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Quantitative Analysis of Phytochemicals and HPTLC Profiling of Methanolic Extract of *Holostemma ada-kodien* Schult. Root Tuber

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Abstract

The present study is to quantify the phytochemical constituents in various extracts from the root tubers of *Holostemma ada-kodien* and also to establish chemical finger print through HPTLC analysis of methanolic extract. The HPTLC profile can be used as a marker for identifying adulterants in terms of phytochemicals from raw drug. The powdered root sample was extracted by soxhlet using various solvents such as petroleum ether, chloroform, acetone, methanol and water based on the polarity and was used for quantifying various phytochemicals. HPTLC analysis showed the functional groups of diverse phytochemicals in the methanolic extract which may substantiate its medicinal properties.

Keywords:*Holostemmaada-kodien*, Methanolic extract, HPTLC profile.

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1. Introduction

Holostemma ada-kodien Schult. (Syn. *H. annulare* (Roxb.) K. Schum.) of Asclepiadaceae was used as antidiabetic, rejuvenative, aphrodisiac, expectorant, stimulant, treating ophthalmic disorders and for maintaining youthful vigour, strength and vitality in traditional medicine [1-3]. The roots and leaves are used to treat spider poisoning in the form of powder and juice. The roots rubbed into a mash are used in cold milk as a cure to diabetes [4]. The seeds of *H. adakodien* showed a high rate of germination but fruit set was a major problem in multiplying the species. The tuberous roots are used in several Ayurvedic drug preparations and so unscientific way of collection of the root tubers led to acute scarcity of the plant and is listed out as rare [5] and vulnerable in the first red list of medicinal plants of South India [6]. Even though the plant has immense biological potentiality, not much research work has been carried out to explore its phytoconstituents. The present work focuses on the quantification of secondary metabolites from the serial extracts of the root tuber and also the HPTLC profile of the secondary metabolites in the methanolic extract.

2. Materials and Methods

Mature root tuber from the *Holostemma ada-kodien* plant maintained in the Department of Botany, University of Kerala, Kariavattom was the source of material for the study (**Figure 1a**). The accessions collected from localities of Peechi, Thrissur District (76º 18’ East Longitude and 10º 28’ North Latitude. and Altitude 55.00 m), Kerala, India were verified and confirmed by Curator, Department of Botany, University of Kerala, Thiruvananthapuram, Kerala, India and a voucher specimen was deposited at the Department Herbarium with an accession number KUBH 10456. Two year old root tubers were shade dried and powdered and this powder was used for the phytochemical analysis (**Figure1b&c**). Finely powdered root tuber was extracted by soxhlet with various solvents such as hexane, chloroform, acetone, methanol and water. The extract was filtered and dried using a rotary evaporator under vacuum at 45°C.Quantitative analysis of phytochemicals in various extracts was carried out according to the standard protocols.

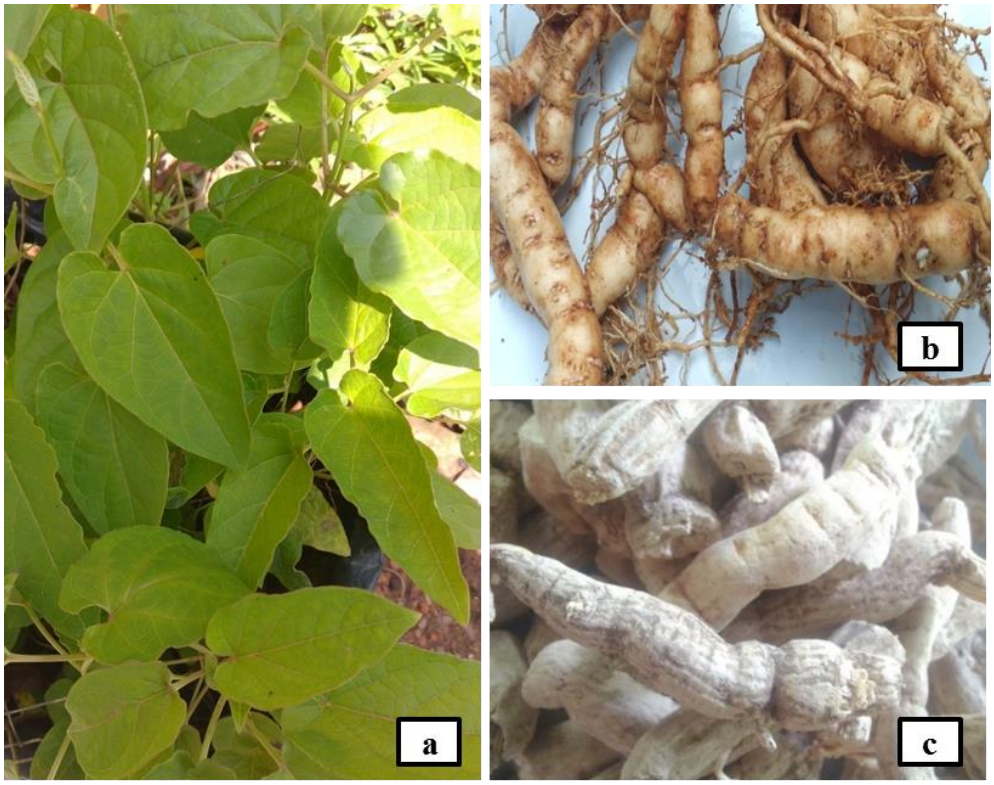


Figure 1.a) *Holstemma ada-kodien* plant,**b)** root tuber and**c)** dried root tuber.

2.1.Quantitative Analysis on Phytochemical constituents in various extracts.

For quantitative analysis, 1mg/ml of extract was prepared using the solvent employed forextraction. The methodology adopted was based on standard protocols. The absorbance under UV-Visible spectrophotometer at particular wavelength was noted.

Terpenoids*:* 1mg/ml extract was mixed well in 9ml ethanol. Add 10 ml of petroleum ether into separating funnel having ethanol extract. Shake well so that terpenoid was leached into petroleum ether. The ether extract was dried and quantified from pre-weighed glass vials [7].

Flavanoid*:* Total flavanoid content was measured by the aluminium chloride colorimetric assay with Quercetin as standard at 510nm. The reaction mixture consists of 1mg/ml of extract and 4 ml of distilled water in a 10 ml volumetric flask. Then, added 0.30 ml of 5 % sodium nitrite and after 5 minutes 0.3 ml of 10 % aluminium chloride were mixed. About 2 ml of 1M Sodium hydroxide was treated and diluted to 10 ml with distilled water after 5 minutes.[8].

Tannin: Content of tannins in sample was determined by Folin-Ciocalteu method. Added 0.5 mL of Folin-Ciocalteu reagent and 35% 1ml sodium carbonate solution. The absorbance at 700nm was measured after the volume made upto 10ml with distilled water [9].

Phenol: The sample was pipetted out and the volume in the tube was made up to 3.0 ml with distilled water against the blank with distilled water. Folin-Ciocalteu reagent (0.5ml) was added and then mixed with 2ml of 20% sodium bicarbonate solution, for one minute the tubes were placed in a boiling water bath. The tubes were cooled and the absorbance was read at 750nm [10].

Glycosides: Glycosides were quantified using the standard digitoxin. Cardiac glycosides showed an orange red colour complex with Baljet's reagent (Picric acid in alkaline medium). The colour intensity produced was proportional to the concentration of glycosides. Cardiac glycosides were quantitatively determined by some modifications [11].

Alkaloid: 1ml of plant extract was dissolved in 1ml dimethyl sulphoxide (DMSO), added 1ml of 2N HCl and filtered. This solution was transferred to a separating funnel and added 5 ml of bromocresol green solution and 5 ml of phosphate buffer. At 1, 2, 3 and 4 ml chloroform with mixture after vigorous shaking were collected in a 10 ml volumetric flask and diluted to the volume with chloroform. Quantification was done at 470nm with atropine as standard [12].

Saponin: For determining total saponin content of plant extract vanillin-sulphuric acid assay was done by incubating 1mg/ml of plant sample extracts, standards or reagent blank with 0.25 mL of 0.8% (w/v) vanillin in ethanol and 2.5 ml of 72% (v/v) sulphuric acid in water for 15 min at 60°C in a shaking water bath. The OD at 544nm was measured for the extract after cooling in water at the ambient temperature for 5 min. and for quantification, the standard diosgenin was used [13].

2.2.HPTLC analysis

The solvent system which gave the maximum resolution was selected as the optimal solvent system for the extract. The optimum separations of constituents were achieved using toluene: ethyl acetate: formic acid (5: 1: 0.1) asmobile phase. The methanol extracts were applied as two tracks of different concentrations of width 8 mm each on silica gel 60 F_{254} pre-coated aluminium sheets through CAMAG micro litre syringe using Automatic TLC Sampler 4 (ATS4). After sample application the plate was introduced vertically in a CAMAG developing chamber (10 cm × 10 cm) pre-saturated with the mobile phase selected and developed to 70mm. The chromatogram were air dried to evaporate solvents from the plate and the plate was kept in CAMAG Visualizer and the images were captured under UV light at 254 nm and 366 nm. At 254 nm and 366 nm the plate was scanned using TLC Scanner 4 and the finger print profiles were documented. The R_f values and finger print data were recorded with win CATS software associated with the scanner. The

plate was derivatised using vanillin-sulphuric acid reagent, heated at 105°C by placing on CAMAG TLC plate heater till the colour of the bands appeared (**Figure 2**). Then the plate was visualized under white light and the chromatograms were documented (**Figure 3** and**4**). The plate was scanned at 575 nm and the Rf values and finger print data were documented (**Figure 5**).

3. Results

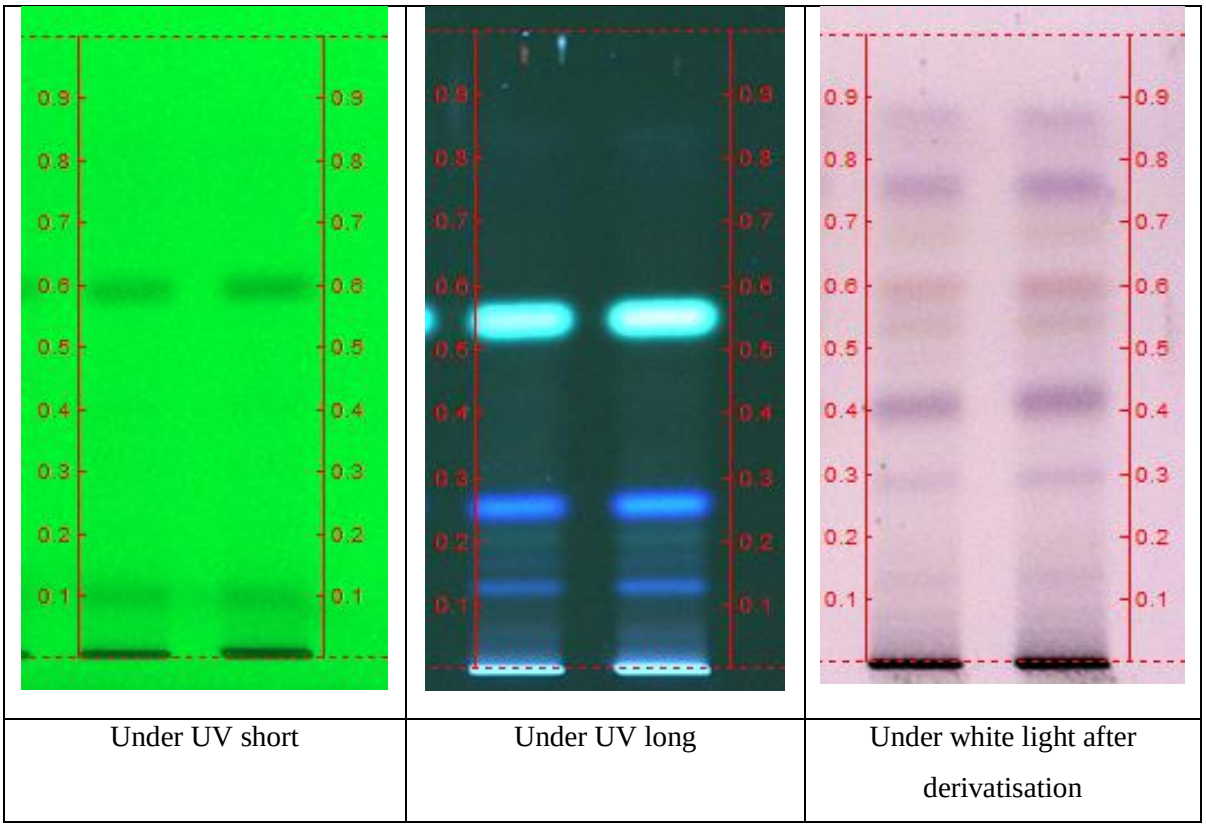
The Phytochemical quantification of alkaloids, phenols, terpenoids, glycosides, flavonoids, saponins, steroids and tannins reveals an idea about the presence of diverse secondary metabolites on the basis of quantity in various solvents. The petroleum ether extract of *Holostemma ada-kodien* root tuber contained higher levels of total terpenoid. From the results, it was noted that, the flavonoids and glycosides were found to be higher in chloroform extract, phenol in methanol extract and alkaloids in acetone extract. Saponin was quantified in chloroform, acetone and aqueous extract, among which aqueous extract shows highest quantity of saponin (**Table 1**).

Table 1. Quantitative phytochemical analysis in µg/mg of root tuber serial extract of *H. adakodien*

Phytochemical Constituents	**Petroleum ether**	**Chloroform**	**Acetone**	**Methanol**	**Aqueous**
Terpenoids	420	310	240	190	180
Alkaloids	110	180	211	159	132
Phenols	9.62	66	84.12	64.75	13.75
Glycosides	12	98	54	6	36
Flavanoids	47.5	280	180	137.5	117.5
Saponin	-	131.83	201.83	-	328.32
Tannin	13	116.67	1651.67	86.67	14.67

On visualizing under 254 nm, methanol extract showed five bands. The first peak with Rf 0.04 start point and 0.14 end point with maximum peak at 0.08 and 22.95% area in track 1 in which

15µl is applied and in second track in which 20 µl maximum peak at 0.07 and 17.55% area. Second peak in first track reveals Rf start position 0.35 and end position 0.41 and maximum peak at 0.38 with an areaof 3.68% and in track II, maximum peak at 0.38 with an areaof 4.81%. Third peak in first track with Rf start position 0.52 and end position 0.63 and maximum peak at 0.57 with an area of 57.65% and in track II, maximum peak at 0.57 with an area of 62.31%. Fourth peak with Rf start point at 0.78 and end point at 0.87 and maximum peak at 0.79 and area 12.79% in first track and start point at 0.77and end point at 0.86 and maximum peak at 0.79 and area 13.04% in second track. The last peak Rf start position at 0.96 and end position 1.02 with maximum peak at 0.96 with an area of 2.94%,while track II shows start point at 0.96 and end point at 1.00 and maximum peak at 0.98 and area 2.29%.

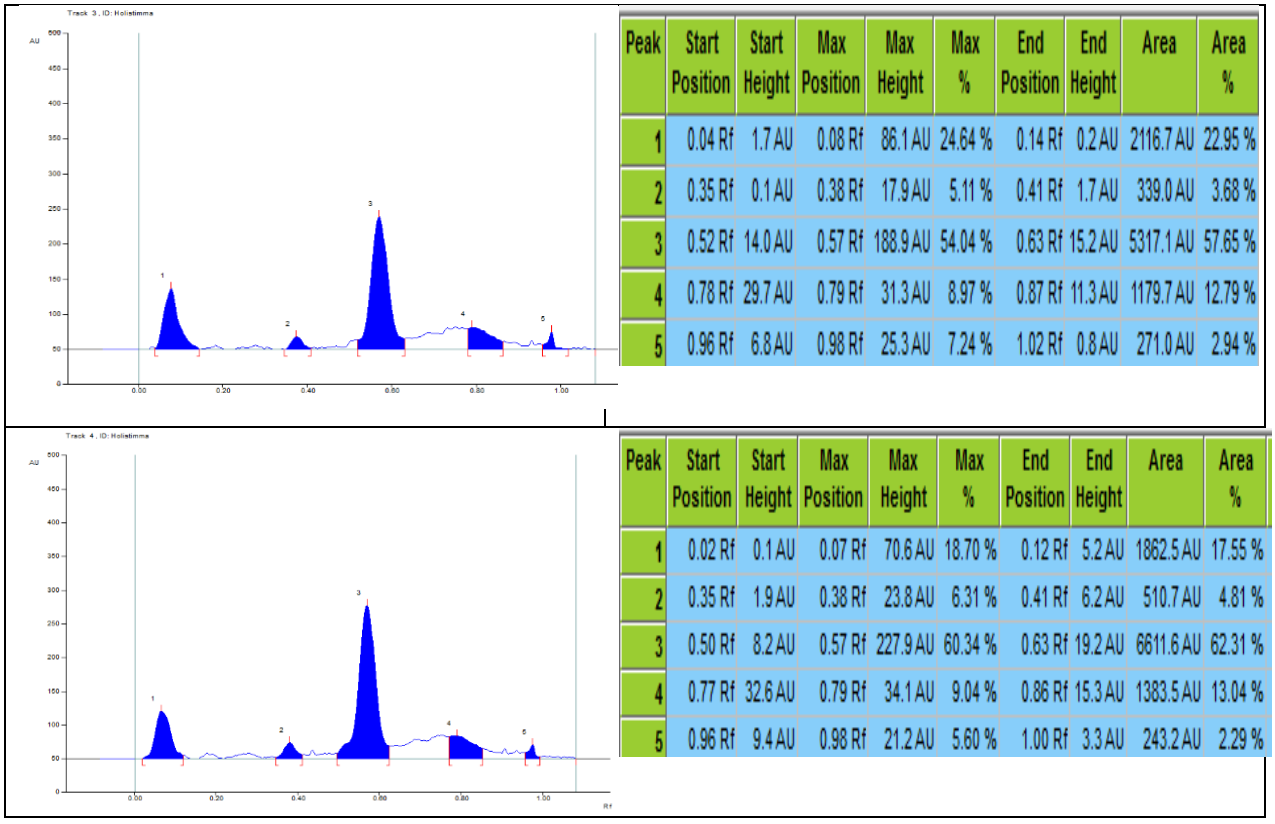


Under UV short	Under UV long	Under white light after derivatisation

Figure2. HPTLC fingerprints under visible light, short UV and long UV respectively in 15 µl and 20 µl of *Holostemma ada-kodien* root tuber methanolic extract.

Under 366 nm, HPTLC plate showed four bands. In track I, in which 15 µl was applied, the first peak with Rf 0.08 start point and 0.14 end point with maximum peak at 0.11 and area of 2.51%

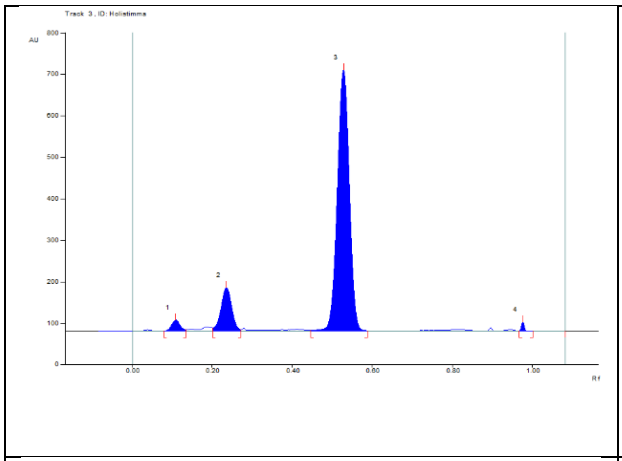
and in second track in which 20 µl maximum peak at 0.11 and 2.68% area. Second peak in first track with Rf start position 0.20 and end position 0.27 and maximum peak at 0.24 with an area of 12.56% and in track II, maximum peak at 0.24 with an area of 12.69%. Third peak with Rf start position 0.45 and end position 0.59 and maximum peak at 0.53 with an area of 84.25% in first track and maximum peak at 0.53with an area of 84.21% in track II. The last peak with Rf start point at 0.97 and end point at 1.00 and maximum peak at 0.98 and area 0.67% in first track and maximum peak at 0.94 and area of0.43% in second track.



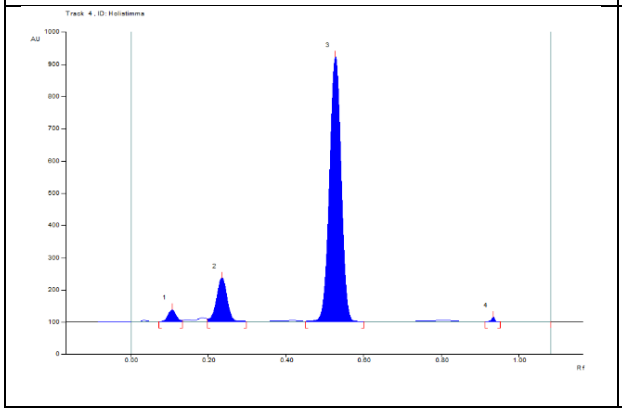
Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.04 Rf	1.7 AU	0.08 Rf	86.1 AU	24.64 %	0.14 Rf	0.2 AU	2116.7 AU	22.95 %
2	0.35 Rf	0.1 AU	0.38 Rf	17.9 AU	5.11 %	0.41 Rf	1.7 AU	339.0 AU	3.68 %
3	0.52 Rf	14.0 AU	0.57 Rf	188.9 AU	54.04 %	0.63 Rf	15.2 AU	5317.1 AU	57.65 %
4	0.78 Rf	29.7 AU	0.79 Rf	31.3 AU	8.97 %	0.87 Rf	11.3 AU	1179.7 AU	12.79 %
5	0.96 Rf	6.8 AU	0.98 Rf	25.3 AU	7.24 %	1.02 Rf	0.8 AU	271.0 AU	2.94 %

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.02 Rf	0.1 AU	0.07 Rf	70.6 AU	18.70 %	0.12 Rf	5.2 AU	1862.5 AU	17.55 %
2	0.35 Rf	1.9 AU	0.38 Rf	23.8 AU	6.31 %	0.41 Rf	6.2 AU	510.7 AU	4.81 %
3	0.50 Rf	8.2 AU	0.57 Rf	227.9 AU	60.34 %	0.63 Rf	19.2 AU	6611.6 AU	62.31 %
4	0.77 Rf	32.6 AU	0.79 Rf	34.1 AU	9.04 %	0.86 Rf	15.3 AU	1383.5 AU	13.04 %
5	0.96 Rf	9.4 AU	0.98 Rf	21.2 AU	5.60 %	1.00 Rf	3.3 AU	243.2 AU	2.29 %

Figure 3. Densitogram, Rf values and peak area showing percentage of secondary metabolites in methanolic root tuber extract under 15 µl and 20 µl of *Holostemma ada-kodien* at 254nm.



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.08 Rf	0.1 AU	0.11 Rf	26.8 AU	3.41 %	0.14 Rf	3.3 AU	394.0 AU	2.51 %
2	0.20 Rf	7.1 AU	0.24 Rf	104.8 AU	13.37 %	0.27 Rf	3.1 AU	1968.4 AU	12.56 %
3	0.45 Rf	1.5 AU	0.53 Rf	630.5 AU	80.42 %	0.59 Rf	0.6 AU	13199.5 AU	84.25 %
4	0.97 Rf	0.0 AU	0.98 Rf	21.9 AU	2.79 %	1.00 Rf	0.1 AU	105.1 AU	0.67 %



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.07 Rf	0.0 AU	0.11 Rf	36.8 AU	3.65 %	0.14 Rf	4.2 AU	544.2 AU	2.68 %
2	0.20 Rf	8.2 AU	0.24 Rf	136.0 AU	13.47 %	0.30 Rf	0.9 AU	2577.1 AU	12.69 %
3	0.45 Rf	0.9 AU	0.53 Rf	821.8 AU	81.43 %	0.60 Rf	0.3 AU	17107.4 AU	84.21 %
4	0.91 Rf	0.4 AU	0.94 Rf	14.6 AU	1.45 %	0.95 Rf	0.2 AU	86.6 AU	0.43 %

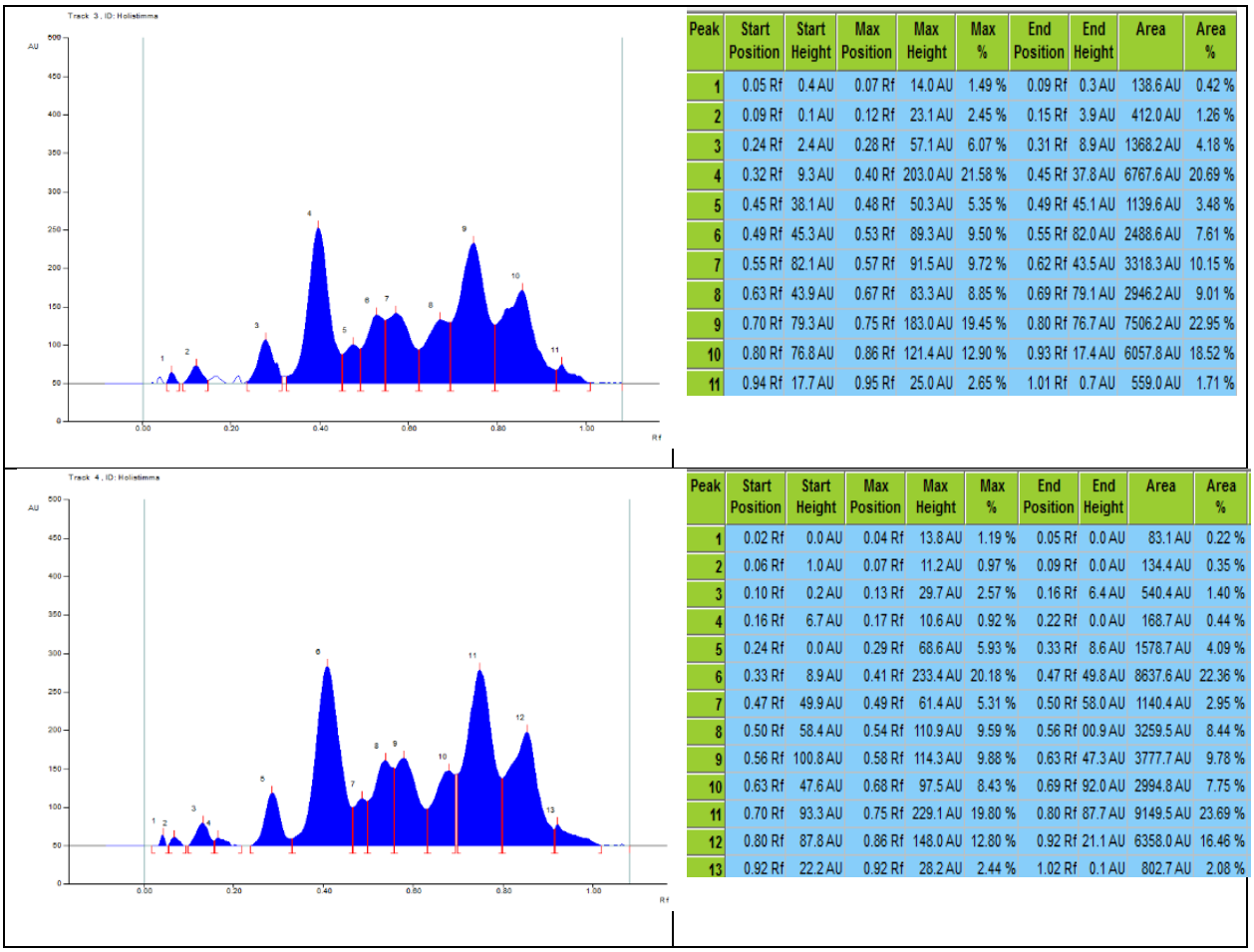
Figure4. Densitogram, Rf values and peak area showing percentage of secondary metabolites in methanolic root tuber extract under 15 µl and 20 µl of *Holostemma ada-kodien* at 366 nm.

HPTLC plate when viewed under 575nm showed eleven bands in which three peaks with maximum area 20.69%, 22.95% and 18.52% having Rf maxima 0.40, 0.75 and 0.86 respectively in track I and in track II three peaks corresponding to track I with maximum area 22.36% , 23.69% and 16.46% having Rf maxima 0.41, 0.75 and 0.86 respectively.

4. Discussions

Plants with therapeutic properties are commonly used by traditional healers in most rural areas of the world's developing countries. [14]. The polarity of the extraction solvent and the extraction procedure have a significant impact on the efficiency (yield) and efficacy (magnitude of bioactivity) of produced extracts. [15]. As the extraction of *H. ada-kodien* root tuber was done on the basis of polarity, the result was used as first hand reference for selecting solvent for isolating a specific metabolite. It was reported that root tuber produce terpenoid sugars that are responsible

for the medicinal properties [16]. But literature on further studies in *Holostemma ada-kodien* do not focuses on above study. Present study confirms the major component in root tuber as terpenoid. Terpenoids were the most widespread, chemically interesting groups of secondary metabolites with over 30,000 known compounds including steroids reported by [17].



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.05 Rf	0.4 AU	0.07 Rf	14.0 AU	1.49 %	0.09 Rf	0.3 AU	138.6 AU	0.42 %
2	0.09 Rf	0.1 AU	0.12 Rf	23.1 AU	2.45 %	0.15 Rf	3.9 AU	412.0 AU	1.26 %
3	0.24 Rf	2.4 AU	0.28 Rf	57.1 AU	6.07 %	0.31 Rf	8.9 AU	1368.2 AU	4.18 %
4	0.32 Rf	9.3 AU	0.40 Rf	203.0 AU	21.58 %	0.45 Rf	37.8 AU	6767.6 AU	20.69 %
5	0.45 Rf	38.1 AU	0.48 Rf	50.3 AU	5.35 %	0.49 Rf	45.1 AU	1139.6 AU	3.48 %
6	0.49 Rf	45.3 AU	0.53 Rf	89.3 AU	9.50 %	0.55 Rf	82.0 AU	2488.6 AU	7.61 %
7	0.55 Rf	82.1 AU	0.57 Rf	91.5 AU	9.72 %	0.62 Rf	43.5 AU	3318.3 AU	10.15 %
8	0.63 Rf	43.9 AU	0.67 Rf	83.3 AU	8.85 %	0.69 Rf	79.1 AU	2946.2 AU	9.01 %
9	0.70 Rf	79.3 AU	0.75 Rf	183.0 AU	19.45 %	0.80 Rf	76.7 AU	7506.2 AU	22.95 %
10	0.80 Rf	76.8 AU	0.86 Rf	121.4 AU	12.90 %	0.93 Rf	17.4 AU	6057.8 AU	18.52 %
11	0.94 Rf	17.7 AU	0.95 Rf	25.0 AU	2.65 %	1.01 Rf	0.7 AU	559.0 AU	1.71 %

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.02 Rf	0.0 AU	0.04 Rf	13.8 AU	1.19 %	0.05 Rf	0.0 AU	83.1 AU	0.22 %
2	0.06 Rf	1.0 AU	0.07 Rf	11.2 AU	0.97 %	0.09 Rf	0.0 AU	134.4 AU	0.35 %
3	0.10 Rf	0.2 AU	0.13 Rf	29.7 AU	2.57 %	0.16 Rf	6.4 AU	540.4 AU	1.40 %
4	0.16 Rf	6.7 AU	0.17 Rf	10.6 AU	0.92 %	0.22 Rf	0.0 AU	168.7 AU	0.44 %
5	0.24 Rf	0.0 AU	0.29 Rf	68.6 AU	5.93 %	0.33 Rf	8.6 AU	1578.7 AU	4.09 %
6	0.33 Rf	8.9 AU	0.41 Rf	233.4 AU	20.18 %	0.47 Rf	49.8 AU	8637.6 AU	22.36 %
7	0.47 Rf	49.9 AU	0.49 Rf	61.4 AU	5.31 %	0.50 Rf	58.0 AU	1140.4 AU	2.95 %
8	0.50 Rf	58.4 AU	0.54 Rf	110.9 AU	9.59 %	0.56 Rf	00.9 AU	3259.5 AU	8.44 %
9	0.56 Rf	100.8 AU	0.58 Rf	114.3 AU	9.88 %	0.63 Rf	47.3 AU	3777.7 AU	9.78 %
10	0.63 Rf	47.6 AU	0.68 Rf	97.5 AU	8.43 %	0.69 Rf	92.0 AU	2994.8 AU	7.75 %
11	0.70 Rf	93.3 AU	0.75 Rf	229.1 AU	19.80 %	0.80 Rf	87.7 AU	9149.5 AU	23.69 %
12	0.80 Rf	87.8 AU	0.86 Rf	148.0 AU	12.80 %	0.92 Rf	21.1 AU	6358.0 AU	16.46 %
13	0.92 Rf	22.2 AU	0.92 Rf	28.2 AU	2.44 %	1.02 Rf	0.1 AU	802.7 AU	2.08 %

Figure 5. Densitogram, Rf values and peak area showing percentage of secondary metabolites in methanolic root tuber extract under 15 µl and 20 µl of *Holostemma ada-kodien* at 575 nm.

HPTLC provides improved resolution and accurate assessment of active components in a shorter amount of time [18]. These analytical techniques provide more accurate and précised data, not only supporting drug discovery and development but also post market surveillance [19]. The chemoprofile can be used to identify the plants from which the extract was obtained. It is a method of chemical standardization of the drug. The fingerprint profile gives the authenticity of the drug.

5. Conclusion

The quantification of secondary metabolites from the serial extract gives a clear idea about the concentration of metabolites in each extract. The HPTLC profile confirmed the presence of a wide variety of bioactive compounds in the species. The presence of phytochemicals in the extract of *Holostemma ada-kodien* proves the ethnomedicinal use of root tuber for various treatments.

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Conflict of Interest: We declare that we have no conflict of interest.

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