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HPTLC FINGERPRINTS AND FTIR ANALYSIS FOR AUTHENTICATION AND QUALITY CONTROL OF *Holostemma ada-kodien* Schult.

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ABSTRACT

Plant-derived pharmaceuticals are gaining increased acceptance in treating various ailments, but ensuring their quality is crucial. Both conventional and modern techniques are employed to validate the quality. High performance thin layer chromatography (HPTLC) and Fourier transform infra-red (FTIR) analysis were conducted to analyse and identify the various phytoconstituents in serial root extract of *Holostemma ada-kodien*. The peaks observed in HPTLC fingerprint profile corresponding to different phytoconstituents served as markers for standardizing drug; while FTIR analysis aids in identifying the major functional groups present. Thirteen bands were noted for hexane extract of *H. ada-kodien* in short UV in track I, while chloroform and methanol extracts showed eight bands each. In track II, the hexane extract exhibited eight bands, while chloroform and methanol extracts showed eleven and eight bands, respectively, under similar conditions. The hexane extract in long UV showed a maximum area of 78.4 and 71.59%, in track 1 and 2, respectively, with respective spanning from Rf 0.61 to 0.75 and 0.51 to 0.73. The methanol extract in visible light in track I showed a maximum area of 46.87%, spanning from Rf 0.73 to 0.98, and in track II, it displayed a maximum area of 36.59%, spanning from Rf 0.75 to 0.96 with 10 µL applied. Methanolic root extract of *H. ada-kodien* showed clear banding patterns in HPTLC fingerprints which seemed to be a dependable technique to detect adulteration while FTIR technique revealed functional groups of methanol root extracts.

Keywords: *Holostemma ada-kodien*, HPTLC, FTIR analysis, authentication

INTRODUCTION

The majority of traditional medicines used in healthcare originate from plants. These medicinal plants possess a variety of therapeutic properties (Chopra and Ananda, 2003). The pharmaceutical industries identify the active principles and thus elevate the status of medicinal plants. Common criteria for drug evaluation include assessing the quality and therapeutic value of bulk drug/ pharmaceutical product, their identification, purity, content, uniformity, chemical and physical stability and biological availability (Gershell and Atkins, 2003; Butler, 2008). Root, stem, leaves, fruits, flowers and seeds of plant possess secondary metabolites and their bioactivities are confirmed through clinical trials. The new phytopharmaceutical regulations permit drug development through advanced techniques of solvent extraction, fractionation, modern formulation development, etc. (Bhatt, 2016).

Holostemma ada-kodien Schult. belongs to family Asclepiadaceae and its roots are used for treating cough, fever, ophthalmic diseases, stomachache, dysentery, tuberculosis, arrested urination, scorpion bite, kidney stones, goitre, etc. (Warrier *et al.*, 1995). Conventionally, *H. ada-kodien* is used

as an astringent to the bowels; curative for ulcers, leucoderma, gonorrhea and ophthalmic disorders (Warrier *et al.,* 1995; Gupta, 1997). Roots are used as an ingredient for the preparation of drug Jivanti (Kolammal, 1979) and root tuber for decoction preparation for body strength by Mullukurumba tribes (Silja *et al.,* 2008). Kani tribes orally consumed root tuber paste in cow milk to promote lactation (Vijayan *et al.,* 2007*).* Jivanti is important ingredients in several traditional polyherbal formulations of Indian system of medicine, such as Ashoka Ghrita, Ashwagandhadi Ghrita, Anu Thaila, etc. (Vasu, 2017). It also possesses some proven medicinal activities *viz*., anthelmintic (Sadasivam *et al.,* 2014), antioxidant (Shanmugham *et al.,* 2011), hepatoprotective (Sunil *et al.,* 2015) activities. The plant root tuber has antidiabetic and antipyretic action (Sunil *et al.,* 2016), antibacterial (Irimpan *et al.,* 2011), anthelmintic (Sadasivam *et al.,* 2014) and anti-inflammatory (Smitha Devi *et al.,* 2023) activities. The phytochemical analysis of column extract has revealed that the extract is active antioxidant and by using 1H NMR, 13C NMR and mass spectrometry seven therapeutically active compounds have been isolated (Deepak *et al.,* 2019).

High performance thin layer chromatography (HPTLC) is a popular method for assessing the quality control of herbal products/ medicines. It is widely used for separation, qualitative and quantitative estimation of marker compounds present in herbal drugs. HPTLC fingerprint profile is suitable for the standardization of components followed by determination of specific bioactive phyto-constituents from plant materials and for quality control development of herbal formulations. The major advantage of HPTLC is its ability to analyse several samples simultaneously using a small quantity of sample (Khandelwal, 2002; Qureshi, 2018). HPTLC is one of the best methods for validating the composition of complex botanical products with respect to their quality control, purity, stability and identity of compounds, so is the final coherent step in the evaluation of analytical applications in natural products (Nicoletti, 2011). The chromatographic chemical profiles offer sufficient data and parameters for thorough identification of medication, its qualitative and quantitative evaluation, and comparison of main active ingredient in samples under study so as to ensure quality control, and guarantee therapeutic efficacy (Chandrakar, 2018). To prevent fraudulent or inadvertent mislabeling and adulteration of expensive substances, medicinal plant providers authenticate their products based on the commodity, variety, and place of origin of their products. The degree of success in differentiating the real and fraudulent products varies depending on the region of electromagnetic spectrum used and the chemometric techniques applied to the spectra. The authentic good's finger-prints are believed to give their complete chemical composition that might detect adulteration. An effective technique for confirming these claims is FTIR spectroscopy (Rodriguez-Saona and Allendorf, 2011).

The major Southern Indian pharmacies annually require at least 150 metric tons of *H. ada-kodien* root tubers for preparing ayurvedic medicine (Sunil *et al.*, 2016). The plant has become rare because of indiscriminate root tuber extraction as raw material for ayurvedic drugs (Dan and Shanavaskhan, 1991; Gowthami *et al.,* 2021). The plant is a vulnerable species and listed in the first red list of medicinal plants of southern India (CAMP, 1995). Plant based remedies are seriously affected by incorrect plant species identification; and contaminated herbal drug products might exacerbate health issues (Luis *et al.,* 2019). Adulteration and substitution not only reduce the therapeutic potential but also pose risk to the customers' health (Ekor, 2014). The quality control is, therefore, extremely important in terms of public health because of adulteration and substitution issues and hence HPTLC and FTIR data may serve as an authenticated tool. The conventional macroscopic and microscopic techniques do not suffice quality control criteria; instead chemical evaluation plays a crucial role in standardization (Klein-Junior *et al.,* 2021). The HPTLC chromatogram and FTIR spectrum developed can be used as a ready reference for the quality control of plant material even in the powdered form. HPTLC is a useful tool with high sensitivity, better separation and reproducibility not only for the detection of adulteration with different species but also by other synthetic compounds (Nicoletti, 2011; El-Ahmady and Ashour, 2016). The present study was aimed to establish the quality control measures for identifying the adulterants in the root tuber of *Holostemma ada-kodien*, a RET species used for Ayurvedic purposes.

MATERIALS AND METHODS

Preparation of extract

Holostemma ada-kodien plants were collected from various localities of Peechi, Thrissur district (76º 18' east longitude and 10º 28' north latitude. and altitude 55.00 m), Kerala, India. Two-year old root tuber of *H. ada-kodien* (Fig. 1) were shade dried, powdered and used for phytochemical analysis. Finely powdered root tuber was extracted by Soxhlet with various solvents such as hexane, chloroform and methanol (Redfern *et al.*, 2014). The collected extract was filtered and dried using a rotary evaporator under vacuum at 45°C.

Fig. 1: *Holostemma ada-kodien* plant, (left hand side); Two years old root tubers (right hand side)

HPTLC analysis

The HPTLC analysis of extracts was carried out as per Do *et al.*, (2021). The hexane, chloroform and methanolic (HPTLC grade Sigma-Aldrich) extracts were applied in two tracks of different concentrations of width 8 mm each on silica gel 60 F254 pre-coated aluminium sheets through CAMAG microlitre syringe using automatic TLC sampler (ATS4 CAMAG, Switzerland). For hexane extract, optimum separations of constituents were achieved by using toluene: ethyl acetate: formic acid (5.0: 1.5: 0.1), while for chloroform and methanolic extracts, toluene: ethyl acetate: formic acid (5: 3: 0.1) was used in mobile phase. After sample application the plate was introduced vertically in a CAMAG developing chamber (10 cm × 10 cm) pre-saturated with mobile phase selected and developed to 70 mm. The developed chromatograms were air-dried to evaporate solvents from the plates and then the plates were kept in CAMAG® TLC Visualizer Swizz and the images captured under UV light at 254 (D2 lamp, absorption mode) and 366 nm (Hg lamp, fluorescence mode). The plates were scanned at 254 and 366 nm using TLC Scanner 4 for obtaining densitograms (Scanner 4 with win CATS software) and the finger print profiles were documented. The Rf values and finger print data were recorded with win CATS software associated with the scanner. The plates were derivatised using vanillin-sulphuric acid reagent, heated at 105°C by placing on CAMAG TLC plate heater till the colour of bands appeared. For hexane, chloroform and methanolic extracts three replica were tried and the plates were visualized under white light and chromatograms scanned at 575 nm (W lamp, absorption mode) and Rf values and finger print data were documented. This comprehensive approach ensured accurate characterization and comparison of the phytoconstituents present in the extracts, facilitating their identification and quality assessment for pharmaceutical applications.

Fourier transform infra-red (FTIR) analysis

The FTIR analysis was performed as per Berthomieu and Hienerwadel (2009). Dried methanolic extract (1 mg) was mixed with potassium bromide (KBr) powder to form a pellet. Typically, 10 mg KBr was used per 1 mg sample extract. The mixture was then pressed into a translucent pellet or disc using a hydraulic press. This pellet ensured uniformity and allowed accurate IR measurements. The prepared sample disc was placed into the Nicolet iS50 FTIR spectrometer (Nicolet Raman, Thermofisher Scientific}, with a scan range from 15 to 27,000 cm^{-1} with a resolution of 0.09 cm^{-1}). The instrument operates on Fourier transform infrared spectroscopy, which measures the absorption of infrared light by the sample. The scan range covers a wide spectrum from 15 cm^{-1} to 27,000 cm^{-1}, allowing for detailed analysis of functional groups present in sample. The resolution of 0.09 cm^{-1} ensured high precision during absorption peaks measurement. The FTIR spectrometer collects the

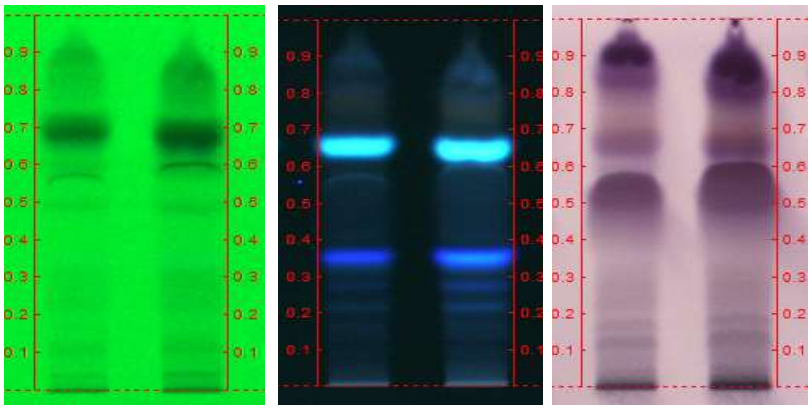
data on how the sample absorbs infrared radiation at different wavelengths. and processes the collected data, providing spectral information and allowing for the analysis of functional groups present in the methanolic extract. Specific absorption peaks corresponded to the characteristic vibrations of functional groups (such as C-H, O-H, C=O, etc.) in the extract were detected.

RESULTS AND DISCUSSION

HPTLC fingerprint of hexane, chloroform and methanolic root extract of *H. ada-kodien* at 254, 366 and 575 nm showed clear banding pattern (Fig. 2A, 3A and 4A). Upon visualizing under short UV, the hexane extract of *H. ada-kodien* in track I showed 13 bands, while chloroform and methanol extracts each showed 8 bands. In track II, the hexane extract displayed 8 bands, the chloroform extract showed 11 bands, and the methanol extract revealed 8 bands. The hexane extract exhibited a peak with a maximum area of 47.26%, spanning from Rf 0.64 to 0.79 in track I, and a peak with a maximum area of 43.04%, spanning from Rf 0.64 to 0.77 in track II with 10 µL applied (Fig. 2B). The chloroform extract in track I showed a maximum area of 40.26%, from Rf 0.67 to 0.94, peaking at 0.72 with 5 µL applied. In track II, the chloroform extract exhibited a peak with a maximum area of 28.78%, from Rf 0.67 to 0.79, peaking at 0.72 with 10 µL applied (Fig. 3B). The methanol extract in track I showed a maximum area of 26.17%, spanning from Rf 0.27 to 0.33, and in track II, it exhibited a maximum area of 23.43%, spanning from Rf 0.27 to 0.33, peaking at 0.29 with 10 µL applied (Fig. 4B).

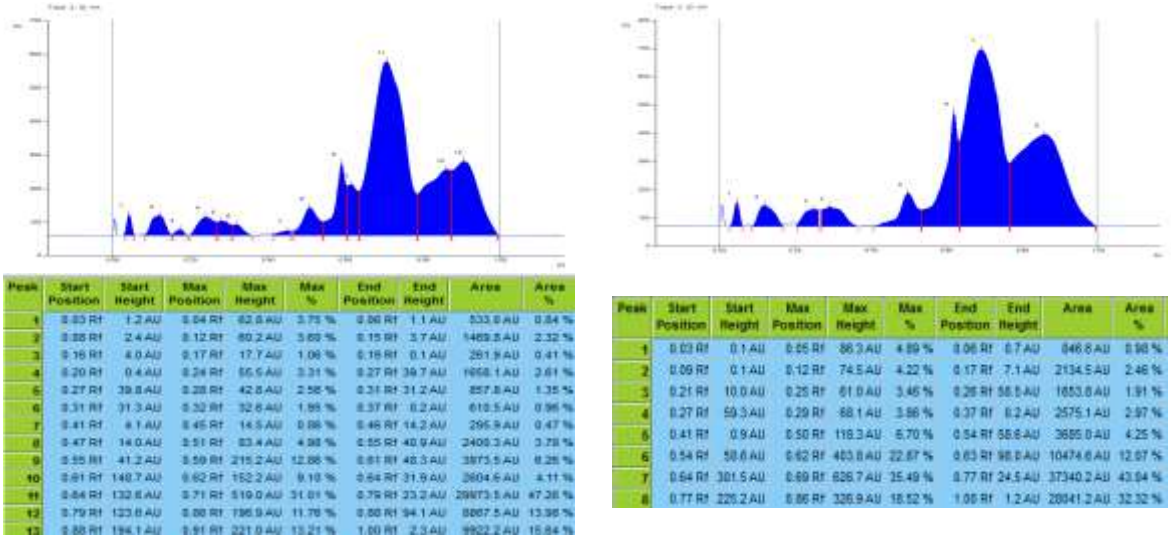
Under long UV visualization, the hexane extract of *H. ada-kodien* in track I exhibited eight bands, while chloroform extract showed ten bands and methanol extract showed eight bands. In track II, the hexane extract displayed eight bands, the chloroform extract had ten bands, and the methanol extract showed eight bands. The hexane extract demonstrated a peak with a maximum area of 78.42%, spanning from Rf 0.61 to 0.75 in track I, and in track II with a maximum area of 71.59%, spanning from Rf 0.51 to 0.73 with 10 µL applied (Fig. 3B). The chloroform extract in track I showed a maximum area of 31.98%, spanning from Rf 0.14 to 0.20, peaking at 0.72 with 5 µL applied. In track II, chloroform extract showed a peak with a maximum area of 27.43%, spanning from Rf 0.15 to 0.24 with 10 µL applied (Fig. 3C). The methanol extract in track I showed a maximum area of 43.36%, spanning from Rf 0.01 to 0.84, and in track II, it displayed a maximum area of 23.88%, spanning from Rf 0.27 to 0.33, peaking at 0.03 with 10 µL applied (Fig. 3D).

Under visible light, the hexane, chloroform, and methanol extracts of *H. ada-kodien* in track I showed 5, 10 and 9 bands, respectively. In track II, hexane extract displayed 6 bands, chloroform extract showed 5 bands, and methanol extract had 9 bands. The hexane extract demonstrated a peak with a maximum area of 43.04%, spanning from Rf 0.64 to 0.77 in track I and 47.60% with Rf 0.73 to 1.00 in track II (Fig. 4B). The chloroform extract in track I showed a maximum area of 45.87%, spanning from Rf 0.74 to 0.98 with 5 µL applied. In track II, chloroform extract exhibited a peak with a maximum area of 47.81%, spanning from Rf 0.74 to 0.97



Under UV short Under UV long Under white light

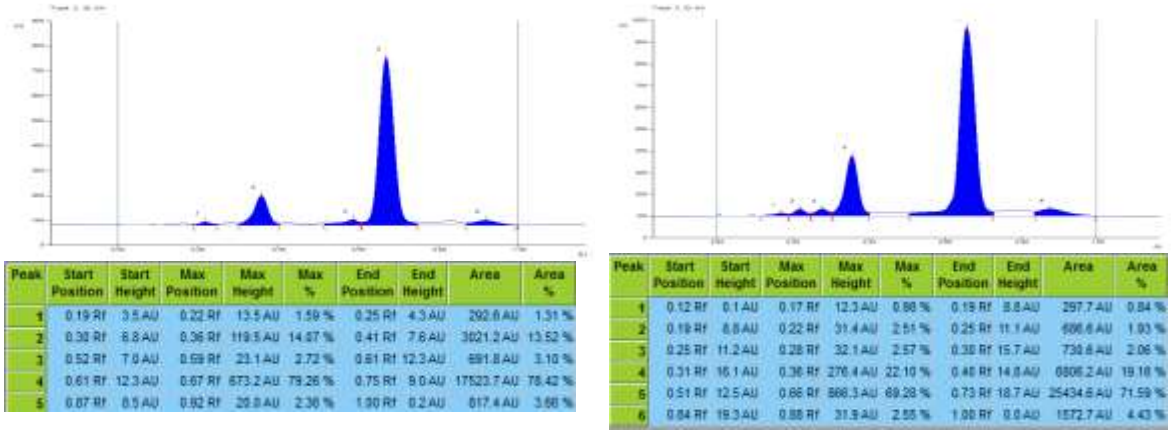
Fig. 2 A: HPTLC fingerprint of hexane root extract of *H. ada-kodien* at 254, 366 and 575 nm



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.03 Rf	1.2 AU	0.04 Rf	62.8 AU	3.75 %	0.06 Rf	1.1 AU	533.0 AU	0.84 %
2	0.08 Rf	2.4 AU	0.12 Rf	80.2 AU	3.69 %	0.15 Rf	3.7 AU	1469.5 AU	2.32 %
3	0.16 Rf	4.0 AU	0.17 Rf	17.7 AU	1.06 %	0.18 Rf	0.1 AU	261.9 AU	0.41 %
4	0.20 Rf	0.4 AU	0.24 Rf	55.5 AU	3.31 %	0.27 Rf	39.7 AU	1658.1 AU	2.61 %
5	0.27 Rf	39.8 AU	0.28 Rf	42.8 AU	2.56 %	0.31 Rf	31.2 AU	857.8 AU	1.35 %
6	0.31 Rf	31.3 AU	0.32 Rf	32.6 AU	1.95 %	0.37 Rf	0.2 AU	610.5 AU	0.96 %
7	0.41 Rf	0.1 AU	0.45 Rf	14.5 AU	0.86 %	0.46 Rf	14.2 AU	295.9 AU	0.47 %
8	0.47 Rf	14.0 AU	0.51 Rf	83.4 AU	4.98 %	0.55 Rf	40.9 AU	2400.3 AU	3.78 %
9	0.55 Rf	41.2 AU	0.59 Rf	215.2 AU	12.86 %	0.61 Rf	48.3 AU	3975.5 AU	6.26 %
10	0.61 Rf	148.7 AU	0.62 Rf	152.2 AU	9.10 %	0.64 Rf	31.9 AU	2604.6 AU	4.11 %
11	0.64 Rf	132.6 AU	0.71 Rf	519.0 AU	31.01 %	0.79 Rf	23.2 AU	29973.5 AU	47.26 %
12	0.79 Rf	123.6 AU	0.80 Rf	196.9 AU	11.76 %	0.86 Rf	94.1 AU	8867.5 AU	13.98 %
13	0.88 Rf	196.1 AU	0.91 Rf	221.0 AU	13.21 %	1.00 Rf	2.3 AU	9922.2 AU	15.64 %

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.03 Rf	0.1 AU	0.05 Rf	86.3 AU	4.89 %	0.06 Rf	0.7 AU	846.8 AU	0.98 %
2	0.09 Rf	0.1 AU	0.12 Rf	74.5 AU	4.22 %	0.17 Rf	7.1 AU	2134.5 AU	2.46 %
3	0.21 Rf	10.0 AU	0.25 Rf	61.0 AU	3.46 %	0.28 Rf	58.5 AU	1653.6 AU	1.91 %
4	0.27 Rf	59.3 AU	0.29 Rf	68.1 AU	3.86 %	0.37 Rf	0.2 AU	2575.1 AU	2.97 %
5	0.41 Rf	0.9 AU	0.50 Rf	118.3 AU	6.70 %	0.54 Rf	58.6 AU	3685.0 AU	4.25 %
6	0.54 Rf	58.6 AU	0.62 Rf	403.8 AU	22.87 %	0.63 Rf	98.8 AU	10474.6 AU	12.07 %
7	0.64 Rf	301.5 AU	0.69 Rf	626.7 AU	35.49 %	0.77 Rf	24.5 AU	37340.2 AU	43.04 %
8	0.77 Rf	225.2 AU	0.86 Rf	326.9 AU	18.52 %	1.00 Rf	1.2 AU	28041.2 AU	32.32 %

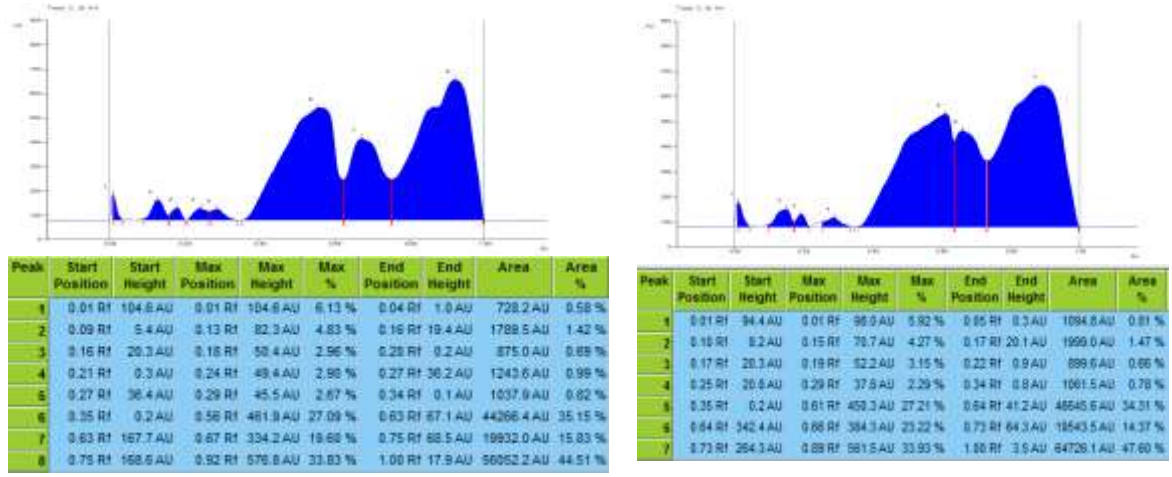
Fig. 2B: HPTLC densitogram of *H. ada-kodien* hexane root extract in 5 (left hand side) and 10 (right hand side) µL at 254 nm.



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.19 Rf	3.5 AU	0.22 Rf	13.5 AU	1.59 %	0.25 Rf	4.3 AU	292.6 AU	1.31 %
2	0.30 Rf	8.8 AU	0.36 Rf	119.5 AU	14.07 %	0.41 Rf	7.6 AU	3021.2 AU	13.52 %
3	0.52 Rf	7.0 AU	0.59 Rf	23.1 AU	2.72 %	0.61 Rf	12.3 AU	691.8 AU	3.10 %
4	0.61 Rf	12.3 AU	0.67 Rf	673.2 AU	79.26 %	0.75 Rf	9.0 AU	17523.7 AU	78.42 %
5	0.87 Rf	8.5 AU	0.92 Rf	20.8 AU	2.36 %	1.00 Rf	0.2 AU	817.4 AU	3.66 %

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.12 Rf	0.1 AU	0.17 Rf	12.3 AU	0.88 %	0.19 Rf	8.8 AU	297.7 AU	0.84 %
2	0.19 Rf	8.8 AU	0.22 Rf	31.4 AU	2.51 %	0.25 Rf	11.1 AU	696.6 AU	1.93 %
3	0.25 Rf	11.2 AU	0.28 Rf	32.1 AU	2.57 %	0.30 Rf	15.7 AU	730.8 AU	2.06 %
4	0.31 Rf	16.1 AU	0.36 Rf	276.4 AU	22.10 %	0.40 Rf	14.8 AU	6806.2 AU	19.18 %
5	0.51 Rf	12.5 AU	0.66 Rf	866.3 AU	69.28 %	0.73 Rf	18.7 AU	25434.6 AU	71.59 %
6	0.84 Rf	19.3 AU	0.88 Rf	31.9 AU	2.55 %	1.00 Rf	0.0 AU	1572.7 AU	4.43 %

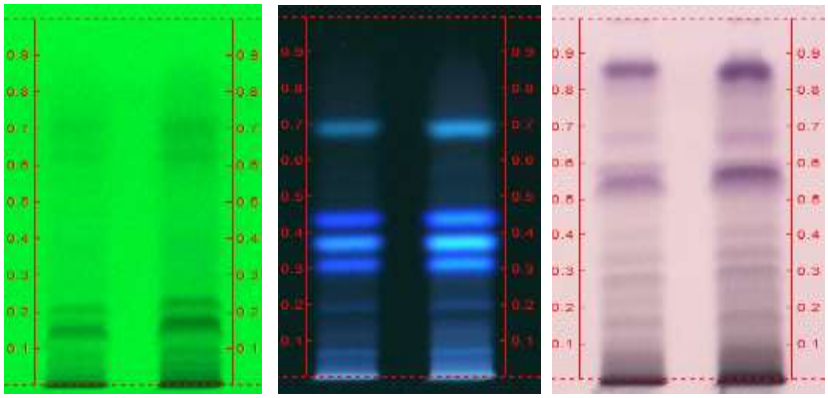
Fig. 2C: HPTLC densitogram of *H. ada-kodien* hexane root extract in 5 (left hand side) and 10 (right hand side) µL at 366 nm



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.01 Rf	104.6 AU	0.01 Rf	104.6 AU	6.13 %	0.04 Rf	1.0 AU	728.2 AU	0.58 %
2	0.09 Rf	5.4 AU	0.13 Rf	82.3 AU	4.83 %	0.16 Rf	19.4 AU	1788.5 AU	1.42 %
3	0.16 Rf	20.3 AU	0.18 Rf	50.4 AU	2.96 %	0.20 Rf	0.2 AU	875.0 AU	0.69 %
4	0.21 Rf	0.3 AU	0.24 Rf	49.4 AU	2.90 %	0.27 Rf	36.2 AU	1243.6 AU	0.99 %
5	0.27 Rf	36.4 AU	0.29 Rf	45.5 AU	2.67 %	0.34 Rf	0.1 AU	1037.9 AU	0.82 %
6	0.35 Rf	0.2 AU	0.56 Rf	481.9 AU	27.09 %	0.63 Rf	67.1 AU	44266.4 AU	35.15 %
7	0.63 Rf	167.7 AU	0.67 Rf	334.2 AU	19.66 %	0.75 Rf	68.5 AU	19932.0 AU	15.83 %
8	0.75 Rf	168.6 AU	0.92 Rf	576.8 AU	33.93 %	1.00 Rf	17.9 AU	56052.2 AU	44.51 %

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.01 Rf	94.4 AU	0.01 Rf	96.0 AU	5.92 %	0.05 Rf	0.3 AU	1094.8 AU	0.81 %
2	0.10 Rf	8.2 AU	0.15 Rf	70.7 AU	4.27 %	0.17 Rf	20.1 AU	1999.0 AU	1.47 %
3	0.17 Rf	20.3 AU	0.19 Rf	52.2 AU	3.15 %	0.22 Rf	0.9 AU	899.6 AU	0.66 %
4	0.25 Rf	20.6 AU	0.29 Rf	37.9 AU	2.29 %	0.34 Rf	0.8 AU	1061.5 AU	0.78 %
5	0.35 Rf	0.2 AU	0.61 Rf	459.3 AU	27.21 %	0.64 Rf	41.2 AU	46545.6 AU	34.31 %
6	0.64 Rf	342.4 AU	0.66 Rf	384.3 AU	23.22 %	0.73 Rf	64.3 AU	19543.5 AU	14.37 %
7	0.73 Rf	264.3 AU	0.89 Rf	581.5 AU	33.93 %	1.00 Rf	3.5 AU	64726.1 AU	47.60 %

Fig. 2D: HPTLC densitogram of *H. ada-kodien* hexane root extract in 5 (left hand side) and 10 (right hand side) µL at 575 nm



Under UV short Under UV long Under white light

Fig. 3 A: HPTLC fingerprint of chloroform root extract of *H. ada-kodien* at 254, 366 and 575 nm

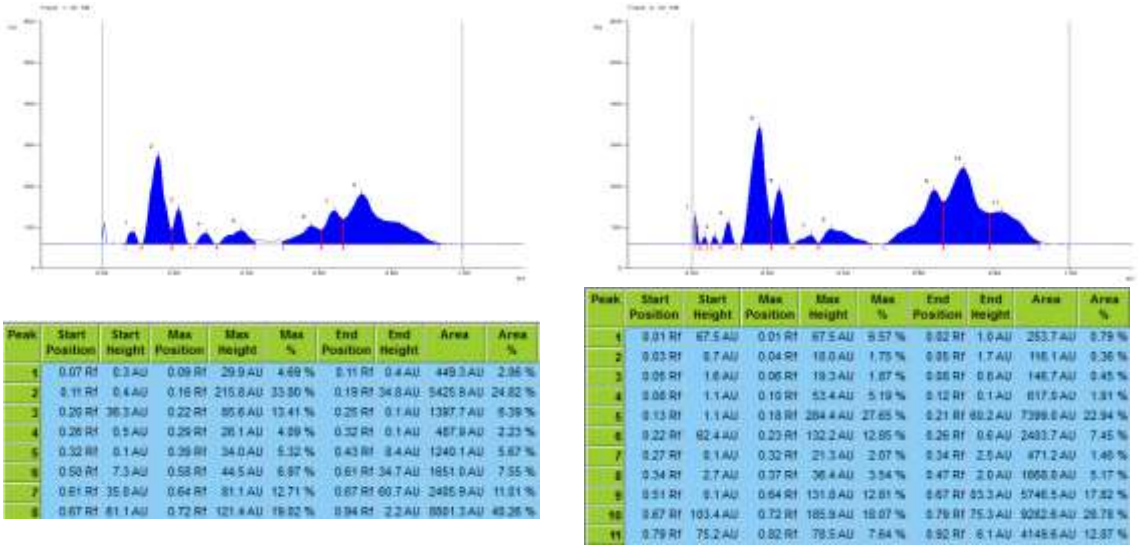


Fig. 3B: HPTLC densitogram of *H. ada-kodien* chloroform root extract in 5 (left hand side) and 10 (right hand side) µL at 254 nm

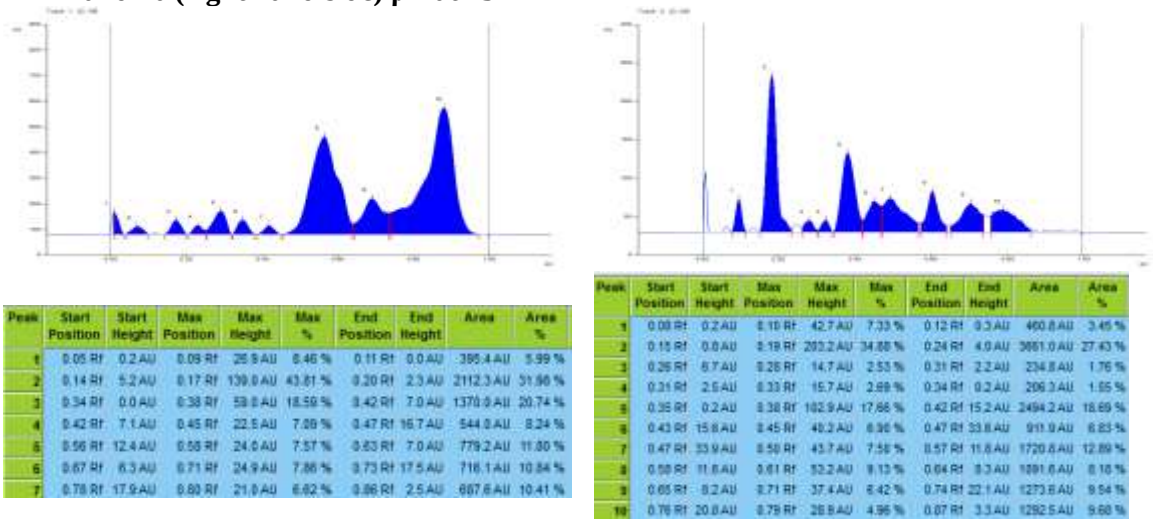
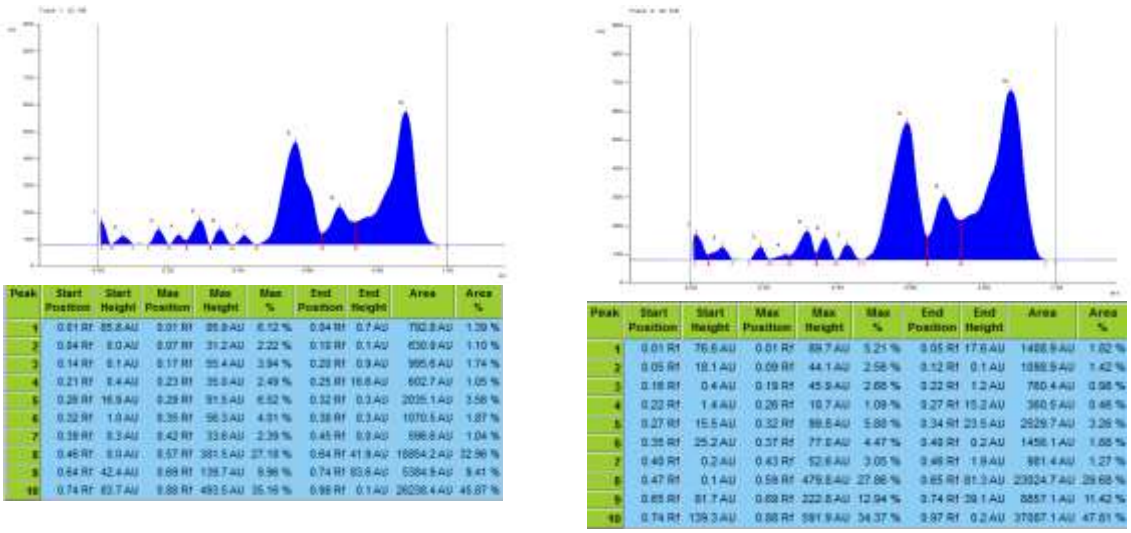


Fig. 3C: HPTLC densitogram of *H. ada-kodien* chloroform root extract in 5 (left hand side) and 10 (right hand side) µL at 366 nm

with 10 µL applied (Fig. 4C). Methanol extract in track I showed a maximum area of 46.87%, spanning from Rf 0.73 to 0.98, and in track II, it displayed a maximum area of 36.59%, spanning from Rf 0.75 to 0.96 with 10 µL applied (Fig. 4D).

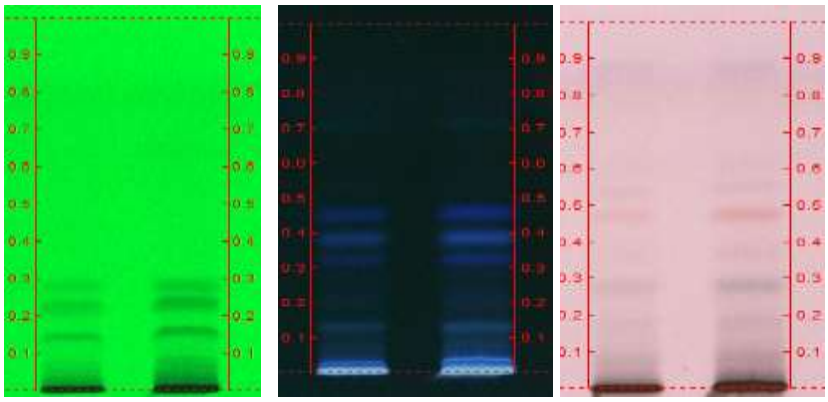
The HPTLC is a crucial modern analytical method used extensively in pharmaceutical industries for method development, identification and detection of adulterants



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.01 Rf	85.8 AU	0.01 Rf	85.8 AU	6.12 %	0.04 Rf	0.7 AU	782.8 AU	1.39 %
2	0.04 Rf	0.0 AU	0.07 Rf	31.2 AU	2.22 %	0.10 Rf	0.1 AU	630.9 AU	1.10 %
3	0.14 Rf	0.1 AU	0.17 Rf	55.4 AU	3.94 %	0.20 Rf	0.9 AU	995.6 AU	1.74 %
4	0.21 Rf	0.4 AU	0.23 Rf	35.0 AU	2.49 %	0.25 Rf	16.6 AU	602.7 AU	1.05 %
5	0.28 Rf	16.9 AU	0.28 Rf	91.5 AU	6.52 %	0.32 Rf	0.3 AU	2035.1 AU	3.56 %
6	0.32 Rf	1.0 AU	0.35 Rf	56.3 AU	4.01 %	0.38 Rf	0.3 AU	1070.6 AU	1.87 %
7	0.38 Rf	0.3 AU	0.42 Rf	33.6 AU	2.39 %	0.45 Rf	0.9 AU	596.8 AU	1.04 %
8	0.46 Rf	0.9 AU	0.57 Rf	381.5 AU	27.18 %	0.64 Rf	41.9 AU	10854.2 AU	22.96 %
9	0.64 Rf	42.4 AU	0.69 Rf	139.7 AU	9.96 %	0.74 Rf	83.6 AU	5384.9 AU	9.41 %
10	0.74 Rf	83.7 AU	0.88 Rf	493.5 AU	35.16 %	0.98 Rf	0.1 AU	26236.4 AU	45.87 %

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.01 Rf	76.6 AU	0.01 Rf	89.7 AU	5.21 %	0.05 Rf	17.6 AU	1488.9 AU	1.82 %
2	0.05 Rf	18.1 AU	0.09 Rf	44.1 AU	2.56 %	0.12 Rf	0.1 AU	1166.9 AU	1.42 %
3	0.16 Rf	0.4 AU	0.19 Rf	45.9 AU	2.66 %	0.22 Rf	1.2 AU	780.4 AU	0.95 %
4	0.22 Rf	1.4 AU	0.26 Rf	18.7 AU	1.09 %	0.27 Rf	15.2 AU	380.5 AU	0.46 %
5	0.27 Rf	15.5 AU	0.32 Rf	99.8 AU	5.80 %	0.34 Rf	23.5 AU	2629.7 AU	3.20 %
6	0.35 Rf	25.2 AU	0.37 Rf	77.0 AU	4.47 %	0.40 Rf	0.2 AU	1456.1 AU	1.66 %
7	0.40 Rf	0.2 AU	0.43 Rf	52.6 AU	3.05 %	0.46 Rf	1.9 AU	981.4 AU	1.27 %
8	0.47 Rf	0.1 AU	0.59 Rf	479.8 AU	27.86 %	0.65 Rf	81.3 AU	23924.7 AU	29.68 %
9	0.65 Rf	81.7 AU	0.69 Rf	222.8 AU	12.94 %	0.74 Rf	29.1 AU	8857.1 AU	11.42 %
10	0.74 Rf	139.3 AU	0.88 Rf	591.9 AU	34.37 %	0.97 Rf	0.2 AU	37067.1 AU	47.81 %

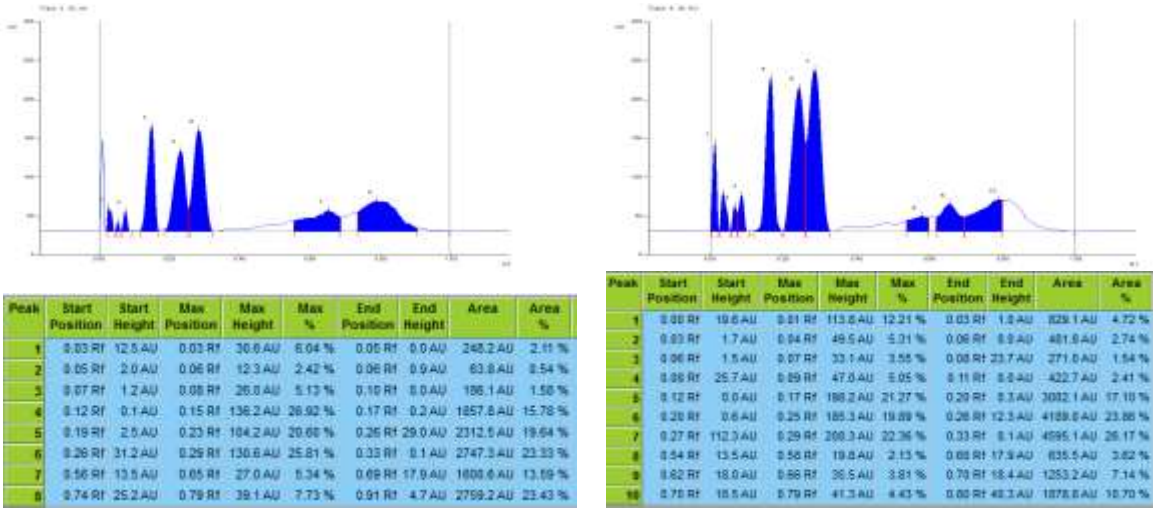
Fig. 3D: HPTLC densitogram of *H. ada-kodien* chloroform root extract in 5 (left hand side) and 10 (right hand side) µL at 575 nm



Under UV short Under UV long Under white light

Fig. 4 A. HPTLC fingerprint of methanol root extract of *H. ada-kodien* at 254, 366 and 575 nm

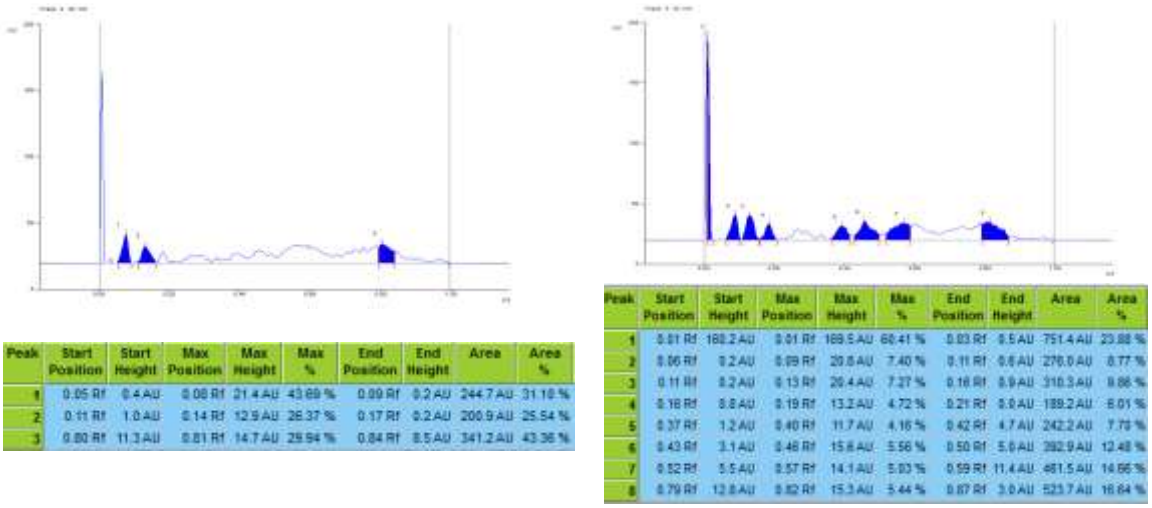
and substituents in Ayurvedic formulations (Kumar *et al.*, 2010). Traditional macroscopic and microscopic techniques are inadequate for comprehensive quality control and standardization. Chemical evaluation is essential in this context, and HPTLC serves as a valuable tool due to its high sensitivity, superior separation, and reproducibility. This method is effective not only for detecting adulteration with



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.03 Rf	12.5 AU	0.03 Rf	30.6 AU	6.04 %	0.05 Rf	0.0 AU	246.2 AU	2.11 %
2	0.05 Rf	2.0 AU	0.06 Rf	12.3 AU	2.42 %	0.06 Rf	0.9 AU	63.8 AU	0.54 %
3	0.07 Rf	1.2 AU	0.08 Rf	26.0 AU	5.13 %	0.10 Rf	0.0 AU	186.1 AU	1.58 %
4	0.12 Rf	0.1 AU	0.15 Rf	136.2 AU	26.92 %	0.17 Rf	0.2 AU	1857.8 AU	15.78 %
5	0.19 Rf	2.5 AU	0.23 Rf	104.2 AU	20.60 %	0.26 Rf	29.0 AU	2312.5 AU	19.64 %
6	0.26 Rf	31.2 AU	0.29 Rf	130.6 AU	25.81 %	0.33 Rf	0.1 AU	2747.3 AU	23.33 %
7	0.56 Rf	13.5 AU	0.65 Rf	27.0 AU	5.34 %	0.69 Rf	17.9 AU	1600.6 AU	13.59 %
8	0.74 Rf	25.2 AU	0.79 Rf	39.1 AU	7.73 %	0.91 Rf	4.7 AU	2759.2 AU	23.43 %

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.00 Rf	19.6 AU	0.01 Rf	113.6 AU	12.21 %	0.03 Rf	1.9 AU	825.1 AU	4.72 %
2	0.03 Rf	1.7 AU	0.04 Rf	49.5 AU	5.31 %	0.06 Rf	0.9 AU	481.6 AU	2.74 %
3	0.06 Rf	1.5 AU	0.07 Rf	33.1 AU	3.55 %	0.08 Rf	23.7 AU	271.0 AU	1.54 %
4	0.08 Rf	25.7 AU	0.09 Rf	47.0 AU	5.05 %	0.11 Rf	0.0 AU	422.7 AU	2.41 %
5	0.12 Rf	0.0 AU	0.17 Rf	198.2 AU	21.27 %	0.20 Rf	3.3 AU	3032.1 AU	17.10 %
6	0.20 Rf	0.6 AU	0.25 Rf	185.3 AU	19.89 %	0.26 Rf	12.3 AU	4189.6 AU	23.86 %
7	0.27 Rf	112.3 AU	0.29 Rf	209.3 AU	22.36 %	0.33 Rf	0.1 AU	4595.1 AU	26.17 %
8	0.54 Rf	13.5 AU	0.56 Rf	19.6 AU	2.13 %	0.60 Rf	17.9 AU	635.5 AU	3.62 %
9	0.62 Rf	18.0 AU	0.66 Rf	36.5 AU	3.81 %	0.70 Rf	18.4 AU	1253.2 AU	7.14 %
10	0.76 Rf	18.5 AU	0.79 Rf	41.3 AU	4.43 %	0.80 Rf	48.3 AU	1878.6 AU	10.70 %

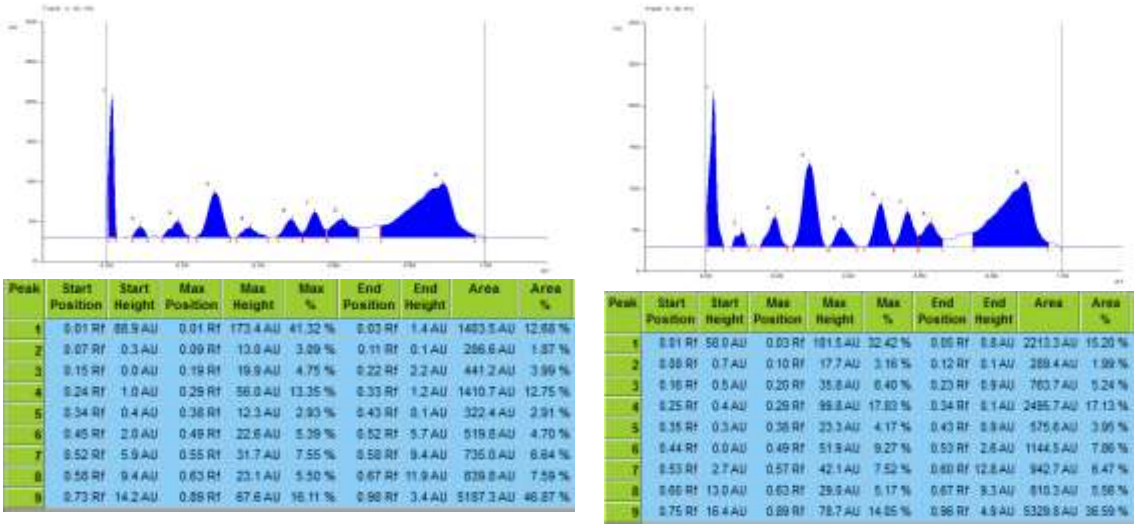
Fig. 4B: HPTLC densitogram of *H. ada-kodien* methanol root extract in 5 (left hand side) and 10 (right hand side) µL at 254 nm



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.05 Rf	0.4 AU	0.08 Rf	21.4 AU	43.69 %	0.09 Rf	0.2 AU	244.7 AU	31.10 %
2	0.11 Rf	1.0 AU	0.14 Rf	12.9 AU	26.37 %	0.17 Rf	0.2 AU	200.9 AU	25.54 %
3	0.80 Rf	11.3 AU	0.81 Rf	14.7 AU	29.94 %	0.84 Rf	8.5 AU	341.2 AU	43.36 %

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.01 Rf	168.2 AU	0.01 Rf	169.5 AU	60.41 %	0.03 Rf	8.5 AU	751.4 AU	23.88 %
2	0.06 Rf	0.2 AU	0.09 Rf	20.8 AU	7.40 %	0.11 Rf	0.6 AU	276.0 AU	8.77 %
3	0.11 Rf	0.2 AU	0.13 Rf	20.4 AU	7.27 %	0.16 Rf	8.9 AU	310.3 AU	9.86 %
4	0.16 Rf	0.8 AU	0.19 Rf	13.2 AU	4.72 %	0.21 Rf	8.9 AU	189.2 AU	6.01 %
5	0.37 Rf	1.2 AU	0.40 Rf	11.7 AU	4.16 %	0.42 Rf	4.7 AU	242.2 AU	7.70 %
6	0.43 Rf	3.1 AU	0.46 Rf	15.6 AU	5.56 %	0.50 Rf	5.6 AU	392.9 AU	12.48 %
7	0.52 Rf	5.5 AU	0.57 Rf	14.1 AU	5.03 %	0.59 Rf	11.4 AU	461.5 AU	14.66 %
8	0.79 Rf	12.8 AU	0.82 Rf	15.3 AU	5.44 %	0.87 Rf	3.0 AU	523.7 AU	16.64 %

Fig. 4C. HPTLC densitogram of *H. ada-kodien* methanol root extract in 5 (left hand side) and 10 (right hand side) µL at 366 nm



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.01 Rf	88.9 AU	0.01 Rf	173.4 AU	41.32 %	0.03 Rf	1.4 AU	1483.5 AU	12.68 %
2	0.07 Rf	0.3 AU	0.09 Rf	13.0 AU	3.09 %	0.11 Rf	0.1 AU	286.6 AU	1.87 %
3	0.15 Rf	0.0 AU	0.19 Rf	19.9 AU	4.75 %	0.22 Rf	2.2 AU	441.2 AU	3.99 %
4	0.24 Rf	1.0 AU	0.29 Rf	56.0 AU	13.35 %	0.33 Rf	1.2 AU	1410.7 AU	12.75 %
5	0.34 Rf	0.4 AU	0.38 Rf	12.3 AU	2.93 %	0.43 Rf	0.1 AU	322.4 AU	2.91 %
6	0.45 Rf	2.0 AU	0.48 Rf	22.6 AU	5.39 %	0.52 Rf	5.7 AU	519.6 AU	4.70 %
7	0.52 Rf	5.9 AU	0.55 Rf	31.7 AU	7.55 %	0.58 Rf	9.4 AU	735.0 AU	6.64 %
8	0.58 Rf	9.4 AU	0.63 Rf	23.1 AU	5.50 %	0.67 Rf	11.9 AU	839.8 AU	7.59 %
9	0.73 Rf	14.2 AU	0.89 Rf	67.6 AU	16.11 %	0.98 Rf	3.4 AU	5157.3 AU	46.67 %

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.01 Rf	58.0 AU	0.03 Rf	161.5 AU	32.42 %	0.05 Rf	8.8 AU	2213.3 AU	15.20 %
2	0.08 Rf	0.7 AU	0.10 Rf	17.7 AU	3.16 %	0.12 Rf	8.1 AU	289.4 AU	1.99 %
3	0.16 Rf	0.5 AU	0.20 Rf	35.8 AU	6.40 %	0.23 Rf	8.9 AU	763.7 AU	5.24 %
4	0.25 Rf	0.4 AU	0.29 Rf	95.8 AU	17.03 %	0.34 Rf	8.1 AU	2485.7 AU	17.13 %
5	0.35 Rf	0.3 AU	0.38 Rf	23.3 AU	4.17 %	0.43 Rf	8.9 AU	575.6 AU	3.95 %
6	0.44 Rf	0.0 AU	0.49 Rf	51.9 AU	9.27 %	0.53 Rf	2.6 AU	1144.5 AU	7.86 %
7	0.53 Rf	2.7 AU	0.57 Rf	42.1 AU	7.52 %	0.60 Rf	12.8 AU	942.7 AU	6.47 %
8	0.66 Rf	13.0 AU	0.63 Rf	29.8 AU	5.17 %	0.67 Rf	9.3 AU	810.3 AU	5.56 %
9	0.75 Rf	16.4 AU	0.89 Rf	78.7 AU	14.05 %	0.96 Rf	4.9 AU	5329.8 AU	36.59 %

Fig. 4D: HPTLC densitogram of *H. ada-kodien* methanol root extract in 5 (left hand side) and 10 (right hand side) µL at 575 nm

standardized quality control measures (Shinde *et al.*, 2009). The methanol extract, for instance, showed clear separation of bands in chromatograms and fingerprints, which can be used for quality

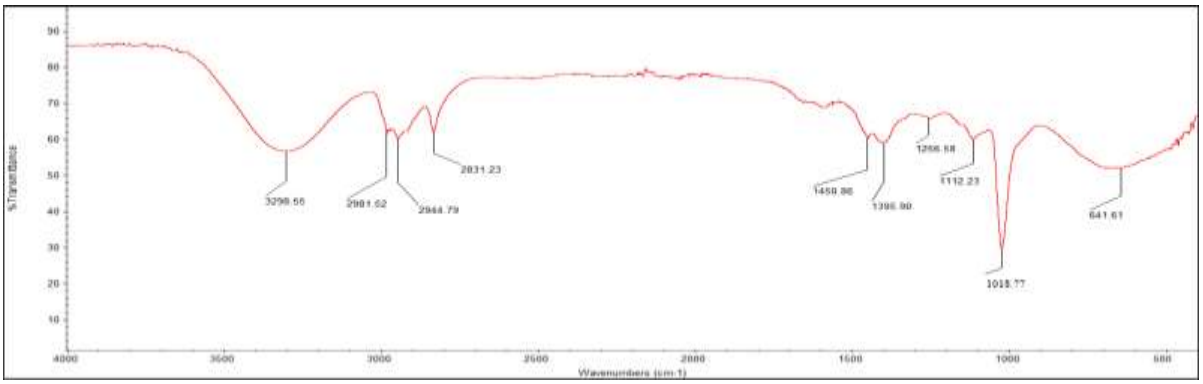


Fig. 5: FT-IR spectrum of methanolic extract of *H. adakodien*

Table 1: Functional groups and vibrations based on peak frequencies of *H. adakodien* in FTIR analysis

Peak frequency (cm^{-1})	Functional group	Name of the functional group	Type of vibration
3298.55	OH	Alcohol and hydroxy	OH stretch
2981.62	C-H	Methyl	Sym. Stretch
2944.79	CH	Methyne	C-H stretch
2831.23	CH3	Methoxy, methyl ether	O-CH_3, C-H stretch
1450.86	CH2	Methylene	C-H bend
1396.90	OH	Alcohol and Hydroxy Compound	O-H bend
1256.58	R-O-R	Aromatic ethers, aryl	O stretch
1112.23	CH	Aromatic	C-H bend
1018.77	CN	Primary amine	CN stretch
641.61	OH	Alcohol	OH out of plane bend

assessment in *H. ada-kodien*. Reports are there for chemical profiling by HPTLC studies on *Fumaria parviflora* helps in the identification of bioactive compounds and markers (Bhargava et al., 2021). In *H. ada-kodien,* methanol extract shows a clear separation of bands in chromatograms and fingerprints, which can be used as a methodology for quality confirmation. The HPTLC fingerprinting profile of *Nelumbo nucifera* seeds has been employed as a tool for establishing quality standards, authentication, and identification (Maharana et al., 2022) supports the present data.

The FTIR analysis clearly revealed that the root tuber of *H. ada-kodien* had characteristic peaks indicating the presence of various functional group (Table 1) and FTIR spectrum (Fig. 3). FT-IR gave a broad peak at 3298.55 cm^{-1}, which indicated an O-H stretching. A strong peak at 1018.77 cm^{-1} showed C-N stretch indicating the presence of primary amine. The peak at 641.61 cm^{-1} indicated the presence of CH bends out of the plane. The peak at 2981.62 cm-1 pointed to symmetric stretch indicating the presence of methyl group. The peak at 1256.58 cm-1 indicated O stretch pointing the presence of aromatic ethers or aryl group. In *Ichnocarpus frutescens,* the detected elements and functional groups in ethanol extract of whole plant using FTIR spectroscopic method is reported (Liu et al., 2006). Many researchers applied the FTIR spectrum as a tool for distinguishing closely associated plants and other organisms (Rebuffo et al., 2006; Sahoo, 2011). FTIR spectroscopy has been used to analyze various extracts of *Grewia tilifolia* (Vahl) leaves and was recommended to be used as a pharmacognostic marker to identify the medicinally significant Grewia species (Devi and Battu, 2019). Enormous bioactive components are found in medicinal plants which depict strong pharmacological effects. The different functional groups contained in the various extracts of samples of medicinal plants obtained are identified using FTIR methods (Wongsa et al., 2022). The FTIR releases peaks and spectra that can act as a fingerprint of a specific molecular structure and chemical bonding (Nandiyanto et al., 2023)

Conclusions: Plants contain numerous bioactive phytocompounds, and this complexity presents a significant challenge to phytoscientists in identifying which compounds have the potential to cure specific diseases. Without screening the active compounds from particular plant, the researcher cannot develop new medicinal drugs to cure a particular disease. Standard procedures using sophisticated modern analytical techniques may help in improving the acceptance level by developed nations. HPTLC fingerprint profile obtained by various chromatographic techniques and functional group detection using FTIR analysis play important role in standardization of Ayurvedic formulations. This study can be commercially exploited to enhance the reliance on phytomedicines. al drugs.

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Conflict of interest: The authors declare no conflict of interest.

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