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SIMULTANEOUS QUALIFICATION AND QUANTIFICATION OF WITHAFERIN A, WITHANOLIDE A AND WTHANILODE B FROM THE AERIAL PARTS OF *LEPTADENIA PYROTECHNICA* (FORSSK) DECNE COLLECTED FROM HOT DESERT REGIONS OF INDIA

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ABSTRACT

Leptadenia pyrotechnica (Forssk.) Decne is a desert herb belonging to family Asclepiadaceae. (Synonym- *L. Spartinum* Wigh) locally known as Khimp or Kheep (Rajasthan), Khimparlo, Thahawar, Ranser (Gujarat), Broom bush (English). In the present investigation HPTLC fingerprint profile of Withaferin A, Withanolide A and Wthanilode B in aerial parts of three populations of *Leptadenia pyrotechnica* (Forssk.) Decne, collected from wild locations of Churu, Bikaner and Ajmer is reported. The separation of the compounds was done on the aluminium pre-coated silica gel $60F_{254}$ plates. All the three compounds were separated simultaneously by using Toluene: Ethlyacetate: formic acid: ethanol (6:3:0.1:0.6 v/v) as solvent system and derivatizing in freshly prepared *p*-anisaldehyde sulphuric acid. The calibration curve was found to be linear between 2 µl to 10µl/band with correlation coefficient of 0.986. The amount of Withaferin A and Withanolide A are more in population collected from Churu and the amount of Withanolide B is more populations collected from Ajmer.

Keywords: HPTLC, Withaferin A, Withanolide A, Withanolide B, Xerophytic plants

I. INTRODUCTION

L. pyrotechnica is a wonderful desert plant, of which almost every plant part is used in traditional medicinal system to treat various disorders. It is an erect broom-like shrub up to 1.5 to 3 m in height with green stem and pale green bushy branches with watery sap. Leaves are rarely found if any, they are small deciduous. This species holds variety of bioactive constituents that trigger healing properties, has been reported to possess anti-tumour and anticancer activity. This plant possesses antifungal, antibacterial, anticancer, antioxidant, wound healing, anthelmintic, anti-atherosclerotic, hypolipidemic, anti-diabetic and hepatoprotective activities coupled with other multifarious uses. Almost all plant parts are used in the traditional medicinal system to treat various disorders. *L. pyrotechnica* (Forssk.) Decne is found throughout the desert habitats of the state. This plant species

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is an important component of an arid ecosystem and good source of medicines, forage and fiber. It is one of the botanical Sources of the Ayurvedic drug *Jivanti*[1]. *Jivanti* is one of the important *rasayana* drugs in Ayurveda. It is used as an ingredient in formulations like *Jivantadya taila, Jivantadya Rasa, Jivantadya Ghrita, Ashwagandhadi ghritha, Anuthaila, Chandanadi thaila* these formulations are effective in the treatments of dieases like haemorrhage, tuberculosis, emaciation, fever and cardiac ailments etc [2]. Plants like *Leptadenia reticulate* (Retz.) Wt. & Arn. and *Holostemma ada-kodien* Schultes from Asclepiadaceae and *Trema orientalis* Blume from Ulmaceae are members of *Jivanti* sources [3]. Its fibers are used as antihistaminic and expectorant . It is rich in fatty acid, amino acids, flavonoids, alkaloids, sterols-sitosterol, triterpenes, steroidal glycosides, polyoxypregnane derivatives and cardenolides [4-10]. Whole plant sap is applied on the skin to cure skin diseases and also given to diabetic patients. Plants show antibacterial activity against *Staphylococcus aureus* and *Bacillus subtitlis* [11-12]. Latex is used to remove thorns and whole plant infusion mixed with butter milk is given in case of stomach disorder and to cure constipation [13]. The aim of the present contribution is to provide database for *L. pyrotechnica* (Forssk.) Decne, which is highly used by local tribal people of Rajasthan. The results are promising and show the presence of three marker compounds (Withaferin A, Withanolide A & Withanolide B) which are attracting many researchers for its anti-cancerous properties.

II. METHODOLOGY

2.1 Collection and identification of plant material

L. pyrotechnica (Forssk.) Decne was collected from Churu, Bikaner and Ajmer districts of Rajasthan and voucher specimens were submitted in the Herbarium, Department of Botany, Punjabi University, Patiala (PUN). Identification of plant was done by Botanical Survey of India, Jodhpur, Rajasthan.

2.2 Sample extraction

The plant material was shade dried and coarsely powdered before Soxhlet apparatus application. 10g of each dried and powdered aerial plant parts was applied to the methanolic extraction independently in Soxhlet apparatus. The extracts were concentrated using rota-evaporator and then lypholyzed. Powdered extracts was weighed and 5mg of each was dissolved in 5ml of methanol to obtain 1mg/1ml concentration.

2.3 Drugs and Chemicals

Reference standard Withaferin A, Withanolide A and Withanolide B were obtained from Sigma Aldrich Ltd. All the standards were prepared in concentration of 1mg/1ml. Analytical grade solvents Toluene, Ethlyacetate, formic acid and ethanol were obtained from Merck, Germany. Percolated silica gel $60F_{254}$ Merck plates were obtained from the E. Merck Ltd. (Mumbai India).

2.4 Preparation of standard solutions and Linearity

The stock solutions of the marker compounds were prepared by dissolving accuretly weighed of each marker compound (1mg/1ml). The aliquots 2,4,6,8 and10 µL were applied on the pre-coated silica gel plates.

2.5 Etnobotanical studies

It is one of most important fibre yielding plants of Thar Desert. It is well adapted to harsh edapho-climatic conditions of hot arid region. *Pyrotechnica* means fire-making. Local people use this plant as a good supply of fiber. Medicinally it is used to remove kidney stones, in joint pains, fever and for sciatica.

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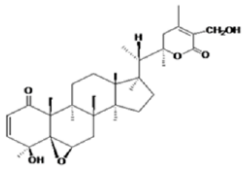
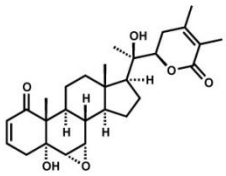
2.6 Phytochemical studies

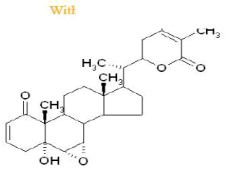
Aerial parts of three populations of *L. pyrotechnica* (Forssk.) Decne were extracted with methanol by using Soxhlet apparatus. HPTLC analysis were spotted in the form of bands of width of 8mm with a Camag 100µL syringe using Camag HPTLC system equipped with Camag Linomat V sample applicator (muttenz, Switzerland), Camag TLC scanner 3 and CATS 4 software for interpretation of data. Absorbent used was pre-coated silica gel aluminium plates $60F_{254}$ with 200µm thickness from Merck Comapany (Darmstadt, Germany). The linear ascending development was carried out in a CAMAG twin trough chamber (10cm X 10 cm), which was pre-saturated with 25 ml mobile phase. All the three compounds were simultaneously separated by using Toluene: Ethlyacetate: formic acid: ethanol (6:3:0.1:0.6 v/v) as solvent system on a distance of 7cm. After drying the plate in stream of warm air the plates were derivatized using freshly prepared *p*-anisaldehyde sulphuric acid solution. Then the plate was air dried for 5 minutes and kept in hot air oven at 110 ºC for 3 minutes. The image of the plate is captured under white light. Derivatization and determination of the plate was performed under controlled environmental conditions (27±2ºC and 35-40% relative humidity). The peak areas of the chromatograms were determined using winCATS software. The peak areas of all the three marker compounds were considered as an experimental response for finding the optimal extraction conditions.



Structure of Withaferin A



Structure of Withanolide A



Structure of Withanolide B

2.7 Specificity

The specificity of the method was ascertained by examining the marker compounds bands and comparing them to the corresponding band in the sample. The R*f* values of Withaferin A, Withanolide A and Withanolide B were confirmed by comparing them to R*f* values of samples.

2.8 Sensitivity

The sensitivity of the method was determined with the respect of limit of detection and limit of quantification. Determination of LOD was done by lowest concentration reported by the instrument and LOQ was determined by the lowest concentration quantified in the samples. These were determined as analyte concentrations giving rise to signal-to-noise ratios of 2 to 10 respectively (Table 2).

III. RESULTS AND DISCUSSION

India has a vast history of using traditional knowledge of plants. All the ethno-botanical information and data of folk medicines which is available among the diverse ethnic communities should be complied before the traditional culture is completely lost. Imposing knowledge of medicinal plants plays a vital role in primary health care and has a great potential for the discovery of new drugs. Present paper adds to existing information in the stream of identification of various compounds in *L. pyrotechnica* (Forssk.) Decne a typical xerophytic plant with medicinal properties. Chromatographic techniques are used for the isolation and separation of various compounds from the raw plant material. In the present study, different compounds have been separated and their

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R*f* value have been noted. Simultaneous chromatographic separation of three components from methanol extractions of whole plant powders of *L. pyrotechnica* (Forssk.) Decne was performed on TLC aluminum plates precoated with silica gel $60F_{254}$ using suitable mobile phases. The densitometric scanning was done after derivatization at λ=235nm for Withaferin A, Withanolide A and Withanolide B. HPTLC methods revealed presence of various compounds among which R*f* values of Withaferine A, Withanolide A and Withanolide B are found to match with the R*f* values of the metanolic extracts of aerial parts of the plant.

IV. CONCLUSION

In the present study, developed method is simple and reliable to screen and quantify different withanolides in the plant species.

V. ACKNOWLEDGMENTS

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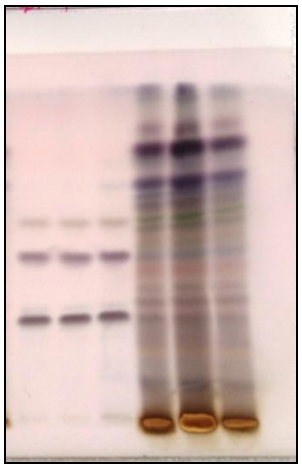


Fig. 1: High performance thin layer (HPTLC) fingerprint profile of methanolic extracts of steroids of aerial parts of *Leptadenia pyrotechnica* (Forssk.) Decne seen at visible light after derivatization.

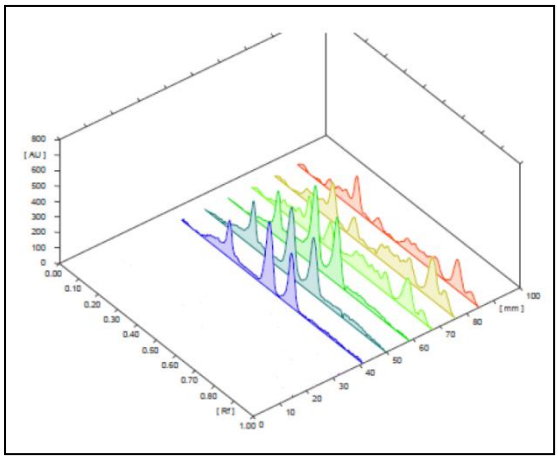
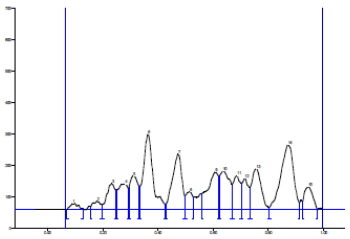


Fig. 2: Three-dimensional plot of fingerprint of Withaferin A, Withanolide A and Withanolide A in methanolic extracts of aerial parts of *Leptadenia pyrotechnica* (Forssk.) Decne. at 235nm.

A)



							End Height	Area	Area %	Assigned substance
						13	3.7	494.5	1.12	unknown *
						20	17.9	608.9	1.38	unknown *
						25	63.5	1961.4	4.43	unknown *
4	0.25	63.6	0.28	83.8	5.02	0.29	69.3	2272.2	5.14	unknown *
5	0.30	72.0	0.31	108.1	6.47	0.33	75.2	2534.4	5.73	unknown *
6	0.34	75.4	0.36	240.6	14.41	0.43	18.3	6561.3	14.83	unknown *
7	0.43	18.7	0.47	177.7	10.64	0.50	46.3	4734.2	10.70	unknown *
8	0.50	46.8	0.52	58.0	3.47	0.53	41.0	1131.8	2.56	unknown *
9	0.56	50.6	0.61	120.9	7.24	0.62	108.8	3731.6	8.43	unknown *
10	0.62	109.6	0.64	124.2	7.44	0.67	82.6	3451.8	7.80	unknown *
11	0.67	83.7	0.69	108.8	6.52	0.70	85.8	2199.2	4.97	unknown *
12	0.71	85.8	0.72	100.5	6.02	0.73	70.9	1743.5	3.94	unknown *
13	0.74	72.6	0.76	131.5	7.88	0.80	8.6	3502.3	7.92	unknown *
14	0.81	9.3	0.87	208.3	12.48	0.91	23.9	7523.9	17.01	unknown *
15	0.93	27.1	0.95	73.5	4.40	0.98	1.0	1792.1	4.05	unknown *

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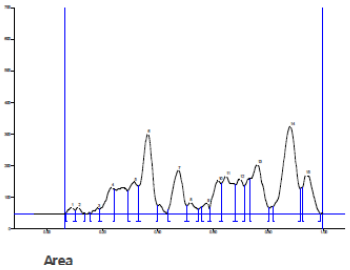
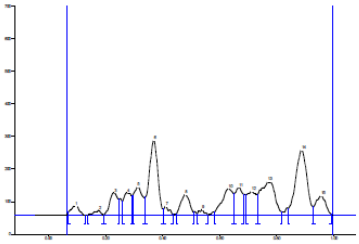
B)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.07	2.7	0.09	19.3	1.22	0.10	9.9	305.7	0.72	unknown *
2	0.10	10.0	0.11	19.2	1.21	0.14	0.4	247.1	0.58	unknown *
3	0.16	2.3	0.19	18.0	1.13	0.19	15.7	257.9	0.60	unknown *
4	0.19	15.9	0.23	83.5	5.26	0.24	76.4	1896.9	4.44	unknown *
5	0.29	74.3	0.32	100.3	6.32	0.33	87.0	2343.7	5.49	unknown *
6	0.33	87.5	0.37	253.4	15.95	0.40	25.4	6854.0	16.05	unknown *
7	0.44	13.4	0.48	137.1	8.63	0.51	25.1	3678.6	8.62	unknown *
8	0.51	25.6	0.52	35.4	2.23	0.55	13.9	710.4	1.66	unknown *
9	0.56	20.9	0.58	33.7	2.12	0.59	15.3	538.2	1.26	unknown *
10	0.59	18.3	0.62	104.5	6.58	0.63	94.3	2167.9	5.08	unknown *
11	0.63	95.2	0.65	119.7	7.53	0.68	92.6	3500.3	8.20	unknown *
12	0.68	91.6	0.70	110.0	6.92	0.71	87.0	2199.7	5.15	unknown *
13	0.73	110.5	0.76	155.6	9.79	0.80	19.4	4878.8	11.43	unknown *
14	0.82	23.2	0.88	276.3	17.39	0.92	78.8	9891.2	23.17	unknown *
15	0.93	83.4	0.94	122.6	7.72	0.99	0.6	3224.1	7.55	unknown *

C)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.07	9.5	0.09	27.3	2.27	0.13	0.0	615.6	1.98	unknown *
2	0.14	0.2	0.18	14.7	1.22	0.19	0.3	244.1	0.79	unknown *
3	0.20	0.9	0.23	68.8	5.71	0.25	49.3	1541.3	4.96	unknown *
4	0.26	42.1	0.28	69.3	5.75	0.29	60.0	1456.1	4.69	unknown *
5	0.30	60.4	0.31	85.6	7.10	0.34	54.3	2092.7	6.74	unknown *
6	0.34	54.9	0.37	229.3	19.03	0.40	18.3	5311.4	17.10	unknown *
7	0.40	18.9	0.41	27.9	2.32	0.44	1.9	378.6	1.22	unknown *
8	0.45	3.1	0.48	64.5	5.36	0.51	9.5	1527.8	4.92	unknown *
9	0.52	4.0	0.54	17.2	1.43	0.56	0.5	238.9	0.77	unknown *
10	0.58	8.8	0.63	80.9	6.71	0.65	65.3	2387.0	7.68	unknown *
11	0.65	65.4	0.67	84.8	7.04	0.69	64.1	1710.9	5.51	unknown *
12	0.69	62.8	0.71	73.4	6.09	0.73	62.9	1874.1	6.03	unknown *
13	0.74	63.7	0.77	102.6	8.51	0.82	7.7	3968.8	12.78	unknown *
14	0.84	20.0	0.89	199.1	16.52	0.93	25.1	6170.8	19.86	unknown *
15	0.93	25.6	0.96	59.6	4.95	0.99	3.4	1547.9	4.98	unknown *

Fig. 3: (A-C) HPTLC chromatographs of three populations of *Leptadenia pyrotechnica* (Forssk.) Decne aptivate at 230nm wherein Withaferin A at end R*f* 0.37, Withanolide A at end R*f* 0.57 and Withanolide B at end R*f* 0.67 has been detected.

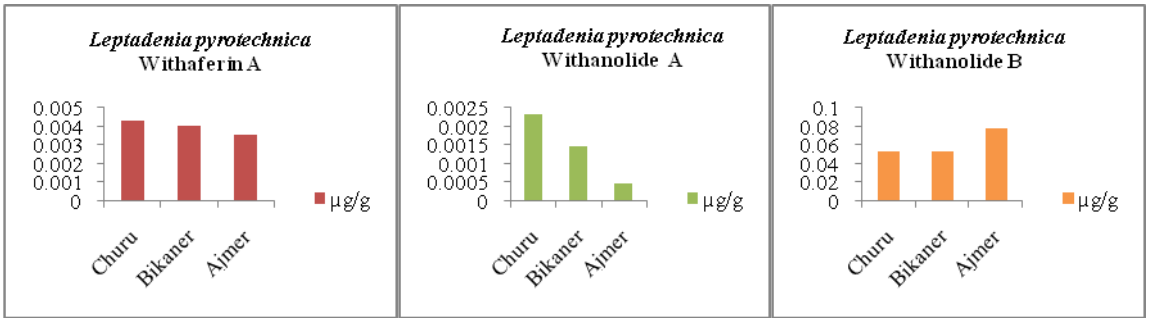


Fig. 4: Bar graphs showing simultaneous analysis of Withaferin A, Withanolide A and Withanolide B in three populations of *Leptadenia pyrotechnica* (Forssk.) Decne. from different areas of Rajasthan.

Fig. 5: Field pictures of *Leptadenia pyrotechnica* (Forssk.) Decne.

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Table 1: Data of location and amount of marker compounds in three different populations of *Leptadenia pyrotechnica* (Forssk.) Decne.

Sr. no.	Location with altitude (m)	Dry weight of the plant	Amount of Withaferin A (µg/g)	Amount of Withanolide A (µg/g)	Amount of Withanolide B (µg/g)
1.	Churu (300m)	30g	0.0044	0.0025	0.0538
2.	Bikaner (250m)	30g	0.0040	0.0015	0.0311
3.	Ajmer (200m)	30g	0.0036	0.0004	0.0538

Table 2: Validation and Quantification of results.

Parameter	Withaferin A	Withanolide A	Withanolide B
LOD	3.8	4.4	2.7
LOQ	11.7	10.5	8.1
Linear dynamic range (LDR) (2-10µL/spot)	2-10 µL	2-12 µL	2-10 µL
Linearity equation	y=599.6x	y=523.4x	1878.x
Coefficient of determination (r^2)	0.976	0.983	0.988