HPTLC and GC MS Analysis of Bark of Stereospermum colais

S. Prema1, K. Chitra[1], V. Gopal[2], C. C. Uma Maheswara Reddy[1]

[1]Faculty of pharmacy, Sri Ramachandra University, Porur, Chennai-600116, Tamil Nadu, India.
[2]Mother Theresa Institute of Health Sciences, A Government of Puducherry Institution, Indira nagar, Puducherry – 605 006.

***Abstract*- Objective:** The present study reports the HPTLC of hexane, chloroform, ethyl acetate and methanol extract of *Stereospermum colais* and GC-MS of an ethyl acetate extract fraction 2. GS-MS chromatogram of the ethyl acetate fraction 2 of study showed 14 peaks in EAESC, besides a number of peaks with very narrow retention time.

Results: There are 13, 10, 14 and 12 polyvalent phytoconstituents and corresponding ascending order of R_f values start from 0.02 to 0.96, 0.11 to 0.87, 0.02 to 0.92 and 0.06 to 0.91 in which highest concentration of the phytoconstituents was found to be 28.80 %, 33.47 %, 30.23 % and 35.04 % and its corresponding R_f value was found to be 0.42, 0.33, 0.35 and 0.52 was recorded in hexane, chloroform, ethyl acetate and methanol extract respectively. GS-MS chromatogram of the ethyl acetate fraction 2 of study showed 14 peaks in EAESC, besides a number of peaks with very narrow retention time.

Conclusions: It can be concluded that GC-MS and HPTLC fingerprint analysis of Bark of *Stereospermum colais* can be used as a diagnostic tool for the correct identification of the plant and it is useful as a phytochemical marker and also a good estimator of genetic variability in plant populations.

***Index Terms*-** GC-MS, HPTLC, Stereospermum colais

I. Introduction

High-performance thin-layer chromatography (HPTLC) is a form of thin-layer chromatography (TLC) that provides superior separation power using optimized coating material, novel procedures for mobile-phase feeding, layer conditioning, and improved sample application. It promotes for higher separation efficiencies, shorter analysis time, lower amounts of mobile phase, and efficient data acquisition and processing. The major parameters that influence separation of the constituents within a mixture are the partition coefficients, retention factor (Rf), and capacity factor of the individual constituents on the plate, selectivity of the mobile and stationary phase to the solutes, and the plate height that decide the separation efficiency as well as resolution of the individual constituents within a mixture. The efficacy of separation of two components of a mixture in a chromatogram is termed as resolution and is influenced by the selectivity of the components between the stationary and the mobile phase, mobile phase flow rate influenced by particle size and solvent strength that influence capacity factors.

A chromatographic fingerprint of a herbal medicine is a chromatographic pattern of the extract of some common chemical components of pharmacologically active and/or chemical characteristics. By using chromatographic fingerprints, the authentication and identification of herbal medicines can be accurately conducted even if the amount and/or concentration of the chemically characteristic constituents is not exactly the same for different samples of drug.

Hence it is very important to obtain reliable chromatographic fingerprints that represent pharmacologically active and chemically characteristic component of the herbal drug. Fingerprint analysis approach using high-performance thin-layer chromatography (HPTLC) has become the most potent tool for quality control of herbal medicines because of its simplicity and reliability. It can serve as a tool for identification, authentication, and quality control of herbal drugs.

Densitometric HPTLC has been widely used for the phytochemical evaluation of the herbal drugs, due to its simplicity and minimum sample clean up requirement. The extract was prepared by using soxhlet extraction method. The normal phase HPTLC method established in this research work uses aluminum backed silica gel 60 F_{254} plates which are less expensive than reversed-phase, preparative plates.

Stereospermum colais is a large straight stemmed deciduous tree 18-30 m in height and 2.8 m in girth found throughout in moist regions of India up to an altitude of about 1200 m, chiefly in deciduous forests (Parrota JA 2001). In English it is known as Yellow snake tree, in Hindi it is padre and in Tamil it is Pathiri. All the parts of the tree are useful in treating many disorders. The leaves are used to treat otalgia, odantalgia, rheumatalgia, malarial fever and wounds. Decoction of the leaves is used as antipyretic and to treat chronic dyspepsia. The root is one of the important ingredients in Dasamula an Ayurvedic formulation. The roots are having bitter, astringent and acrid property. The roots are used as anodyne, appetiser, constipating, diuretic, lithotropic, expectorant, cardio tonic, aphrodisiac, anti-inflammatory, anti bacterial, febrifuge tonic, anti emetic, anti pyretic. The decoction of root is used in the treatment of asthma and cough (Warrier pk et,al, 2002).

In the present study HPTLC and GC-MS of the *Stereospermum colais* bark was performed. The ethyl acetate extract was subjected to column chromatography from which fractions were collected in tared vials and evaporated at 50^o C. The fraction was further subjected to GCMS and HPTLC finger print with the marker as Lapachol.

II. Materials and Method

Fresh stem bark of *S. colais* was collected in September 2010 in Madurai forest, Tamil Nadu state, India. Botanical identification and authentication was done by Botanist Dr. P. Jayaraman, PARC, Chennai and the bark materials were shade dried and coarsely powdered. It is commonly found in India, Myanmar, SriLanka; in the Western Ghats-South, Central and south Maharashtra Sahyadris. (S. Prema et.al, 2013, Warrier pk et al, 2002)

Extraction

The coarsely powdered bark was subjected to successive solvent extraction process with n-hexane, chloroform, ethyl acetate and alcohol. The extracts were then concentrated in vacuum under reduced pressure using rotary flash evaporator and stored in desiccators (Kokate, 1994 C.K. 1994, Harborne, 1998, Pulok .M .Mukerjee, 2005).

III. HPTLC Finger Print of Bark of S. colais Extracts

Materials and methods

Sample Preparation

Hexane, chloroform, ethyl acetate and ethanolic extracts obtained were evaporated under reduced pressure using rotary evaporator. Each extract residue was again dissolved in 1ml of chromatographic grade hexane, chloroform, ethyl acetate and ethanol, which was used for sample application on pre-coated silica gel 60 $F_{254.}$
aluminium sheets.

Developing Solvent System

A number of solvent systems were tried, for extract, but the satisfactory resolution was obtained in the solvent n-Hexane: Ethyl acetate (70: 30) for n-hexane extract, n-Hexane: Ethyl acetate: Formic acid: Acetic acid (60: 40: 2.5: 2.5) for chloroform extract, Chloroform: Methanol: Formic acid: acetic acid (85: 10: 2.5: 2.5) for ethyl acetate extract and Chloroform: Methanol: Formic acid: acetic acid (85: 10: 2.5: 2.5) for ethanol extract.

Sample Application

Application of bands of each extract was carried out (8.0 mm in length and 10 µl in concentration for bark extracts) using spray technique. Sample were applied in duplicate on pre-coated silica gel 60 F_{254} aluminium sheets (5 x 10 cm) with the help of **CAMAG Automatic TLC Sampler 4 (ATS4)**, which was programmed through winCATS software.

Development of Chromatogram

After the application of sample, the chromatogram was developed in Twin Trough Chamber 20x10cm saturated with solvent n-Hexane: Ethyl acetate (70: 30) for n-hexane extract, n-Hexane: Ethyl acetate: Formic acid: Acetic acid (60: 40: 2.5: 2.5) for chloroform extract, Chloroform: Methanol: Formic acid: acetic acid (85: 10: 2.5: 2.5) for ethyl acetate extract and Chloroform: Methanol: Formic acid: acetic acid (85: 10: 2.5: 2.5) for ethanol extract and the chamber was developed for 5 minutes.

Detection of Spots

The air-dried plates were viewed in ultraviolet radiation to mid-day light. The chromatograms were scanned by densitometer at 270 nm. The Rf values and finger print data were recorded by winCATS software.

TABLE HPTLC RESULTS OF EXTRACTS OF *S. COLAIS*

Sample	Solubility	Stationary phase	Mobile phase	Scanning wavelength	Sample concentration	Applied volume	Development mode
Hexane extract	Hexane	silica gel F_{254}	n-Hexane: Ethyl acetate (70: 30)	250 nm	25 mg/ ml	15 µl	ascending mode
Chloroform	Chloroform	silica gel F_{254}	n-Hexane: Ethyl acetate: Formic acid: Acetic acid (60: 40: 2.5: 2.5)	250 nm	25 mg/ ml	10 µl	ascending mode
Ethyl acetate	Ethyl acetate	silica gel F_{254}	Chloroform: Methanol: Formic acid: acetic acid (85: 10: 2.5: 2.5)	250 nm	25 mg/ ml	8 µl	ascending mode
Ethanol	Ethanol	silica gel F_{254}	Chloroform: Methanol: Formic acid: acetic acid (85: 10: 2.5: 2.5)	250 nm	25 mg/ml	8 µl	ascending mode

Isolation

The stationary phase material is suitably moistened with mobile phase and packed sufficiently in the column with a cotton or asbestos pad at the bottom. The extract material or sample to be separated is placed on the top of packed stationary phase with a second cotton or asbestos pad in between.
The mobile phase is poured into the column over the sample. A collecting beaker is placed at the bottom of column near the end to collect the elutes.

IV. Isolation from EAESC

- EAESC were ground with silica beads to be used for column packing using a mortar and pestle.
- The column was packed with silica pre-soaked in 100 % chloroform. After packing, the sample was added to the column and elution was performed after 30 minutes.

- The extract was grounded well with a small amount of silica gel and loaded on the top of the column that was eluted with solvents by increasing polarity.
- About 10 gm of the ethyl acetate extract fraction was chromatographed in 180 gm of silica gel (60 – 120 mesh).
- Increasing gradient of Ethyl acetate in chloroform (20 % - 100 %) followed by increasing gradient of methanol in chloroform (20 % - 100 %) was used for eluting compounds.
- Each fraction was collected, concentrated, tested and examined by TLC for detecting the type of constituents.

GC-MS analysis

GC-MS ANALYSIS EAESC FRACTION

Equipment : Thermo GC - Trace Ultra Ver: 5.0, Thermo MS DSQ II
Column : DB 35 - Ms Capillary Standard Non - Polar Column
Dimension : 30 MTS, ID: 0.25 Mm, Film: 0.25 Mm
Carrier Gas : He, Flow: 1.0 ml/min
Temp Prog : Oven Temp 70^0 C At Raised To 250^0 C at 10 Min Hold

GC-MS analysis was performed using THERMO GC-TRACE ULTRA VER: 5.0 interfaced to a Mass Spectrometer (THERMO MS DSQ II) attached with DB-5-MS capillary standard non-polar column (Length: 30.0 m, Diameter: 0.25 mm, Film thickness: 0.25 mm) which consist of 100 % Dimethyl poly siloxane.

GC-MS Detection

Ionization energy of 70 eV was used in the electron ionization energy system. The carrier gas (Helium gas- 99.999 %) at a constant flow rate of 1.0 ml/ min and volume of 1 ml was injected. Injector temperature was maintained at 200^0 C and the ion-source temperature was at 200^0 C. The oven temperature was programmed between 70^0 C (isothermal for 2 min), to 250^0 C for 10 min. Mass spectra were taken at 70 eV with scan interval of 0.5 s with scan range of 40-1000 m/ z and total GC running time was 38.01 min. Using the average peak area to the total areas was used to find the relative percentage amount of each component. GC MS solution ver: 5.0. was the software adopted to handle mass spectra and chromatograms. (Merlin NJ., et al, 2009; Sumathy H, et al, 2011; Paul John PM, et al, 2012)

Sample preparation

About 1 g of well mixed and ground sample was dissolved in 10 ml of methanol and taken into a screw cap vial. 10µl of this sample was then injected for GC-MS analysis.

Identification of components

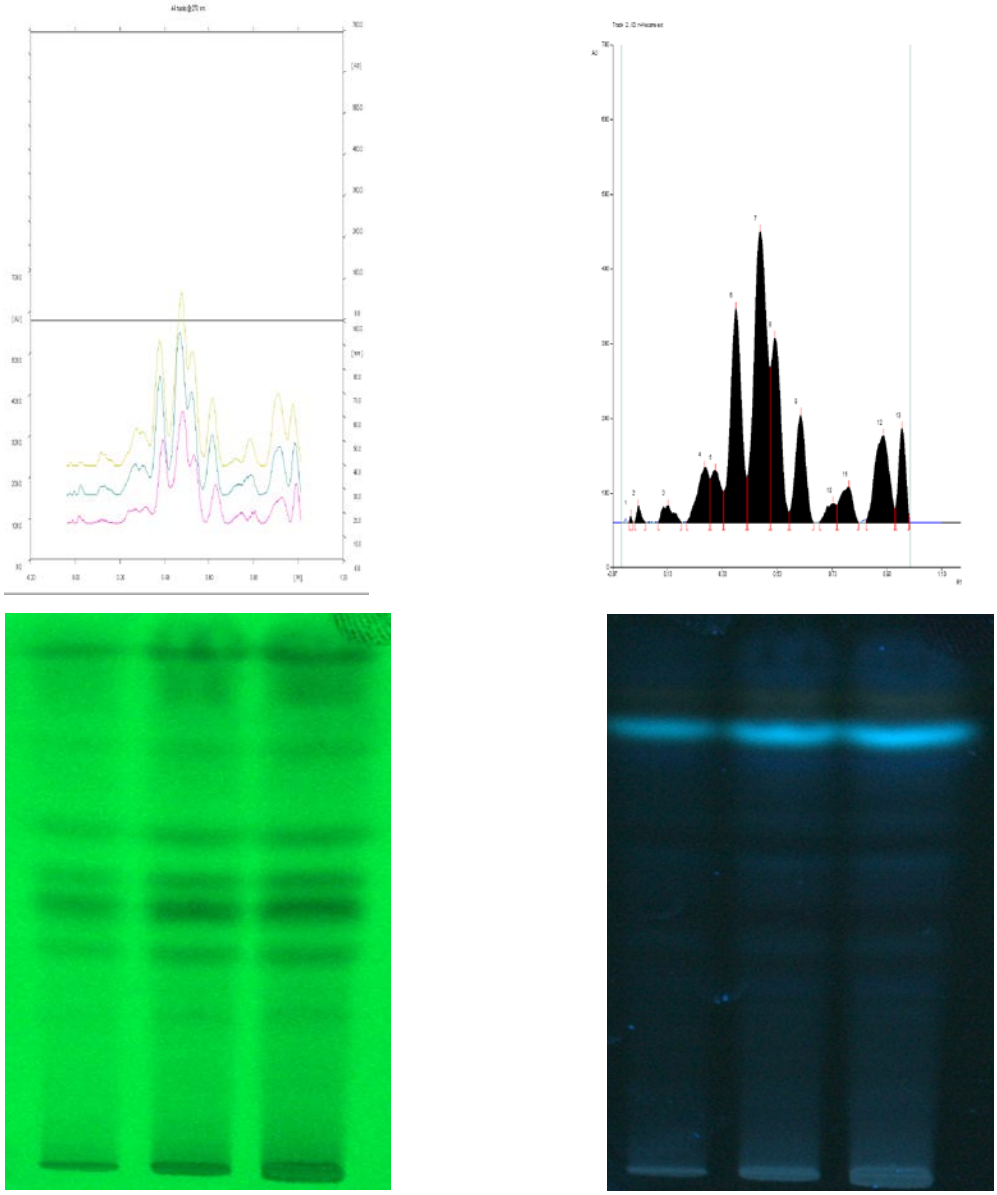
- Using the database of in-built libraries like NIST 8 (National Institute of Standards and Technology) and WILEY 9 having more than 62,000 patterns was done to interpretate on mass spectrum of GC-MS
- The mass spectrum of the unknown component was compared in the WILEY 9 library and NIST 8.
- The name, RT value, molecular weight, percentage peak area and structure of the components were determined.

HPTLC FINGERPRINTING PROFILE OF MARKER COMPOUND (LAPACHOL) IN BARK OF *S. COLAIS*

The HPTLC fingerprinting profile of the ethyl acetate isolate was developed using silica gel F_{254} plates as stationary phase and Hexane: Acetone: Acetic acid in the ratio of 15: 5: 0.3 as mobile phase.

V. RESULTS AND DISCUSSION

Fig 1 HPTLC RESULT OF HEXANE EXTRACT OF BARK OF *S. COLAIS*



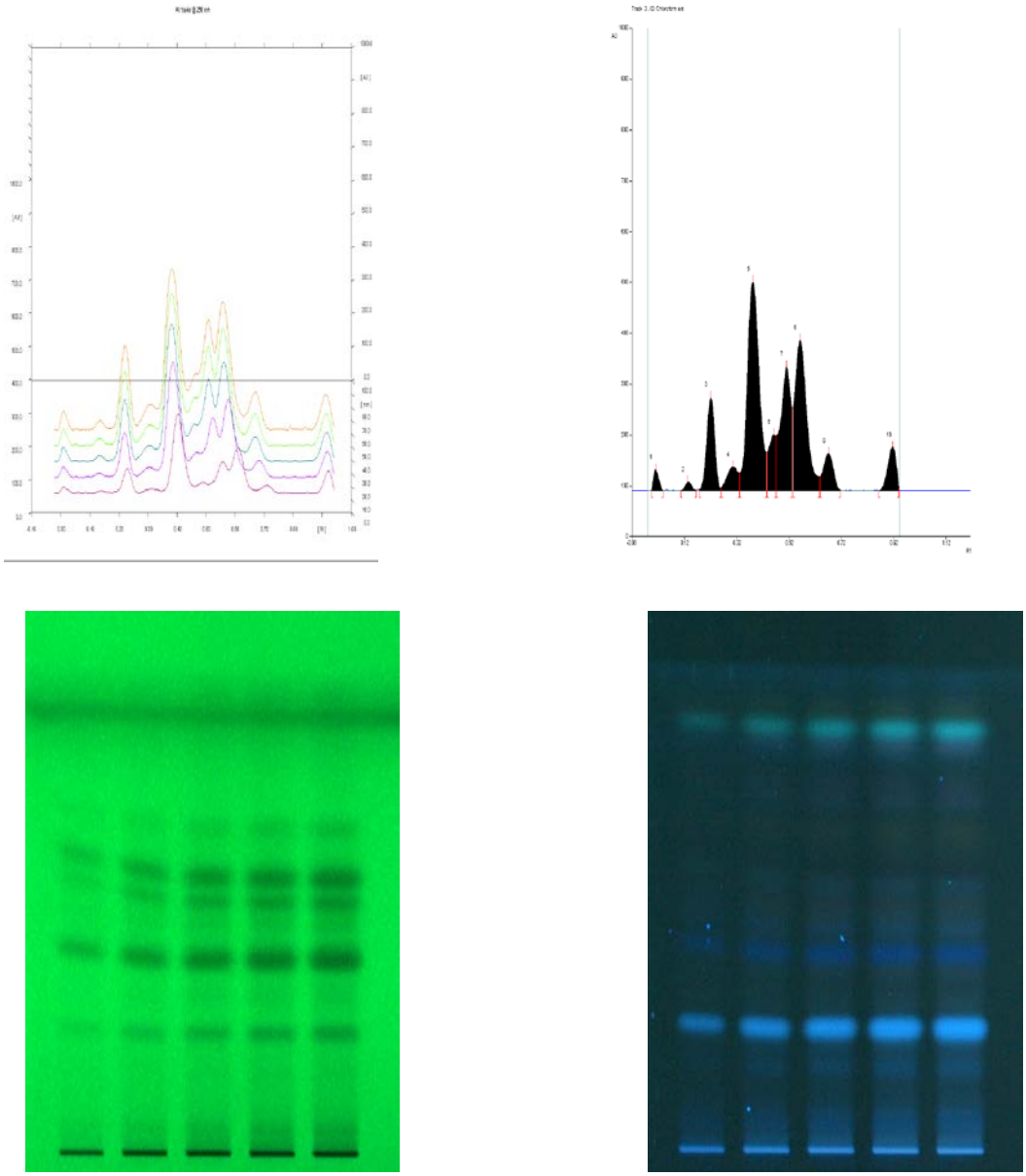
254 nm 366 nm

TABLE 1: R_F VALUES FOR HEXANE EXTRACT OF BARK OF *S. colais*

Peak	Start R_f	Start Height	Max R_f	Max Height	Max %	End R_f	End Height	Area	Area %
1	-0.01	0.5	-0.01	9.0	0.57	0.00	0.5	51.9	0.01
2	0.01	0.5	0.02	22.9	1.44	0.05	0.4	298.6	0.57
3	0.09	0.2	0.13	22.4	1.41	0.18	0.3	802.4	1.52
4	0.20	1.5	0.27	74.4	4.69	0.28	59.3	2542.1	4.81
5	0.28	59.4	0.30	70.3	4.44	0.33	41.6	2162.6	4.10
6	0.33	41.7	0.38	286.9	18.10	0.42	60.8	9586.4	18.16
7	0.42	61.7	0.47	390.5	24.63	0.50	208.0	15206.4	28.80
8	0.50	208.2	0.52	247.5	15.62	0.57	13.9	7312.9	13.85

9	0.57	14.6	0.62	144.7	9.13	0.66	0.0	4463.1	8.45
10	0.68	0.9	0.73	25.8	1.63	0.75	23.6	797.4	1.51
11	0.75	23.7	0.79	47.8	3.01	0.83	0.0	1769.2	3.35
12	0.85	3.8	0.92	116.6	7.35	0.96	19.7	5067.9	9.60
13	0.96	20.9	0.99	126.4	7.97	1.01	10.7	2736.2	5.18

Fig 2 HPTLC RESULT OF CHLOROFORM EXTRACT OF BARK OF *S. colais*



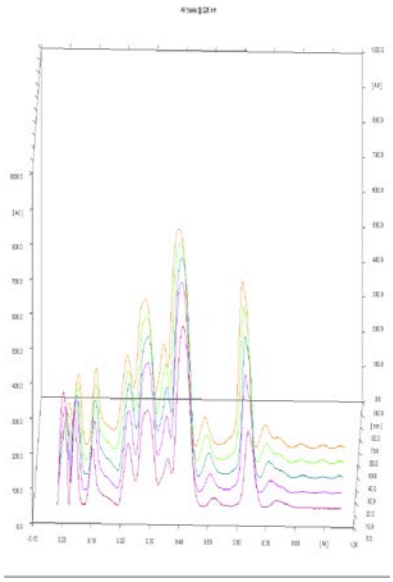
254 nm 366 nm

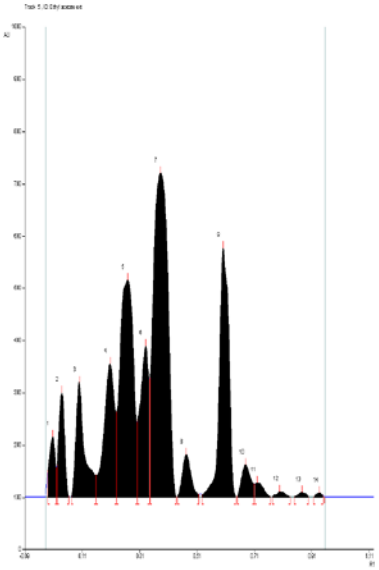
TABLE 2: R_F VALUES FOR CHLOROFORM EXTRACT OF BARK OF *S. colais*

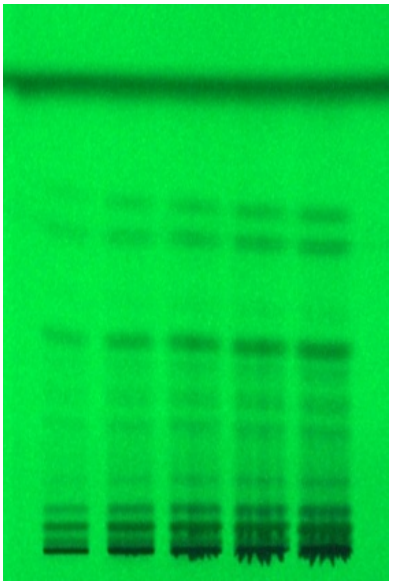
Peak	Start R_f	Start Height	Max R_f	Max Height	Max %	End R_f	End Height	Area	Area %
1	-0.01	0.2	0.01	30.9	2.66	0.04	0.4	461.6	1.42
2	0.11	3.2	0.13	12.6	1.09	0.16	3.1	244.2	0.75
3	0.17	1.6	0.22	133.4	11.47	0.26	5.7	2883.9	8.89
4	0.26	3.2	0.30	30.6	2.63	0.33	21.9	858.5	2.65

5	0.33	21.4	0.39	343.3	29.53	0.44	49.4	10853.8	33.47
6	0.44	50.0	0.47	75.7	6.51	0.48	71.2	1647.1	5.08
7	0.48	71.2	0.52	177.0	15.22	0.55	120.4	5126.1	15.81
8	0.55	121.0	0.58	232.0	19.96	0.65	20.1	7344.8	22.65
9	0.65	20.2	0.68	50.0	4.30	0.73	0.1	1385.5	4.27
10	0.87	1.9	0.92	77.0	6.62	0.94	32.6	1626.8	5.02

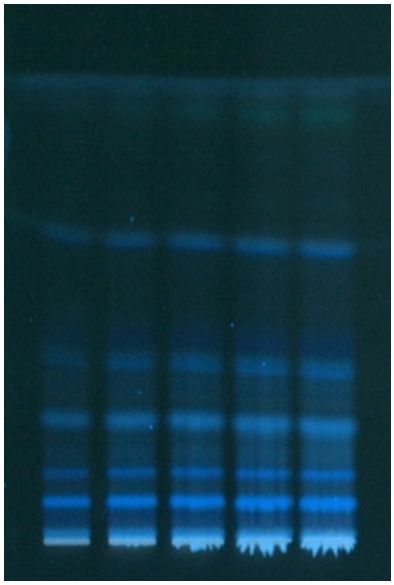
Fig 3 HPTLC RESULT OF ETHYL ACETATE EXTRACT OF BARK OF *S. colais*







254 nm

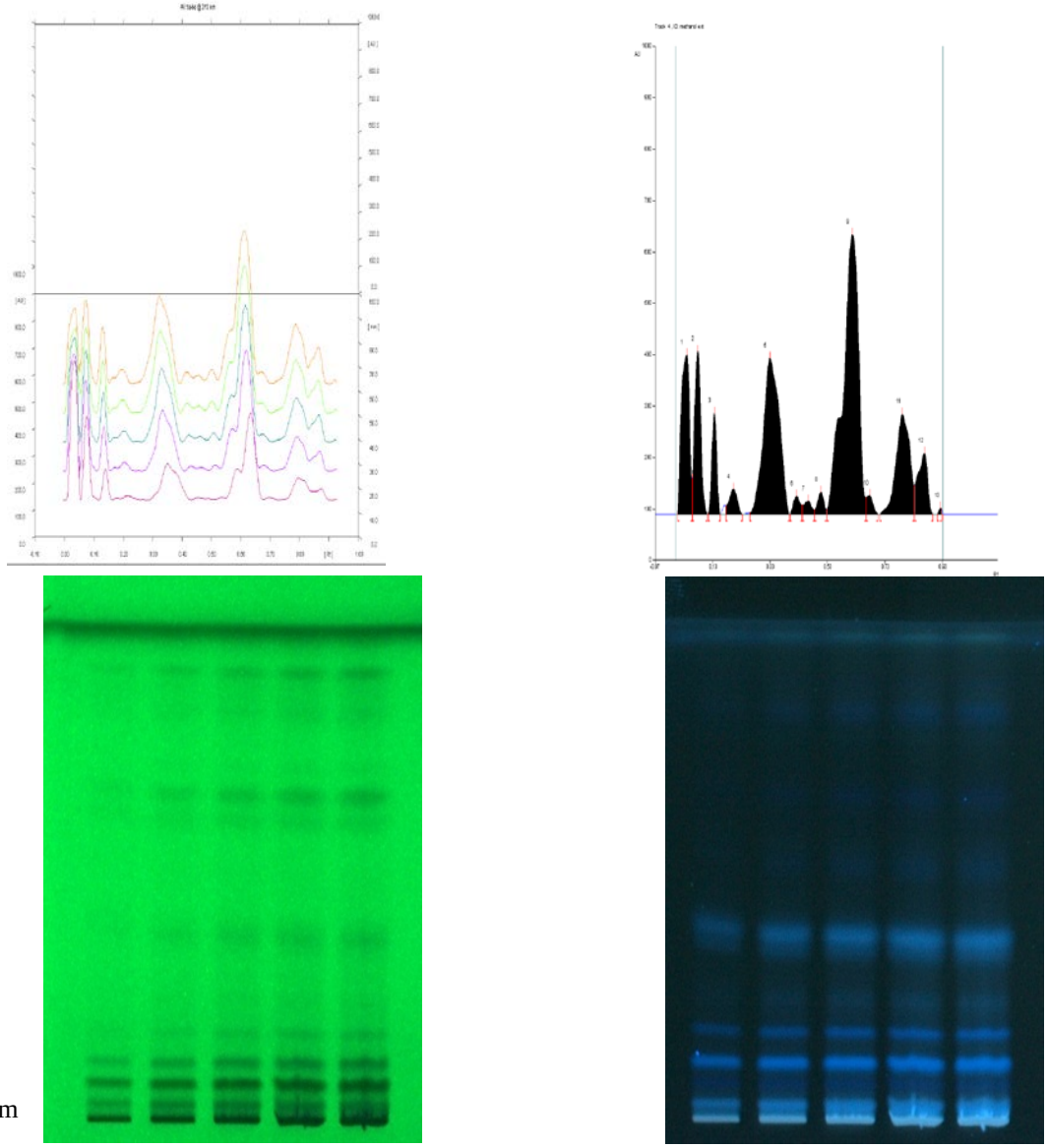


366 nm

TABLE 3: R_F VALUES FOR ETHYL ACETATE EXTRACT OF BARK OF *S. colais*

Peak	Start R_f	Start Height	Max R_f	Max Height	Max %	End R_f	End Height	Area	Area %
1	-0.01	50.9	0.01	116.0	4.15	0.02	55.6	1567.0	2.25
2	0.02	61.2	0.04	200.1	7.17	0.06	0.9	2803.5	4.02
3	0.07	0.9	0.10	218.9	7.84	0.16	42.4	4540.0	6.51
4	0.16	42.5	0.21	255.2	9.14	0.23	161.5	6296.0	9.03
5	0.23	162.5	0.27	415.5	14.88	0.30	143.1	13119.7	18.82
6	0.30	147.2	0.33	289.6	10.37	0.34	224.1	5736.8	8.23
7	0.35	228.6	0.38	620.9	22.24	0.44	0.0	21067.1	30.23
8	0.44	0.5	0.47	81.3	2.91	0.52	5.5	1659.2	2.38
9	0.53	4.6	0.60	477.5	17.10	0.65	0.6	10583.5	15.19
10	0.65	1.0	0.68	61.9	2.22	0.71	25.6	1329.9	1.91
11	0.71	25.6	0.72	27.5	0.99	0.77	0.1	543.4	0.78
12	0.77	0.3	0.80	10.4	0.37	0.83	0.1	202.4	0.29
13	0.85	0.3	0.88	9.4	0.34	0.90	0.2	149.0	0.21
14	0.92	0.1	0.94	7.6	0.27	0.95	2.8	96.3	0.14

Fig 4 HPTLC RESULT OF ETHANOL EXTRACT OF BARK OF *S. colais*



254 nm 366 nm

TABLE 4: R_F VALUES FOR ETHANOL EXTRACT OF BARK OF *S. colais*

Peak	Start R_f	Start Height	Max R_f	Max Height	Max %	End R_f	End Height	Area	Area %
1	0.00	2.6	0.03	310.3	14.20	0.05	68.4	7016.4	10.76
2	0.06	77.3	0.07	316.2	14.48	0.11	0.5	5451.8	8.36
3	0.11	0.7	0.13	196.2	8.98	0.15	0.1	2652.1	4.07
4	0.17	16.3	0.20	48.2	2.21	0.23	0.2	1076.6	1.65
5	0.26	2.2	0.33	303.6	13.90	0.39	0.4	13169.7	20.19
6	0.40	0.3	0.42	34.7	1.59	0.44	17.2	638.3	0.98
7	0.44	16.9	0.46	25.8	1.18	0.48	8.6	544.4	0.83
8	0.48	9.1	0.50	43.0	1.97	0.52	9.5	735.7	1.13
9	0.52	9.7	0.61	544.1	24.91	0.66	31.6	22855.9	35.04
10	0.66	31.7	0.67	36.1	1.65	0.70	0.2	617.0	0.95
11	0.71	0.3	0.79	194.8	8.92	0.83	55.8	7312.5	11.21
12	0.83	56.2	0.86	119.5	5.47	0.89	0.3	3087.9	4.73
13	0.91	0.1	0.92	12.0	0.55	0.92	11.3	73.8	0.11

HPTLC RESULT OF HEXANE EXTRACT OF *S. colais* BARK

The results from HPTLC finger print scanned at wavelength 420 nm for HESC. There are thirteen polyvalent phytoconstituents and corresponding ascending order of R_f values start from 0.02 to 0.96 in which highest concentration of the phytoconstituents was found to be 28.80 % and its corresponding R_f value was found to be 0.42 and was recorded in Table1.

HPTLC RESULT OF CHLOROFORM EXTRACT OF *S. colais* BARK

The results from HPTLC finger print scanned at wavelength 420 nm for CESC. There are ten polyvalent phytoconstituents and corresponding ascending order of R_f values start from 0.11 to 0.87 in which highest concentration of the phytoconstituents was found to be 33.47 % and its corresponding R_f value was found to be 0.33 and was recorded in Table 2.

HPTLC RESULT OF ETHYL ACETATE EXTRACT OF *S. colais* BARK

The results from HPTLC finger print scanned at wavelength 420 nm for EAESC. There are fourteen polyvalent phytoconstituents and corresponding ascending order of R_f values start from 0.02 to 0.92 in which highest concentration of the phytoconstituents was found to be 30.23 % and its corresponding R_f value was found to be 0.35 and was recorded in Table 3.

HPTLC RESULT OF ETHANOL EXTRACT OF *S. colais* BARK

The results from HPTLC finger print scanned at wavelength 420 nm for MESC. There are twelve polyvalent phytoconstituents and corresponding ascending order of R_f values start from 0.06 to 0.91 in which highest concentration of the phytoconstituents was found to be 35.04 % and its corresponding R_f value was found to be 0.52 and was recorded in Table 4.

GC MS OF ETHYL ACETATE FRACTION 2

GS-MS chromatogram of the ethyl acetate fraction 2 of study showed 14 peaks in EAESC, besides a number of peaks with very narrow retention time. The fragmentation patterns for some of the peaks were compared with that of the library of compounds. The ethyl acetate fraction 2 constituents along with their retention time and percentage area and superimposibility obtained from the GCMS analyzer are tabulated in Table 5 and Fig 5

Fig 5 GC MS OF ETHYL ACETATE EXTRACT FRACTION 2

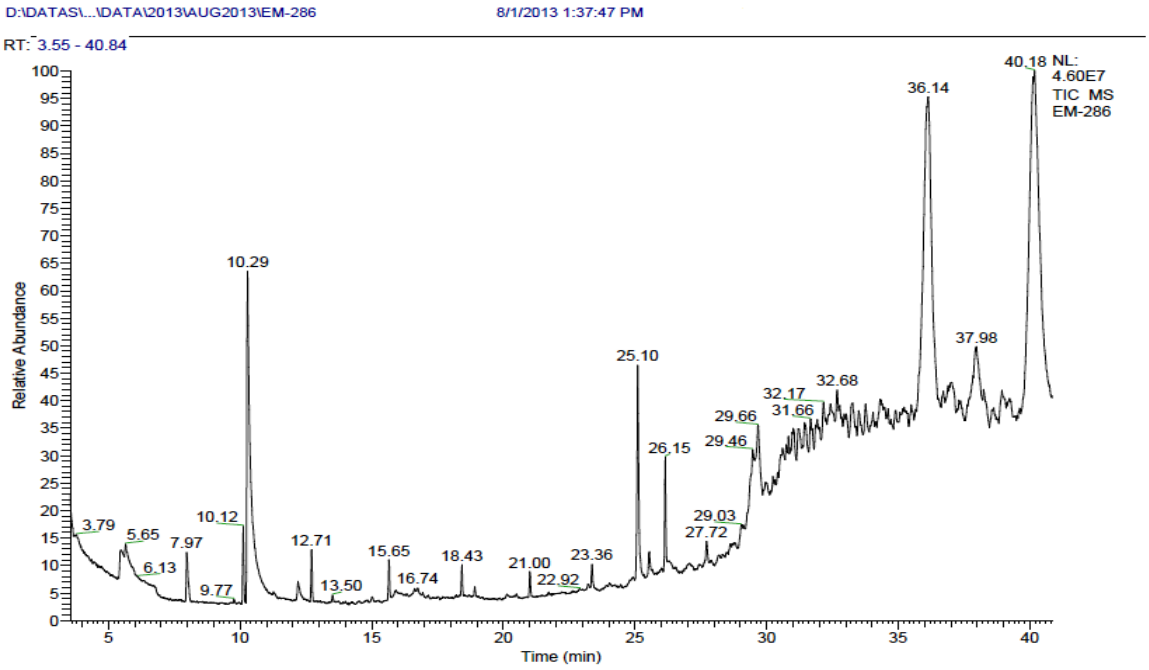


Table 5 GC MS OF ETHYL ACETATE EXTRACT FRACTION 2

S.No	RT	Compound	Area %
1	3.27	Acetic Acid, Ethyl ester	66.90
2	5.65	Cyclopenta siloxane, Deca methyl-	0.91
3	7.99	Cyclohexa siloxane, Dodeca methyl	0.31
4	10.31	2,3-Dihydro-Benzofuran	3.00
5	12.22	**naphthalene-1,4-dione (Lapachol)**	0.18
6	12.71	Hexadeca methyl cyclo octasiloxane	0.20
7	15.65	Cyclonona siloxane, Octadeca methyl	0.18
8	18.43	Cyclodeca siloxane, Eicosa methyl	0.22
9	23.36	Silicone Oil	0.24
10	27.72	**2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one (Quercetine)**	0.28
11	30.58	5-Heptenoic Acid,	0.36
12	33.25	5-Heptenoic Acid, 7-[2- [3- (Methoxy imino)Butyl]-3, 5-Bis[(Trimethylsilyl) Oxy]Cyclop Entyl], Methyl Ester, [1r-(1à,2á,3à,5à)]-	0.27
13	36.12	Stigmast-5-en-3-ol, (3á,24s)-	7.49
14	40.16	Lup-20(29)-en-3-one	8.94

HPTLC FINGERPRINTING PROFILE OF MARKER COMPOUND (LAPACHOL)

A sensitive and reliable densitometric High Performance Thin Layer Chromatography method has been developed for the quantification of Lapachol a flavonoid present in in ethyl acetate extract fraction 2 of bark of *Stereospermum colais.*

Chromatographic analysis was performed using ethyl acetate fraction of *Stereospermum colais* on silica gel F $_{254}$ plates using the solvent system **Hexane: Acetone: Acetic acid (15:5:0.3).** Detection and quantification of Lapachol was done by densitometric scanning at 285 nm and the fraction contains **0.024659 % of Lapachol**

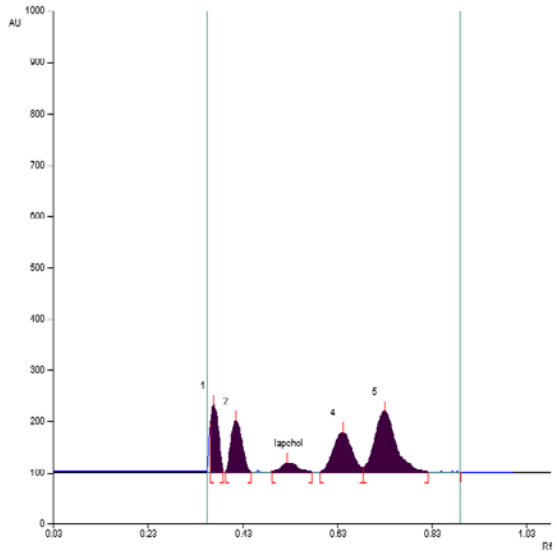
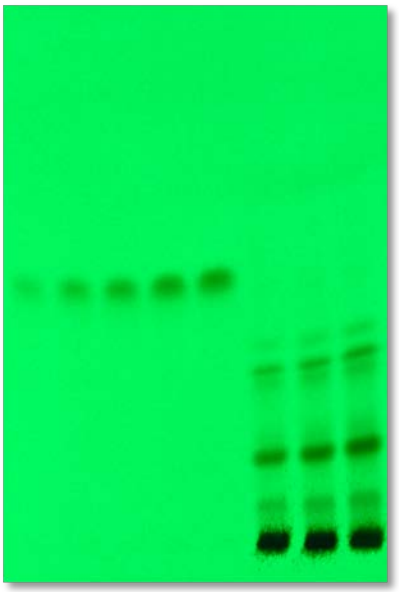


Figure 6 HPTLC Fingerprinting Profile of Marker Compound (Lapachol) In Bark of *Stereospermum colais* at 285 nm



254 nm

366 nm

VI. Conclusion

From the HPTLC studies, it has been found that all extracts contain not a single compound but a mixture of compounds. From the four extracts the ethyl acetate extract had more number of peaks so EAESC were taken for the isolation. The isolates were collected in the tarred vial. The ethyl acetate fraction 2 was performed for the GCMS and HPTLC finger print with a marker Lapachol. Lapachol will be tried to establish the pharmacological activity. The presence of various phytochemicals can contribute to the medicinal activity of the plant. The present work employing HPTLC and GC-MS methods have shown the presence of many phytoconstituents which can be the lead molecule for the treatment of many diseases.

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AUTHORS

First Author – S. Prema, Faculty of pharmacy, Sri Ramachandra University, Porur, Chennai-600116, Tamil Nadu, India.
Second Author – K. Chitra, Faculty of pharmacy, Sri Ramachandra University, Porur, Chennai-600116, Tamil Nadu, India.
Third Author – V. Gopal, Mother Theresa Institute of Health Sciences, A Government of Puducherry Institution, Indira nagar, Puducherry – 605 006.
Fourth Author – C. C. Uma Maheswara Reddy, Faculty of pharmacy, Sri Ramachandra University, Porur, Chennai-600116, Tamil Nadu, India.