Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E: ISSN 2278-4136
P: ISSN 2349-8234
JPP 2014; 3 (3): 80-85
Received: 24-07-2014
Accepted: 13-08-2014

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Finger printing analysis of the flavonoids from *Holoptelea integrifolia* (Roxb.) planch leaves using HPTLC analysis

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Abstract
Objective
The present study was conducted to identify the flavonoids from petroleum ether and methanol extracts of medicinal andeconomicallyuseful leaves of *Holopteleaintegrifolia* (Roxb.) Planch using High Perform ance Thin Layer Chromatography (HPTLC) technique.
Methods
Preliminary phytochemical screening was done and HPTLC studies were carried out. CAMAG HPTLC system equipped with Linomat V applicator (Switzerland). Densitometric scanning was performed with Camag TLC scanner IV in the reflectance absorbance mode at 540 nm and operated by Win CATS software (1.4.6 Camag) with the help of tungsten lamp.
Results
Preliminary phytochemical screening of petroleum extract of *Holoptelea integrifolia* showed the presence of steroids, terpenoids, alkaloids, glycosides, flavonoids, proteins, tannins and carbohydrates, while methanolic extract of *Holoptelea integrifolia* showed the presence of steroids, alkaloids, flavonoids, proteins and carbohydrates. HPTLC fingerPrinting of flavonoids of petr oleum ether extract of leaf revealed five polyvalent phytoconstituents (5 peaks) and corresponding ascen ding order of R_f values in the range of 0.09 to 0.84. While methanol extract of leaf showed eight polyvale nt phytoconstituents (8 peaks) and corresponding ascending order of R_f values in the range of 0.08 to 0.81.
Conclusions
With the above R_f values and preliminary phytochemical analysis we have concluded the presence of flavonoids in both the extracts.

Keywords: *Holoptelea integrifolia* (Roxb.) Planch leaf, Phytochemical Screening, flavonoids, HPTLC Fingerprinting.

1. Introduction
Natural remedies from medicinal plants are found to be safe and effective. Many plant species have been used in folklore medicine to treat various ailments. Even today compounds from plants continue to play a major role in primary health care as therapeutic remedies in many developing countries [1].Standardisation of plant materials is the need of the day. Several pharmacopoeia containing monographs of the plant materials describe only the physicochemical parameters. Hence the modern methods describing the identification and quantification of active constituents in the plant material may be useful for proper standardisation of herbals and it's formulations. Also the WHO has emphasized the need to ensure the quality of medicinal plant products using modern controlled technique and applying suitable standards [2]. High performance thin layer chromatography (HPTLC) is a valuable tool for reliable identification. It can provide chromatographic fingerprints that can be visualized and stored as electronic images [3].
Holoptelea integrifolia belongs to the family ulmaceae commonly called as Indian Elm and commonly used in India by the tribal people for it' s medicinal properties. The mucilaginous bark is boiled and the juice squeezed out and applied to rheumatic swellings [4]. In traditional system of medicine, bark and leaves of *Holoptelea integrifolia* are used as bitter, astringent, acrid, thermogenic, antiinflammatory, digestive, carminative, laxative, anthelmintic, depurative, repulsive, urinary astringent and in rheumatism [5, 6]. The plant *Holoptelea integrifolia* is used traditionally for the treatment of inflammation, gastritis, dyspepsia, colic, intestinal worms, vomiting, wound healing, leprosy, diabetes, hemorrhoids,

dysmenorrhoea and rheumatism [7]. In this present study the Preliminary phytochemical screening of *Holoptelea integrifolia* leaf extract has been done to identify the chemical constituents and HPTLC fingerprinting of *Holoptelea integrifolia* extract has been performed which may be used as markers for quality evaluation and standardization of the drug.

2. Materials and Methods

2.1 Plant material

Leaves of *Holoptelea integrifolia* were collected in the Month of August from the agricultural fields of Tirunelveli district, Tamil Nadu, India. The plant was identified and leaves of *Holoptelea integrifolia* were authenticated and confirmed from Dr. V.Chelladurai, Research Officer, Botany, C.C.R.A.S. (Retired), Govt. of India by compairing morphological features (leaf and stem arrangement, flower/inflorescence arrangement, fruit and seed morphology etc.). The collected plant material was shade dried to retain its vital phytoconstituents and then subjected to size reduction for further extraction process.

2.2 Preparation and Extraction of Plant Material

The powder of *Holoptelea integrifolia* leaves was charged in to the thimble of a Soxhlet apparatus and extracted using petroleum ether. Appearance of colourless solvent in the siphon tube was the indication of exhaustive extraction and based on that the further extraction was terminated. The extract was then transferred into the previously weighed empty beaker and evaporated to a thick paste on the water bath, maintained at 50 ºC to get petroleum ether extract. The extract was finally air dried thoroughly to remove all traces of the solvent and the percentage yield was calculated. The perfectly dried extract was then stored in an air tight container in a refrigerator below 10 ºC. After obtaining the petroleum ether extract the marc was pressed and it is air dried and again it was extracted using methanol. Appearance of colourless solvent in the siphon tube was the indication of exhaustive extraction and based on that the further extraction was terminated. The extract was then transferred into the previously weighed empty beaker and evaporated to a thick paste on the water bath, maintained at 50 ^{0}C to get semi solid mass of methanol extract. The extract was stored in an airtight container in a refrigerator below 10 ^{0}C.

The Petroleum ether and Methanol extracts of *Holoptelea integrifolia* leaves were subjected to the following investigations,

1. Preliminary phytochemical screening.
2. HPTLC Fingerprinting of Flavonoids

2.3 Phytochemical Screening

The phytochemical investigation of the different leaf extracts of *Holoptelea integrifolia* was carried out with standard protocol [8]. The results were presented in Table 1.

2.4 HPTLC Profile (High Performance Thin Layer Chromatography)

HPTLC studies were carried out following the method of Harborne [9] and Wagner [10] *et al.*

2.4.1 Sample Preparation

Petroleum ether and methanol extracts obtained were evaporated under reduced pressure using rotovac evaporator. Each extract residue was re-dissolved in 5 ml of chromatographic grade Petroleum ether and methanol, which was used for sample application on pre-coated silica gel 60F254 aluminium sheets.

2.5 Developing Solvent System

A number of solvent systems were tried, for extracts, but the satisfactory resolution was obtained in the solvent ethyl acetate: formic acid: Glacial acetic acid: water in the volume ratio of 10: 0.5: 0.5: 1.3 (v/v).

2.6 HPTLC Chromatographic conditions, Sample Application, Development of Chromatogram and Detection of Spots

The sample solutions were spotted in the form of bands of width 8.0 mm with a Camag microlitre syringe on precoated silica gel aluminium plate 60F254 (20 cm × 10 cm with 250 µm thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai) using a Camag Linomat V (Switzerland). The plates were activated at 120 °C for 20 min prior to chromatography. A constant application rate of 1.0 µl/s was employed and space between two bands was 5 mm. The slit dimension was kept at 6.0 mm × 0.45 mm and 10 mm/s scanning speed was employed. The slit bandwidth was set at 20 nm, each track was scanned thrice and baseline correction was used. The mobile phase for fingerprinting of flavonoids consisted of ethyl acetate: formic acid: Glacial acetic acid: water in the volume ratio of 10: 0.5: 0.5:1.3 (v/v) and Anisaldehyde Sulphuric acid was used for derivatization of flavonoids. 20 ml of mobile phase was used per chromatography. Linear ascending develop-ment was carried out in 20 cm x 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with filter paper whatman no: 1 in the mobile phase. The optimized chamber saturation time for mobile phase was 20 min at room temperature (25 ^{0}C ± 2) at relative humidity of 60% ± 5. The length of chromatogram run was 8.0 cm. Subsequent to the scanning, TLC plates were dried in a current of air with the help of an air dryer. Densitometric scanning was performed with Camag TLC scanner IV in the reflectance absorbance mode at 540 nm and operated by Win CATS software (1.4.6Camag) with the help of tungsten lamp. Subsequent to the development; TLC plate was dipped in Anisaldehyde sulphuric acid reagent followed by drying in oven at 110 ^{0}C. Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light. Evaluation was carried out by comparing peak areas with linear regression [11-19].

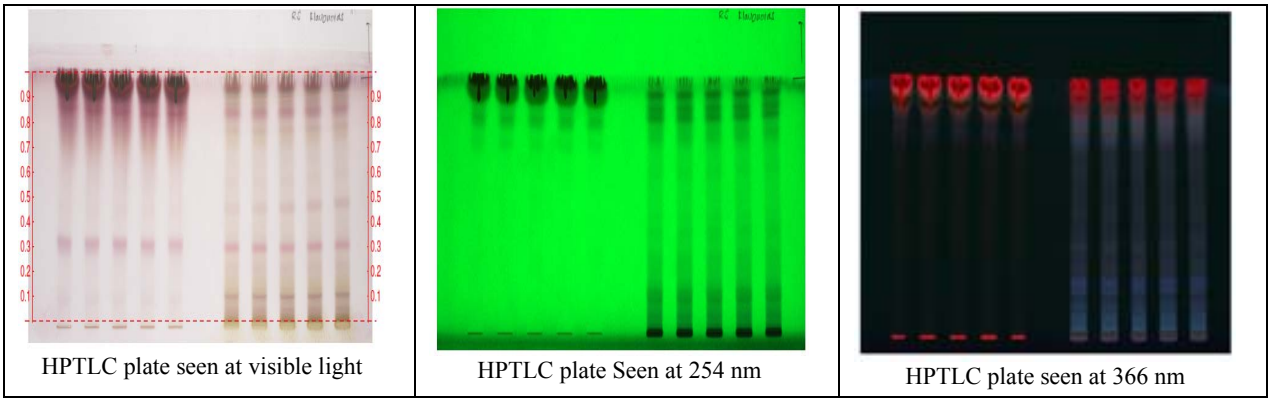
3. Results and Discussion

Preliminary phytochemical analysis of petroleum extract of *Holoptelea integrifolia* showed the presence of steroids, terpenoids, alkaloids, glycosides, flavonoids, proteins, tannins and carbohydrates while methanolic extract of *Holoptelea integrifolia* showed the presence of steroids, alkaloids, flavonoids, proteins and carbohydrates (Table 1). The chromatograms shown in fig.1 indicate that all sample constituents were clearly separated without any tailing and diffuseness.

Table 1: Preliminary Phytochemical Screening of petroleum ether and methanol extracts of *Holoptelea integrifolia* (Roxb.) Planch leaves

Plant Constituents	Test performed	*Holoptelea integrifolia* Leaves	
		Petroleum ether extract	Methanolic extract
Test for Steroids	Salkowski Reaction Liebermann-Burchard Reaction	++ ++	+ +
Test for Triterpenoids		++	-
Test for Glycosides	Balget's test Keller-Killiani test Legals test Borntrager's test	++ + + +	- - + +
Tests for Saponin	Foam Test	-	-
Tests for Carbohydrates	Molisch's test Barfoed's test Fehling's test Benedict's test	++ ++ ++ ++	++ ++ ++ ++
Test for Alkaloids	Mayer's Reagent Hager's Reagent Dragendorff's Reagent	+ - +	- + -
Tests for Flavonoids	Ferric-chloride test Shinoda test	++ ++	+ +
Test for Tannins	$FeCl_3$ Solution Gelatin test	+ +	- -
Test for Proteins	Millon's test Xanthoproteic test Biuret test Ninhydrin test	+ + + +	+ + + +

++ Higher concentration, + Present, - Absent



HPTLC plate seen at visible light | HPTLC plate Seen at 254 nm | HPTLC plate seen at 366 nm

Track 1-5: Petroleum ether extract
Track 7-11: Methanol extract
Note: There was no data available for track 6

Fig 1: HPTLC fingerprint profile of flavonoids of leaf extract of *Holoptelea integrifolia* (Roxb.) Planch

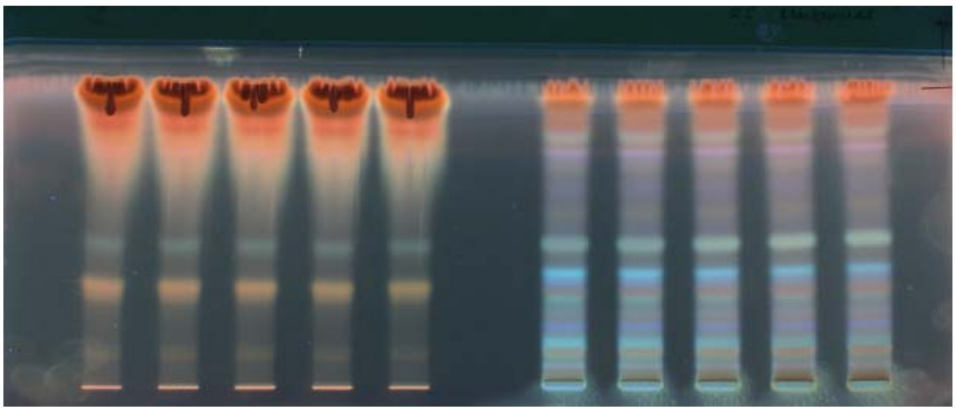


Fig 2: Fingerprint analysis of flavonoids of *Holoptelea integrifolia* (Roxb.) Planch Leaves after derivatization with Anisaldehyde sulphuric acid reagent (ASR) influor-escence at 366 nm.

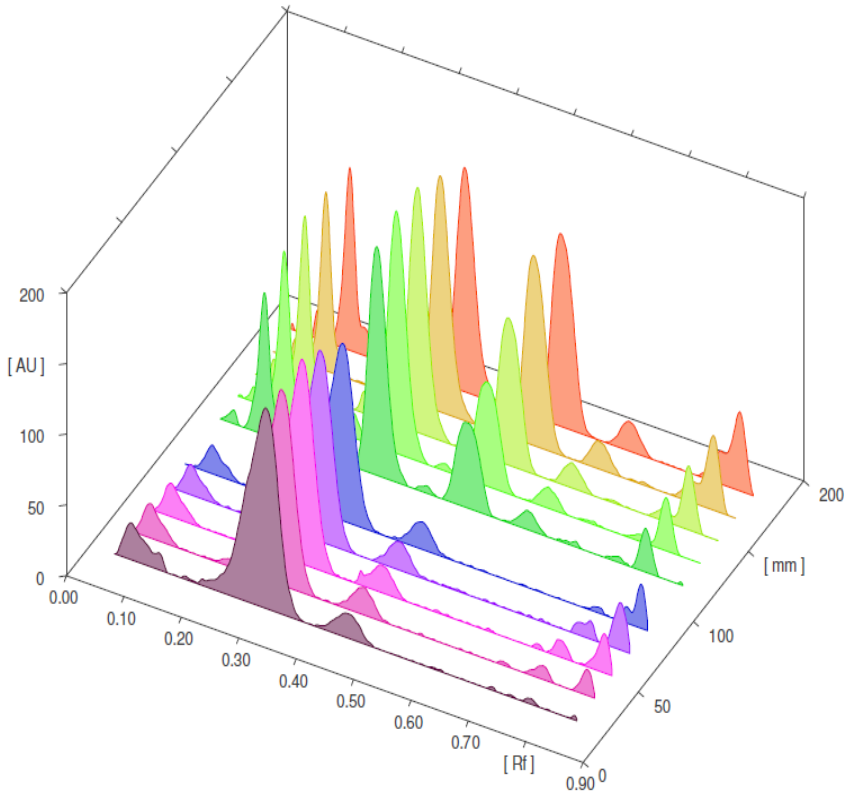


Fig 3: 3-D Plot of Fingerprint of flavonoids of *Holoptelea integrifolia* leaf

The results from HPTLC finger print of flavonoids scanned at wavelength 540 nm for petroleum ether extract of *Holoptelea integrifolia* leaf shows that there are five polyva lent phytoconstituents and corresponding ascending order of R_f values start from 0.09 to 0.84 in which highest Concentration of the phytoconstituents was found to be 60.30% and its corresponding R_f value was found to be 0.32 respectively and was recorded in Table 2. The corresponding HPTLC chromatogram was presented in Figure 4.

The results from HPTLC finger print of flavonoids scannd at wavelength 540 nm for methanol extract of *Holoptelea in tegrifolia* leaf shows that there are eight polyvalent phytoco nstituents and corresponding ascending order of R_f values start from 0.08 to 0.81 in which highest Concentration of the phytoconstituents was found to be 30.34% and its correspond- ding R_f value was found to be 0.33 respectively and was recorded in Table 3. The corresponding HPTLC chromatogram was presented in Figure 5.

Table 2: R_f Values for flavonoids of Petroleum ether extract of *Holoptelea integrifolia* leaf

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.05	0.2	0.09	20.2	9.73	0.14	0.2	531.9	7.69	AutoGenerated2
2	0.23	5.2	0.32	125.2	60.30	0.37	1.1	5272.1	76.19	unknown *
3	0.41	1.6	0.46	19.4	9.36	0.49	2.3	605.1	8.74	unknown *
4	0.80	0.1	0.81	11.7	5.61	0.82	8.9	133.9	1.94	AutoGenerated1
5	0.82	9.0	0.84	31.1	14.99	0.84	21.3	376.8	5.44	unknown *

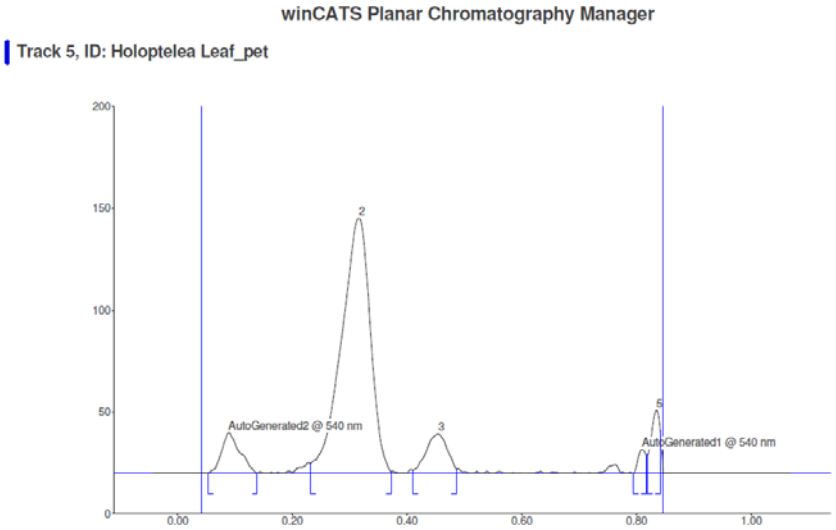


Fig. 4: Chromatogram of flavonoids in petroleum ether extract of *Holoptelea integrifolia* leaf

Table 3: R_f Values for flavonoids in methanol extract of *Holoptelea integrifolia* leaf

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.07	0.3	0.08	14.9	2.71	0.09	0.5	146.5	1.08	unknown *
2	0.10	0.5	0.13	126.0	22.95	0.18	0.4	2250.8	16.65	unknown *
3	0.22	0.1	0.26	19.7	3.59	0.28	0.0	463.1	3.42	AutoGenerated8
4	0.29	0.0	0.33	166.6	30.34	0.40	0.2	4600.1	34.02	unknown *
5	0.45	0.1	0.50	134.1	24.43	0.54	2.0	4123.4	30.50	unknown *
6	0.57	0.6	0.61	19.6	3.58	0.65	0.1	545.1	4.03	unknown *
7	0.73	0.1	0.77	15.5	2.83	0.78	14.7	298.4	2.21	unknown *
8	0.78	14.8	0.81	52.6	9.57	0.83	0.3	1094.1	8.09	AutoGenerated1

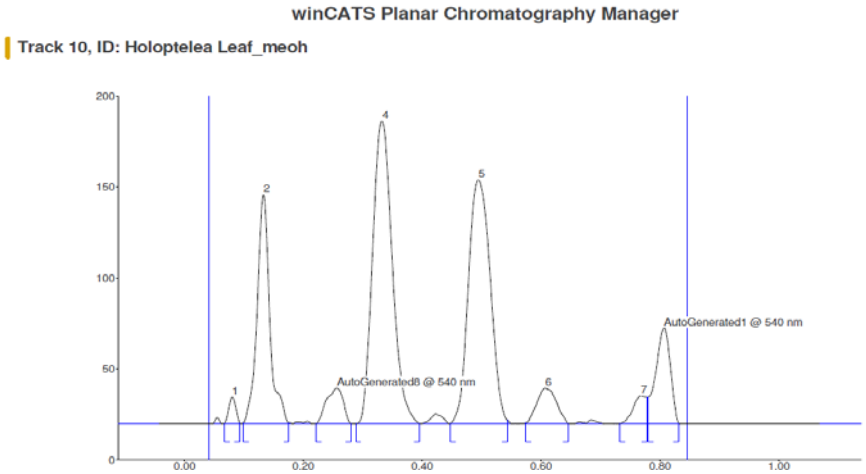


Fig 5: Chromatogram of flavonoids in methanol extract of *Holoptelea integrifolia* leaf

4. Conclusion

Due to the adverse effects of synthetic drugs, in recent years, scientists are in search for alternative medicine. There are some diseases which are chronic and needs a long duration of medication, plant based drugs are less toxic and have no side effects. Since flavonoids have have been implicated in various pharmacological actions it was essential to develop a fingerprint profile of flavonoids of this plant. The developed fingerprint analysis of leaf extract of *Holoptelea integrifolia* will help to isolate and identify new flavonoids, which will offer a possibility to discover lead a molecule for drug development.

5. Acknowledgement

The authors wish to thank Mr. Prashant S. Hande, Application Specialist, Anchrom Lab, Anchrom Test Lab Pvt. Ltd. Mulund (E), Mumbai- 400081, India, for his excellent and generous help in analyzing the HPTLC data.

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