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HPTLC Analysis of the Leaf Extract of *Hydnocarpus macrocarpa* (Beddome) Warb.

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ABSTRACT

The present study was conducted to identify the major phytochemical compounds in the leaves of *Hydnocarpus macrocarpa* (Beddome) Warb., an endemic and endangered species from South India. The methanolic extract of leaf was subjected to phytochemical screening by HPTLC. The analysis revealed that the leaf extract of *H. macrocarpa* is rich in phytochemical compounds like Alkaloids, Essential oils, Flavonoids, Flavonoid glycosides, Phenolics, Saponins, Steroids, Tannins and Triterpenes.

Keywords: *Hydnocarpus macrocarpa*, HPTLC, Phytochemicals compounds, Endemic species.

1. Introduction

Hydnocarpus Gaertn. is an Indo-Malayan genus belonging to the family Flacourtiaceae [1]. Five species of *Hydnocarpus* are reported to occur in India viz., *H. alpina*, *H. kurzii*, *H. macrocarpa*, *H. pentandra* and *H. pendulus* [2-6]. Out of the five species, *H. pentandra*, *H. macrocarpa* and *H. pendulus* are endemic to South India. *H. pentandra* is the most widely distributed species while *H. macrocarpa* is restricted to Ponmudi, Adimali and Sholayar in Kerala and Kanyakumari in Tamil Nadu. It is an underexploited and endangered species [5].

The seeds of *H. Kurzii* [7-8] and other species of *Hydnocarpus* viz., *H. alpina* and *H. pentandra* are the source of 'Chaulmoogra' oil, which is used for the treatment of leprosy. Earlier studies have also revealed the presence of biologically active flavonolignan compounds in the fruit rind of *H. pentandra* (Syn: *H. wightiana*) [9-13]. However, a close examination of literature shows that no studies regarding the phytochemical compounds of *H. macrocarpa* have been done, which necessitates such a requirement. Hence, the present study aims to analyse the major phytochemical compounds in the methanolic extract of *H. macrocarpa*.

2. Materials and Methods

2.1 Collection and Extraction of Plant materials

Fresh and uninfected leaves from the source plant were collected from different locations viz., Ponmudi, Thiruvananthapuram; Adimali, Idukki and Sholayar, Thrissur, Kerala, India. Prior permission was obtained for the collection of plant materials from the Department of Forest and Wild Life, Government of Kerala. The voucher specimen (Voucher No.TD078) is deposited in the CMS herbarium, C.M.S College Kottayam, Kerala, India. The collected materials were thoroughly washed, cleaned, oven dried and powdered.

The powdered leaves (20 g) were extracted in 200 ml 100% methanol in a Soxhlet apparatus for 8 hrs. The extract was filtered and was concentrated using Rotary vacuum evaporator.

2.2 HPTLC method and chromatographic conditions

The HPTLC system (Camag, Muttenz, Switzerland) has Linomat V auto sprayer connected to a nitrogen cylinder; a twin trough chamber (10 x 10 cm) and a derivatization chamber. Pre-coated silica gel 60 F_{254} TLC plates (10 x 10 cm, layer thickness 0.2 mm – E Merck KGaA, Darmstadt, Germany) were used as the stationary phase. TLC plates were prewashed twice with 10 ml of methanol and activated at 80 ^{0}C for 5 minutes prior to sample application. Densitometric analysis was carried out using a TLC scanner III with winCATS software.

2.3 Sample application

7 µl of sample was spotted on pre-coated TLC plate in the form of narrow bands (8 mm) with

with 10 mm from the bottom using Linomat V spotter. Samples were applied under continuous dry stream of nitrogen gas at constant application amount 7 µl

2.4 Mobile phase and migration
The spotted plates were developed using different mobile phases to detect the various classes of phytochemicals. The proportion of the chemicals in the mobile phases is as follows:

Alkaloid - Toluene: Methanol: Diethyl amine (8:1:1)

Essential oils - Toluene: Ethyl acetate (8.5:1.5)

Flavonoid glycosides - Ethyl acetate: Acetic acid: Formic acid: Water (10:1.1:1.1:2.6)

Flavonoids - Toluene: Ethyl acetate: Formic acid (7:3:0.1)

Phenolics - THF: Toluene: Formic acid: Water (16:8:2:1)

Saponins - Chloroform: Acetic acid: Methanol: Water (6.4:3.2:1.2:0.8)

Steroids - Toluene: Methanol: Acetone (6:2:2)

Tannin - Ethyl acetate: Acetic acid: Ether: Hexane (4:2:2:2)

Triterpenes - Toluene: Chloroform: Ethanol (4:4:1)

Linear ascending development was carried out in 10 x 10 cm twin trough glass chamber equilibrated with mobile phase. The optimized chamber saturation time for mobile phase was 20 minutes at 25±2 ^{0}C with a relative humidity of 60±5%. Ten millilitres of the mobile phase (5 ml in trough containing the plate and 5 ml in other trough) was used for the development and allowed to migrate a distance of 70 mm from the point of sample application. After development, TLC plate was dried and the chromatogram was viewed at 254 nm and 366 nm to visualise and detect various phytochemical constituents.

2.5 Derivatization
The TLC plates were derivatized with the following reagents to detect the various classes of phytochemicals.

Alkaloids - Dragendorff reagent

Essential oils, Saponins and **Triterpenes** - Anisaldehyde sulphuric acid

Flavonoids and **Flavonoid glycosides** - NP/PEG Reagent

Phenolics and **Tannin** - Fast blue salt B

Steroids - Vanillin sulphuric acid

2.6 Documentation
The various conditions for documentation were selected based on the recommendations given in the CAMAG TLC Scanner III manual. The plates were photographed in various conditions under UV 254 nm, UV 366 nm and UV 366 nm after derivatization. The plates were subjected to scanning prior to derivatization. Densitometric scanning was performed on CAMAG TLC scanner III in absorbance mode and operated by winCATS planar chromatography version 1.3.4. The source of radiation utilized was Deuterium lamp. The spots were analysed at a wave length of 218 nm. The slit dimensions used in the analysis were of 6 mm length and 0.30 mm width, with a scanning rate of 20 mm/s. It covers 70-90% of the application band length. The monochromator band width was set at 20 mm. Concentration of compound chromatographed were determined on the basis of the intensity of diffusely reflected light and evaluated as peak areas against concentration using linear regression equation.

3. Results and Discussions
The results obtained from HPTLC analysis of the methanolic extract of *H. macrocarpa* with respect to Alkaloids, Essential oils, Flavonoids, Flavonoid glycosides, Phenolics, Saponins, Steroids, Tannin and Triterpenes are given below.

3.1 Alkaloids
The analysis revealed the presence of ten alkaloid bands with specific R_f values which ranges from 0.09 to 0.78. The highest concentration (24.12 %) was noticed with respect to the alkaloid band at R_f 0.13 (Fig. 1, Table 1, Plate 1).

3.2 Essential oil
Ten bands with specific R_f values were obtained with respect to Essential oil components. The R_f values ranges from 0.03 to 0.61. The Essential oil component with highest concentration (30.36%) was detected at R_f 0.61 (Fig. 2, Table 2, Plate 2).

3.3 Flavonoid Glycosides
HPTLC analysis revealed the presence of six flavonoid glycoside bands in the leaf extract. The R_f values ranges from 0.09 to 0.63. The highest concentration (30.05%) was detected at R_f 0.63 (Fig. 3, Table 3, Plate 3).

3.4 Flavonoids
Fourteen prominent Flavonoid bands with specific R_f values were identified from the leaf extract. The R_f values ranges from 0.08 to 0.81. The flavonoid band with R_f value 0.19 shows the highest (14.68%) concentration (Fig. 4, Table 4, Plate 4).

3.5 Phenolics
Thirteen Phenolic bands were identified. The R_f values ranges from 0.12 to 0.76 in which the highest concentration (20.35%) was noticed at R_f 0.34 (Fig. 5, Table 5, Plate 5).

3.6 Saponins
As revealed from the analysis, six Saponin bands were detected. The R_f values ranges from 0.08 to 0.68 and the highest concentration (35.61%) was detected at R_f 0.68 (Fig. 6, Table 6, Plate 6).

3.7 Steroids
Twelve Steroid compounds were detected in the present analysis. The R_f values of these compounds ranges from 0.05 to 0.72. The steroidal compound with R_f 0.54 shows the highest (21.02%) concentration (Fig. 7, Table 7, Plate 7).

3.8 Tannins
With respect to tannin three bands were detected with different R_f values that ranges from 0.03 to 0.24. The tannin band with highest concentration (61.57%) was identified at R_f 0.10 (Fig. 8, Table 8, Plate 8).

3.9 Triterpenes

Thirteen Triterpene bands with R_f values 0.12 to 0.76 were detected in the leaf extract of *H. macrocarpa*. The bands detected with respect to R_f 0.34 (20.35%) represent the prominent triterpene compound (Fig. 9, Table 9, Plate 9).

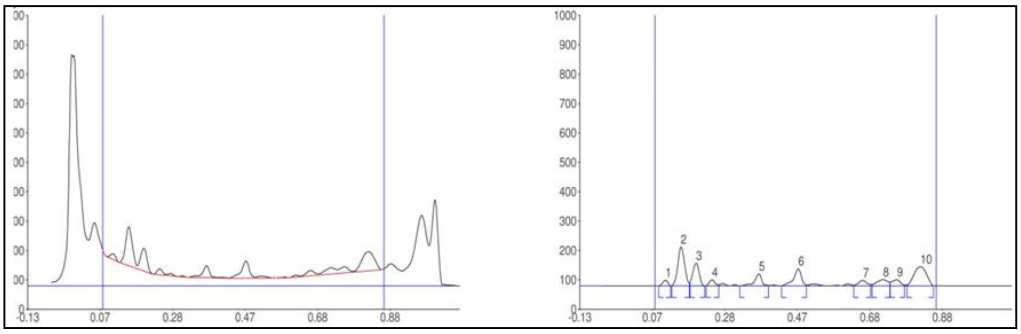

Fig 1: Chromatogram of Alkaloid in the Methanolic leaf extract of *H. macrocarpa*.

Table 1: Result of HPTLC scanning with reference to Alkaloid.

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.09	0.3	0.11	19.7	4.13	0.13	0.1	243.8	2.69
2	0.13	0.2	0.15	132.5	27.87	0.18	10.5	2186.3	24.12
3	0.18	11.9	0.20	77.1	16.22	0.22	0.3	1187.2	13.10
4	0.22	0.3	0.24	20.9	4.40	0.26	3.5	286.1	3.16
5	0.32	0.0	0.37	40.6	8.54	0.40	1.1	705.8	7.79
6	0.43	0.1	0.48	58.2	12.25	0.50	3.2	1098.1	12.11
7	0.63	2.1	0.66	18.6	3.91	0.68	4.8	373.5	4.12
8	0.68	5.0	0.71	21.4	4.50	0.73	12.8	570.0	6.29
9	0.73	12.9	0.75	21.2	4.45	0.77	2.8	435.9	4.81
10	0.78	3.2	0.82	65.2	13.72	0.85	0.4	1977.2	21.81

Plate 1: HPTLC profile with reference to Alkaloid.

Plate 2: HPTLC profile with reference to Essential oils.

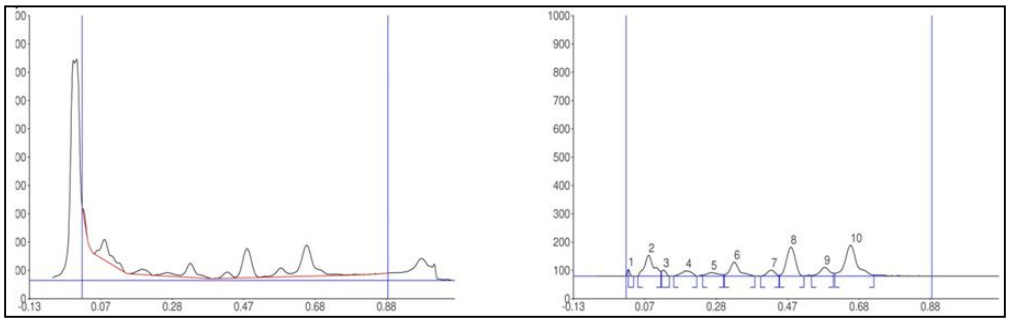

Fig 2: Chromatogram of Essential oils in the Methanolic leaf extract of *H. macrocarpa.*

Table 2: Result of HPTLC scanning with reference to Essential oils.

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.03	24.2	0.03	24.2	5.22	0.04	0.0	83.6	0.78
2	0.06	0.7	0.09	73.6	15.87	0.12	17.2	1710.1	16.03
3	0.12	17.5	0.13	21.2	4.57	0.14	0.0	222.1	2.08
4	0.16	0.0	0.19	18.9	4.08	0.22	0.1	498.4	4.67
5	0.24	3.1	0.26	12.4	2.68	0.30	5.1	378.1	3.54
6	0.30	5.1	0.33	49.4	10.64	0.38	0.0	1157.2	10.85
7	0.40	0.4	0.43	21.3	4.58	0.45	2.4	413.6	3.88
8	0.45	2.6	0.48	102.5	22.09	0.52	0.9	2226.3	20.87
9	0.54	1.6	0.58	31.2	6.73	0.60	10.7	741.3	6.95
10	0.61	10.1	0.65	109.3	23.54	0.72	4.5	3239.0	30.36

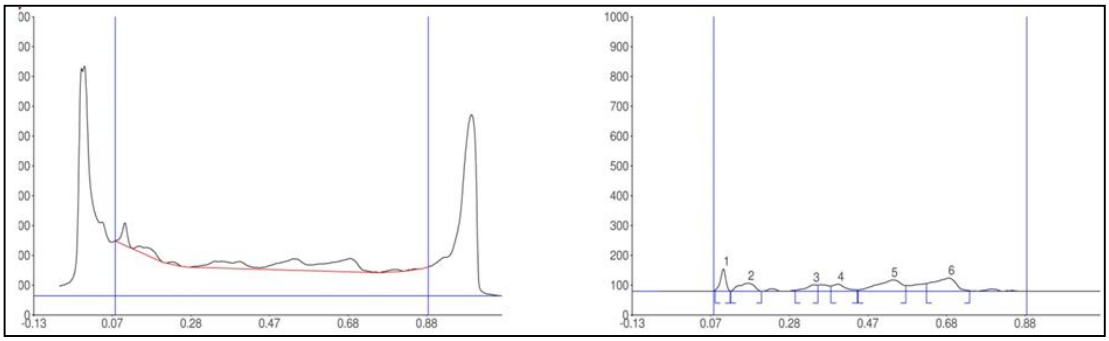

Fig 3: Chromatogram of Flavonoid glycosides in the Methanolic leaf extract of *H. macrocarpa.*

Table 3: Result of HPTLC scanning with reference to Flavonoid glycosides.

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.09	4.6	0.11	75.6	32.78	0.12	1.1	904.9	11.99
2	0.13	1.1	0.17	26.9	11.65	0.20	0.0	958.9	12.70
3	0.29	3.3	0.34	21.7	9.42	0.35	21.5	582.7	7.72
4	0.38	18.0	0.40	23.7	10.29	0.45	4.4	717.5	9.50
5	0.45	4.4	0.54	37.9	16.42	0.57	19.3	2116.9	28.04
6	0.63	26.3	0.68	44.8	19.44	0.74	2.0	2268.2	30.05

Plate 3: HPTLC profile with reference to Flavonoid glycosides.

Plate 4: HPTLC profile with reference to Flavonoids.

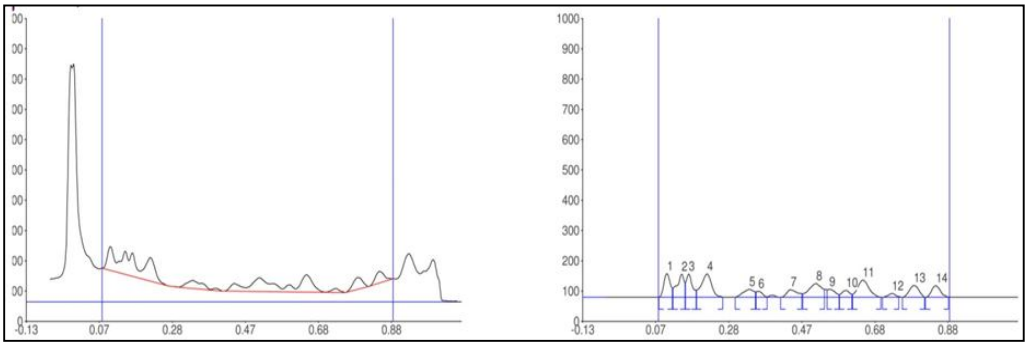

Fig 4: Chromatogram of Flavonoids in the Methanolic leaf extract of *H. macrocarpa.*

Table 4: Result of HPTLC scanning with reference to Flavonoids.

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.08	0.7	0.11	78.2	12.59	0.12	30.1	1205.7	9.21
2	0.12	30.1	0.15	76.3	12.29	0.15	48.3	1307.0	9.99
3	0.16	48.5	0.17	76.8	12.37	0.19	23.9	1128.3	8.62
4	0.19	24.0	0.22	77.3	12.45	0.26	0.2	1920.4	14.68
5	0.29	0.2	0.33	26.6	4.28	0.35	17.8	689.6	5.27
6	0.35	17.8	0.36	19.4	3.13	0.38	1.6	297.7	2.28
7	0.42	0.0	0.45	25.1	4.05	0.48	12.3	650.0	4.97
8	0.48	11.9	0.52	45.5	7.33	0.54	25.9	1409.9	10.77
9	0.54	25.3	0.55	26.3	4.24	0.58	9.3	521.3	3.98
10	0.58	9.4	0.60	22.9	3.69	0.61	9.8	443.8	3.39
11	0.61	10.0	0.64	56.8	9.14	0.69	0.3	1563.1	11.95
12	0.70	0.3	0.72	12.2	1.96	0.74	2.9	226.2	1.73
13	0.75	0.0	0.78	39.2	6.32	0.81	0.4	904.9	6.92
14	0.81	0.0	0.84	38.1	6.14	0.88	0.7	817.4	6.25

Fig 5: Chromatogram of Phenolics in the Methanolic leaf extract of *H. macrocarpa.*

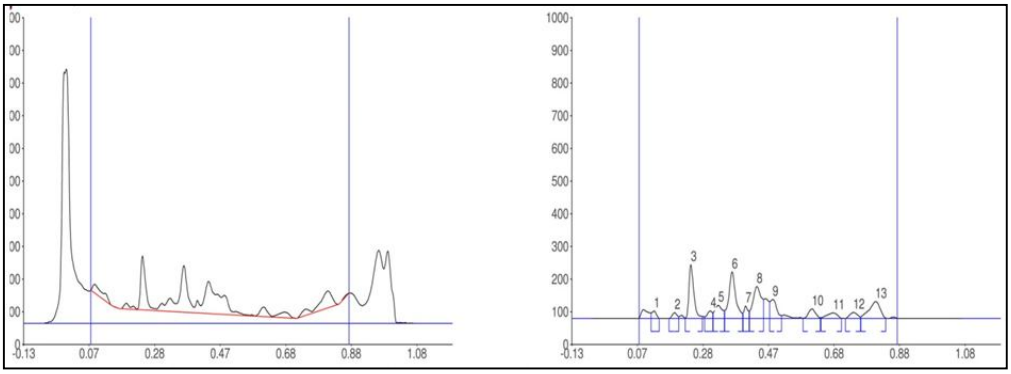

Table 5: Result of HPTLC scanning with reference to Phenolics.

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.12	17.5	0.13	22.9	3.17	0.14	0.3	292.5	2.21
2	0.17	0.1	0.19	17.9	2.48	0.20	5.7	230.8	1.74
3	0.22	1.8	0.24	165.1	22.83	0.27	6.6	2173.4	16.41
4	0.28	5.3	0.30	23.1	3.19	0.30	17.8	311.1	2.35
5	0.31	18.1	0.32	40.3	5.57	0.34	24.7	819.3	6.19
6	0.34	24.7	0.36	142.7	19.72	0.40	16.5	2695.7	20.35
7	0.40	17.0	0.41	38.0	5.26	0.42	22.6	418.8	3.16
8	0.42	23.1	0.44	98.0	13.55	0.46	58.7	2244.5	16.95
9	0.48	51.8	0.49	58.3	8.06	0.52	9.4	1058.8	7.99
10	0.58	0.9	0.61	29.3	4.05	0.63	2.4	569.4	4.30
11	0.64	2.8	0.67	17.2	2.38	0.70	0.6	489.5	3.70
12	0.71	0.0	0.73	18.8	2.60	0.76	4.5	390.5	2.95
13	0.76	4.9	0.80	51.7	7.15	0.83	1.7	1550.5	11.71

Plate 5: HPTLC profile with reference to Phenolics.

Plate 6: HPTLC profile with reference to Saponins.

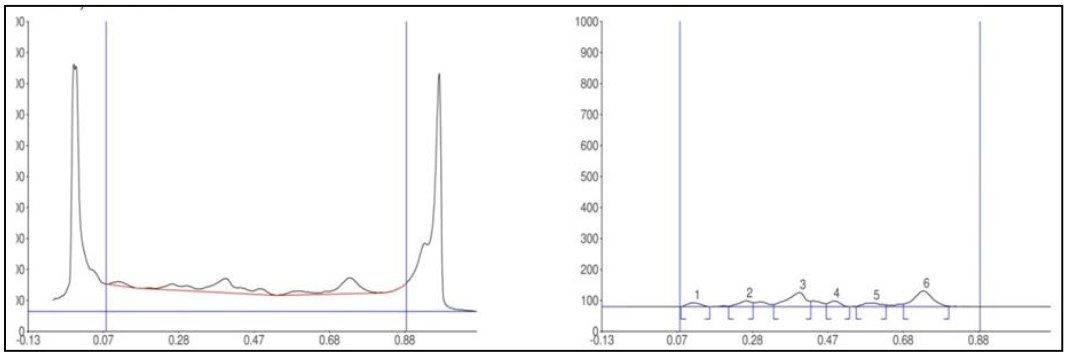

Fig 6: Chromatogram of Saponins in the Methanolic leaf extract of *H. macrocarpa*.

Table 6: Result of HPTLC scanning with reference to Saponins.

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.08	0.2	0.12	13.4	8.27	0.16	0.1	423.4	6.75
2	0.21	2.0	0.26	19.7	12.17	0.28	13.8	592.4	9.44
3	0.33	7.8	0.40	46.0	28.44	0.43	17.7	1983.3	31.61
4	0.47	10.4	0.49	19.1	11.84	0.53	0.1	512.1	8.16
5	0.55	1.5	0.60	12.6	7.78	0.63	5.8	528.1	8.42
6	0.68	9.0	0.73	51.0	31.50	0.80	2.2	2234.0	35.61

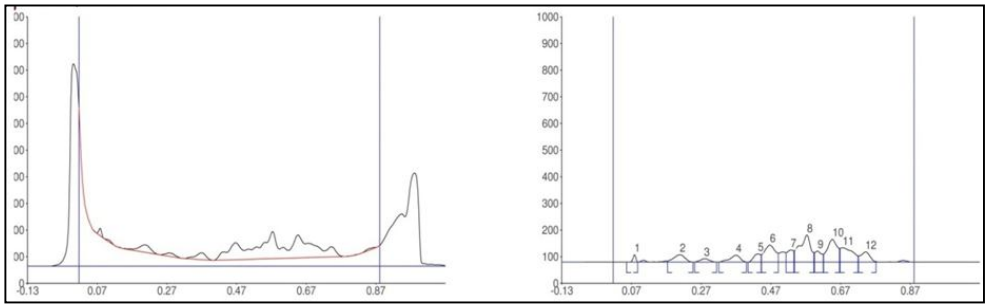

Fig 7: Chromatogram of Steroids in the Methanolic leaf extract of *H. macrocarpa*

Table 7: Result of HPTLC scanning with reference to Steroids.

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.05	0.0	0.08	28.1	5.05	0.09	0.5	200.3	1.50
2	0.17	4.1	0.21	27.5	4.94	0.24	0.4	790.1	5.93
3	0.25	0.1	0.28	12.8	2.30	0.31	0.1	299.0	2.24
4	0.32	0.4	0.37	25.9	4.66	0.40	0.7	683.2	5.12
5	0.40	0.1	0.43	30.7	5.53	0.44	27.7	546.9	4.10
6	0.44	28.3	0.47	64.0	11.51	0.49	34.0	1756.6	13.18
7	0.51	35.6	0.53	45.7	8.22	0.53	42.5	758.0	5.69
8	0.54	43.0	0.57	101.5	18.26	0.59	36.0	2802.3	21.02
9	0.59	36.4	0.60	41.4	7.45	0.62	29.8	717.2	5.38
10	0.62	30.1	0.65	86.2	15.50	0.67	51.9	2151.6	16.14
11	0.67	52.0	0.67	53.8	9.69	0.72	23.4	1723.0	12.92
12	0.72	23.5	0.74	38.3	6.89	0.77	2.2	904.1	6.78

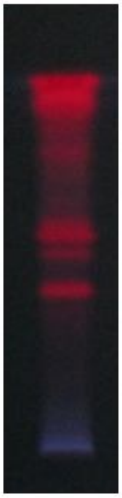

Plate 7: HPTLC profile with reference to Steroids.

Plate 8: HPTLC profile with reference to Tannins.

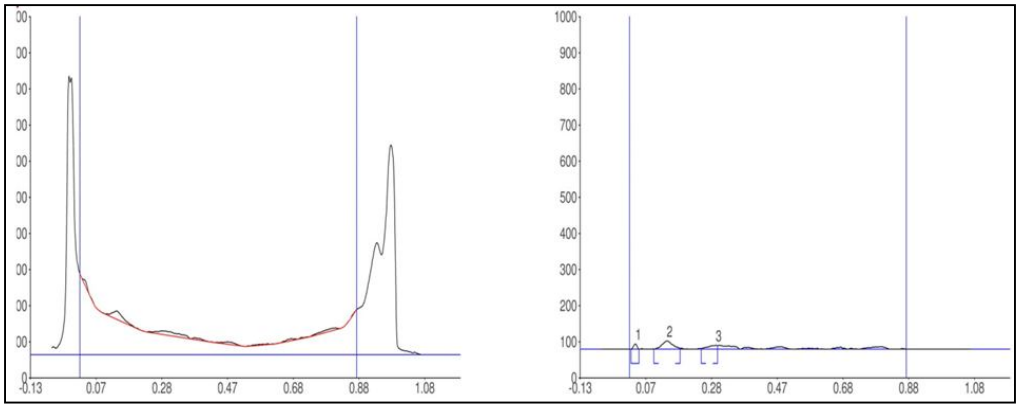

Fig 8: Chromatogram of Tannins in the Methanolic leaf extract of *H. macrocarpa.*

Table 8: Result of HPTLC scanning with reference to Tannins.

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.03	0.2	0.04	14.7	29.99	0.05	0.4	137.3	12.58
2	0.10	0.6	0.14	23.3	47.52	0.18	2.8	671.7	61.57
3	0.24	2.1	0.29	11.0	22.49	0.29	9.7	282.0	25.85

Fig 9: Chromatogram of Triterpenes in the Methanolic leaf extract of *H. macrocarpa*

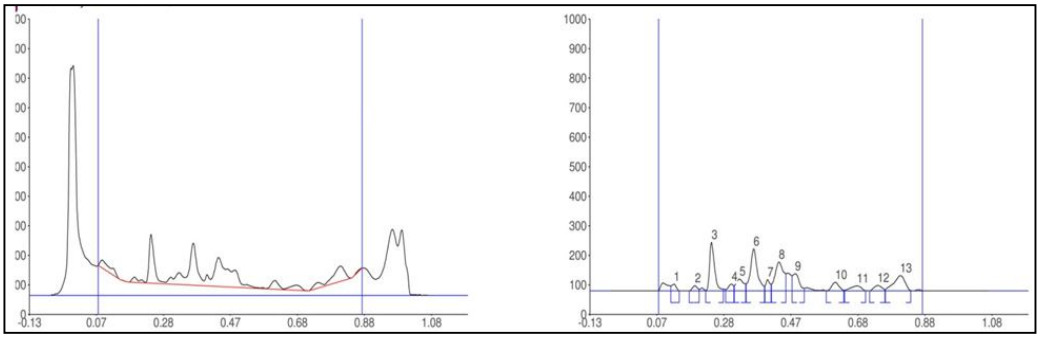

Table 9: Result of HPTLC scanning with reference to Triterpenes.

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.12	17.5	0.13	22.9	3.17	0.14	0.3	292.5	2.21
2	0.17	0.1	0.19	17.9	2.48	0.20	5.7	230.8	1.74
3	0.22	1.8	0.24	165.1	22.83	0.27	6.6	2173.4	16.41
4	0.28	5.3	0.30	23.1	3.19	0.30	17.8	311.1	2.35
5	0.31	18.1	0.32	40.3	5.57	0.34	24.7	819.3	6.19
6	0.34	24.7	0.36	142.7	19.72	0.40	16.5	2695.7	20.35
7	0.40	17.0	0.41	38.0	5.26	0.42	22.6	418.8	3.16
8	0.42	23.1	0.44	98.0	13.55	0.46	58.7	2244.5	16.95
9	0.48	51.8	0.49	58.3	8.06	0.52	9.4	1058.8	7.99
10	0.58	0.9	0.61	29.3	4.05	0.63	2.4	569.4	4.30
11	0.64	2.8	0.67	17.2	2.38	0.70	0.6	489.5	3.70
12	0.71	0.0	0.73	18.8	2.60	0.76	4.5	390.5	2.95
13	0.76	4.9	0.80	51.7	7.15	0.83	1.7	1550.5	11.71

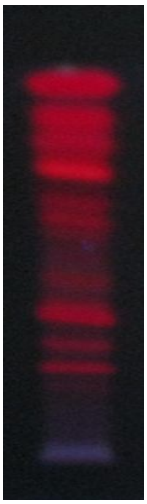

Plate 9: HPTLC profile with reference to Triterpenes.

Various phytochemical compounds are used as potential therapeutic drug in Ayurveda, Siddha, Unani, Homeopathy, Tribal and modern medicines. A related species of *H. macrocarpa,* namely *H. pentandra* show many biological activities. Research studies on the species H. *pentandra* have revealed the pharmacological importance of Flavonoids and Flavonolignan compounds [14]. The antidiabetic and antioxidant activity of the ethanolic extract of H. *pentandra* in mice was also reported [15]. The compounds like Hydnowightin, Hydnocarpin and neohydnocarpin have shown significant biological activity in reducing serum cholesterol and triglyceride level [14]. It also acts against human colon adenocarcinoma and Hela S uterine/ murine L-1210 leukemia growth and is also as an anti-inflammatory agent [14]. Antimicrobial activity of Hydnocarpic acid in *H. pentandra* has been reported by earlier researchers and the former acts by being an antagonist of biotin [16]. Hence, the richness of various phytochemical compounds in *H. macrocarpa* namely flavonoids (14 compounds), triterpenes (13 compounds) phenolics (13 compounds) and steroids (12 compounds) offer ample scope for the characterisation of biologically active phytochemicals.

4. Conclusion

The present study revealed that the leaves of *H. macrocarpa* contains Alkaloids, Essential oils, Flavonoid glycosides,

Flavanoids, Phenolics, Saponins, Steroids, Tannin and Triterpenes. The diversity and richness of phytochemical compounds in *H. macrocarpa* makes it a source for further phytochemical and pharmacological investigation and being an endangered species the plant needs to be conserved.

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