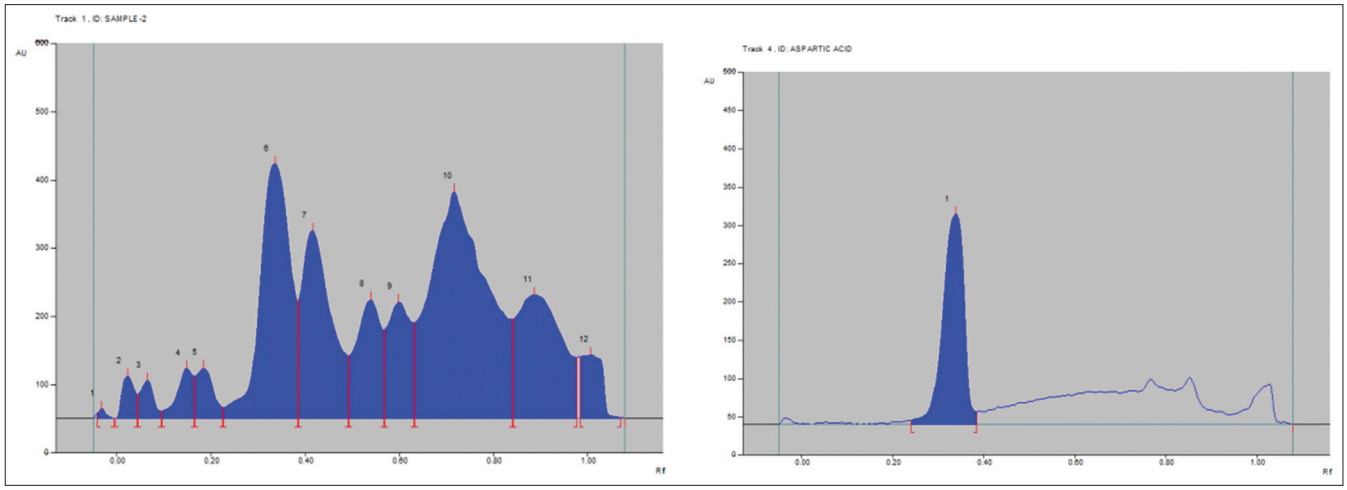

Fig. 6: Comparison of high-performance thin-layer chromatography fingerprint sample with standard amino acid (aspartic acid)

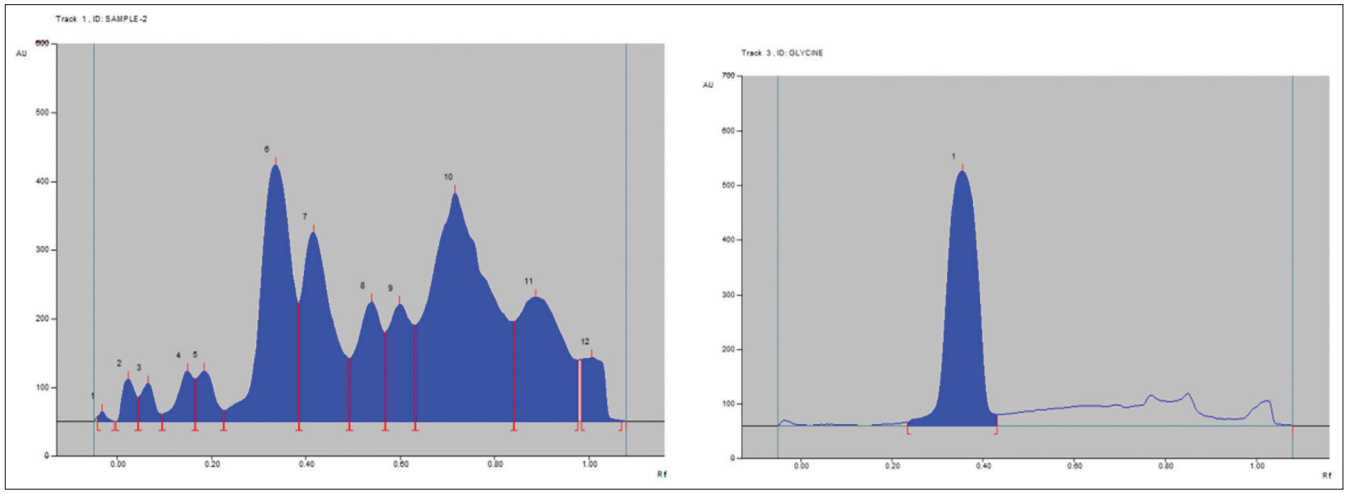

Fig. 7: Comparison of high-performance thin-layer chromatography fingerprint sample with standard amino acid (glycine)

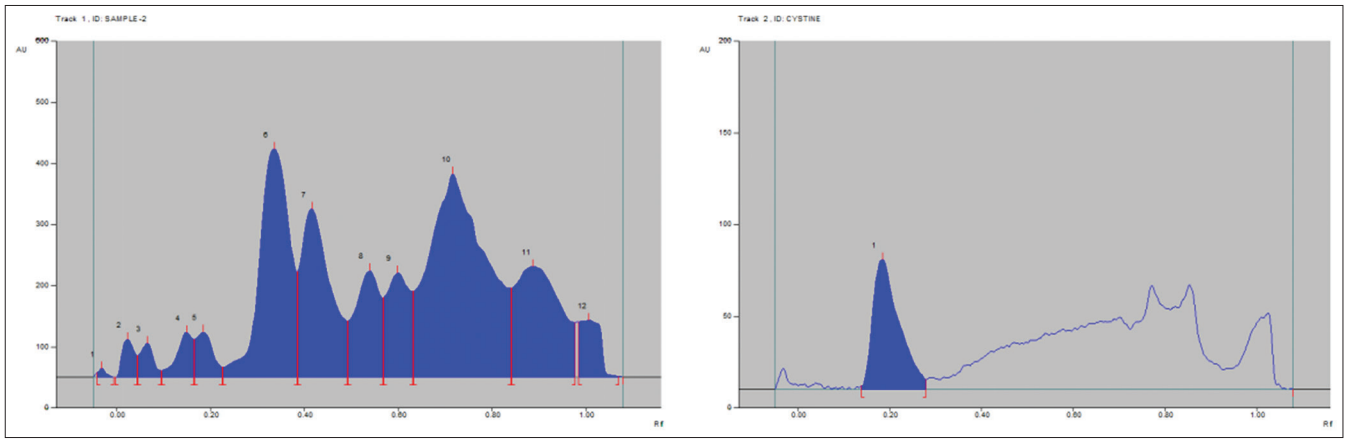

Fig. 8: Comparison of high-performance thin-layer chromatography fingerprint sample with standard amino acid (cysteine)

HPTLC chromatogram was developed; ninhydrin reagent was sprayed and observed. HPTLC fingerprint sample has been compared with standard amino acids - aspartic acid, glycine, cysteine, and proline and reported in Figs. 6-9.

Standard I - Cysteine
Standard II - Glycine
Standard III - Aspartic acid
Standard IV - Proline.

DISCUSSION

Mixture of amino acids has been successfully isolated from the fresh leaves of *A. sessilis* and assumed using paper chromatogram (trial and error method). Based on the paper chromatogram, standard amino acids are decided. From HPTLC, we can conclude the presence of amino acids such as cysteine, aspartic acid, and proline. Hence, plant or plant extract can be given in deficiency of these conditional amino

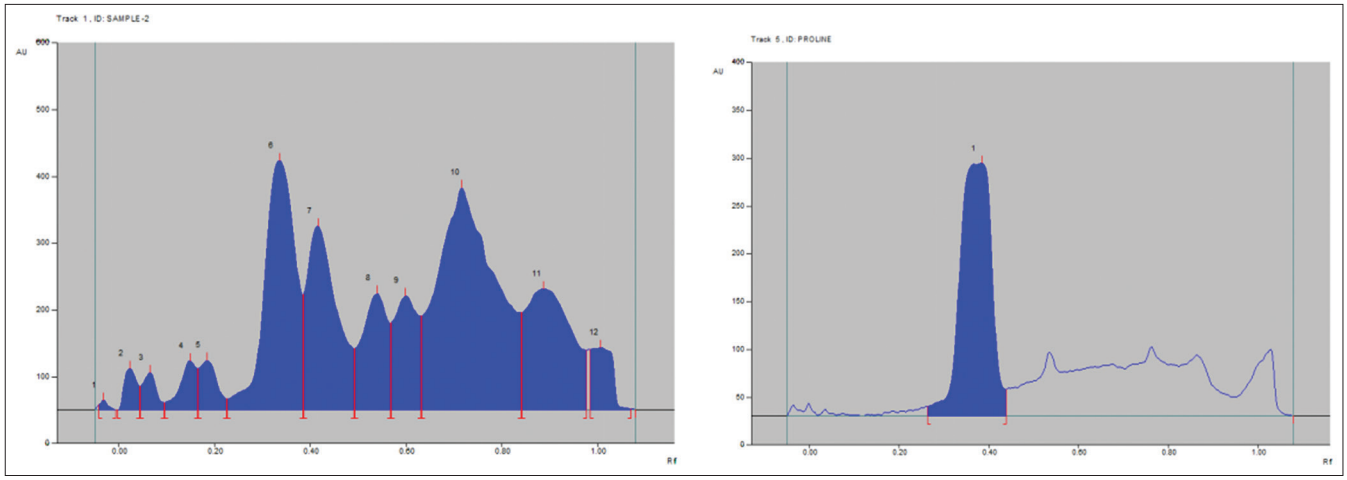

Fig. 9: Comparison of high-performance thin-layer chromatography fingerprint of sample with standard amino acid (proline)

Table 1: HPTLC profile for sample

S.No	R_f value	Peak area
1	0.34	17063.0
2	0.42	12353.7
3	0.54	6528.4
4	0.60	5943.4
5	0.72	28880.4
6	0.89	12464.1
7	1.01	2984.6

HPTLC: High-performance thin-layer chromatography

acids. The result obtained by qualitative evaluation of HPTLC will be helpful in the further quantitative estimation of each and every amino acid and also for standardization of herbal formulations containing this plant.

CONCLUSION

It can be concluded that based on the results obtained from the HPTLC fingerprint analysis will be helpful in identification and standardization of *A. sessilis* and can be utilized as a reference for the identification and quality control of the drug. The present study can be considered as reference and can be used for validation of the plant usage.

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AUTHORS' CONTRIBUTION

All authors have contributed equally to carry out the work.

CONFLICTS OF INTEREST

The authors declared that they have no conflicts of interest.

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