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Comparative Qualitative and Quantitative Phytochemical Evaluation of *Embelia ribes* Burm F Obtained from Different Sources

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Abstract: ***Extracts of the fruits of Embelia ribes collected from different geographical sources were subjected to phytochemical screening in order to identify the nature of constituents present. These extracts were further analysed by HPTLC and HPLC fingerprinting by using embelin as a marker. The Rf values and retention times of constituents were recorded and corresponded peaks were compared with that of the standard embelin.***

Keywords: Embelin, Chromatograhy analysis, Fingerprinting, Phytochemical analysis

1. Introduction

Embelia ribes Burm is a woody climber, belonging to the family *Myrsinaceae*. It is also commonly known as false black pepper or vidanga. *Embelia ribes* grows in semi-evergreen and deciduous forests at an altitude of 1,500 meters, throughout India. It is considered to be vulnerable due to excessive harvesting, because of its many uses (it is used in 75 ayurvedic preparations). *E. ribes* is a highly valuable medicinal plant with anthelmintic, carminative, antibacterial, antibiotic, hypoglycemic, and antifertility properties (Mitra 1995; Anon 2002). *Embelia ribes* has been proven to have great pharmacological potential with a great utility and usage as folklore medicine. Roots, berries and leaves of *Embelia ribes* are used in herbal formulas. *Embelia ribes* has a long history of use in ayurvedic system of medicine in various forms like churna, asava, aristha, lauha and taila. *Embelia ribes* berries contain several chemical constituents like embelin, volatile oil, fixed oil, resin, tannin, christembine (alkaloid), phenolic acids like caffeic acid, vanillic acid, chrorogenic acid, cinnamic acid, o-cumaric acid. (Lal and Mishra 2013)

In the earlier studies, embelin was isolated from the fruits of *Myrsine africana* L. (Myrsinaceae) using analytical methods like high performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC) (Paul et al., 2007). Variation in phenolic content was analyzed among the different market samples of *E. ribes* (Sharadha et al., 2009). Embelin content was estimated in six samples of Embelia collected from different geographical regions of India using HPLC method (Nagamani et al 2013). Validated HPTLC method has been reported for estimation of embelin in methanolic extract of *Embelia ribes* (Saraf et al., 2016). The present study was taken up to find the phytoconstituents of berries of Embelia collected from different sources and select the sample containing highest amount of embelin.

2. Materials and Methods

2.1 Collection Plant material

Samples of the crude drugs were collected from seven different regions which are as follows. Hebsur (Hubli), Ayurvedic medical college (Hubli), Himalayas, Kerala, Rajastan(Ajmeer), Fathepur, Hessarghatta (IIHR). All the samples were authenticated at FRLHT, Bangalore.

2.2 Preparation of extracts

The fruits of *Embelia ribes* were dried in hot air oven at 35^{o}C for three days, powdered to a mesh size of # 40 and stored in air tight containers. The powder was then extracted individually by refluxation for Twenty hours using different solvents(a) Petroleum ether (b) Benzene (c) Chloroform (d) Ethyl acetate (e) Methanol (f) Hexane (g) Acetone. The residues were dried and the % yield was recorded. Results are recorded in **Table-1.**

HPTLC analysis of the extracts
A HPTLC Method for Quantitative Estimation of Embelin was developed using the standard and following parameters.
Instrument : CAMAG HPTLC system
Applicatior : CAMAG Linomat 5
Developing chamber :Camag twin trough chamber
Solvent system : Chloroform: Toluene: Ethyl acetate: Diethylamine(3.5: 3.5: 2: 0.5)
Application volume : 20µl
Development distance: 75mm
Detection : CAMAG TLC scanner 3
Integration software : Wincats

Preparation of sample solution
Sample solution was prepared as per the method described under extraction and TLC identification.

Preparation of standard solution
1mg of standard Embelin was dissolved in 25 ml of methanol. Development chamber and Mobile Phase

The HPTLC chamber (CAMAG twin trough 20 X 10) was saturated with solvent system Chloroform: Toluene: Ethyl acetate: Diethylamine (3.5: 3.5: 2: 0.5).

Sample application
Pre-coated TLC plates (Merck) were spotted with the standard Embelin solution in multiple concentrations (multiple standard levels) and sample preparation in duplicate. The spotting was done by TLC applicator (CAMAG LINOMAT 5). The volume of spotting was decided based on the concentration of the extracts. For very dilute extracts 20µl was spotted on Silica gel GF plate. Development and Scanning After spotting, plates were allowed to develop up to 75mm in previously saturated chamber with solvent systems. The plates were removed from the chamber and dried in an oven at 100^0C for 5 min. subjected for scanning using scanner(CAMAG TLC SCANNER 3) at 268nm. Results of HPTLC analysis are tabulated in **Table-2** and **Fig-1, 2, 3.**

HPLC analysis of the extracts
HPLC method was developed for estimation of active embelin in methanolic extracts of *Embelia ribes*. The peaks were identified using reference standard of Embelin and the content of Embelin in Methanolic Extract was determined using area under the curve of sample and reference standard.

Preparation of standard solution
1mg of standard embelin was dissolved in 25 ml of methanol and sonicated to get the concentration of 0.004%w/v.

Preparation of sample solution
Sample solution was prepared using 25ml of methanol in volumetric flask.

Column:-Lichosorb C18 (4.6 x 250mm, 5µm)
Mobile phase:-Acetonitrile: Buffer (35: 65)
The buffer was prepared by dissolving 6.80 grams of potassium dihydrogenphosphate in 1000 ml HPLC grade water and PH was adjusted to 3.0 with ortho-phosphoric acid.
Detection wavelength:-268 nm
Flow rate:-1.0ml/min

Procedure
20 µl of both standard and sample solution were injected in HPLC and the chromatogram was recorded. The standard peak was identified. The content of embelin was calculated using area under the curve of standard and sample peaks. Results of HPLC analysis are tabulated in **Table-3.** and **Fig-4 .**

Selection of the samples with highest yield of embelin
As per the HPLC & HPTLC analysis, the Ajmer variety was found to contain maximum amount of embelin, hence it was selected for further studies.

Phytochemical screening of extracts
The Phytochemical tests were performed for methanolic extracts of Ajmer variety to identify constituents. Various chemical tests were performed. Results are tabulated in **Table-4.**

3. Results and Discussion

The powdered fruits of *Embelia ribes* were extracted using different solvents. The obtained extracts were evaluated for their % extractive values. Results are tabulated in **Table-1.**

Table 1: % yields of the individual extracts of *Embelia ribes*

Solvents	Ayurvedic Medical College Hubli.	Hebsur	Himalaya	Kerala	**Rajastan (Ajmeer)**	Fathepur	Hessarghatta
Petroleum ether	3.2556	3.2251	3.2332	3.3211	**3.7487%**	3.4214	3.6541
Benzene	4.2315	4.5281	4.6425	4.3251	**4.5311%**	4.4221	42591
Chloroform	3.6215	3.5421	3.6511	3.6281	**4.1057%**	2.3214	3.7251
Ethyl acetate	3.2511	3.2581	3.2541	3.3211	**3.48%**	3.5421	3.3521
Methanol	4.7662	4.7321	4.9321	4.3211	**6.6000%**	5.6321	5.4342
Hexane	3.4561	3.5424	3.5421	3.8522	**4.1577%**	3.9725	3.7241
Acetone	3.1452	3.2154	3.3456	3.4221	**3.6%**	3.5412	3.3415

HPTLC analysis of the extracts
A HPTLC Method for Quantitative estimation of embelin was developed using the standard and parameters as discussed in methodology. The samples exhibited spots which corresponded to the standard drug Embelin. The results of HPTLC analysis and quantification of Embelin in the samples are tabulated in **Table-3** and pictures of chromatograms are depicted in **Fig-1**, **2.**

Table 2: Data showing the peak values of various extracts when compared with embelin

Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area%	Assigned substance
01	0.55	6.5	0.61	156.3	19.36	0.66	2.3	5700.0	38.45	Track 1, ID: EMBILIN 3µl- REFERENCE STD
02	0.52	3.7	0.59	254.9	29.31	0.66	2.2	10074.1	58.23	Track 2, ID: EMBILIN 6 µl REFERENCE STD
03	0.51	4.2	0.58	282.5	34.91	0.65	1.2	11864.3	68.1	Track 13, ID: EMBILIN 9µl- REFERENCE STD
04	0.52	4.9	0.59	315.2	39.31	0.66	1.3	13290.0	78.21	Track 4, ID: EMBILIN 12µl- REFERENCE STD
05	0.50	1.4	0.53	13.2	8.31	0.57	2.2	462.7	16.25	Track 5, ID: EX1206174-TR1(1206219E)3µl- HUBLIC AYUR COLLEGE
06	0.52	3.7	0.59	254.9	29.46	0.66	2.2	10074.1	58.23	Track 6, ID: EX1206175-TR1(1206220E)-6µl-IIHR
07	0.52	3.7	0.59	254.9	29.45	0.66	2.2	10074.1	58.23	Track 7, ID: EX1206176-TR1(1206221E)-6µl-Kerala
08	0.55	6.5	0.61	156.3	14.27	0.66	2.3	5700.0	38.45	Track 8, ID: EX1206177-TR1(1206221E)3µl- Himalaya
09	0.51	4.2	0.58	281.5	25.76	0.65	1.2	11874.3	58.23	Track 9, ID: EX1206178-TR1(1206223E) 9µl- AJMEER
10	0.51	0.1	0.52	21.8	25.76	0.54	0.0	343.2	58.23	Track 10, ID: EX1206179-TR1(1206224E) 3µl- HEBSUR

11	0.55	0.5	0.59	62.8	19.24	0.63	0.2	1514.3	38.45	Track 11, ID: EX1206180-TR1(1206225E)3µl-FATEHPUR SIKRI
12	0.55	6.5	0.61	156.3	19.21	0.66	2.3	5700.0	38.45	Track 12, ID: EMBILIN 3µl- REFERENCE STD
13	0.52	3.7	0.59	254.9	25.76	0.66	2.2	10074.1	58.23	Track 13, ID: EMBILIN-6µl- REFERENCE STD
14	0.51	4.2	0.58	282.5	34.21	0.65	1.2	11864.3	68.1	Track 14, ID: EMBILIN 9µl- REFERENCE STD
15	0.50	1.5	0.57	333.7	42.41	0.65	1.3	14660.1	83.43	Track 15, ID: EMBILIN 12µl- REFERENCE STD

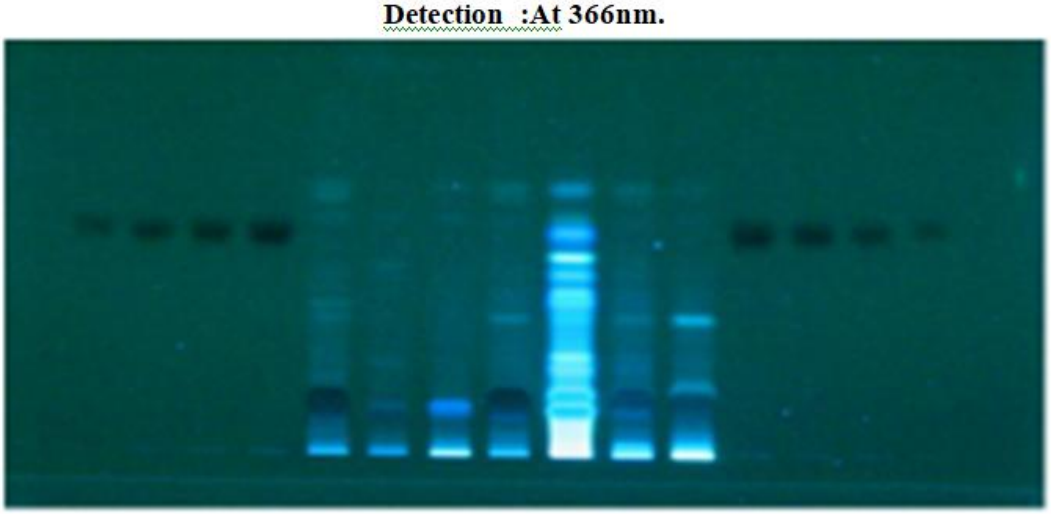
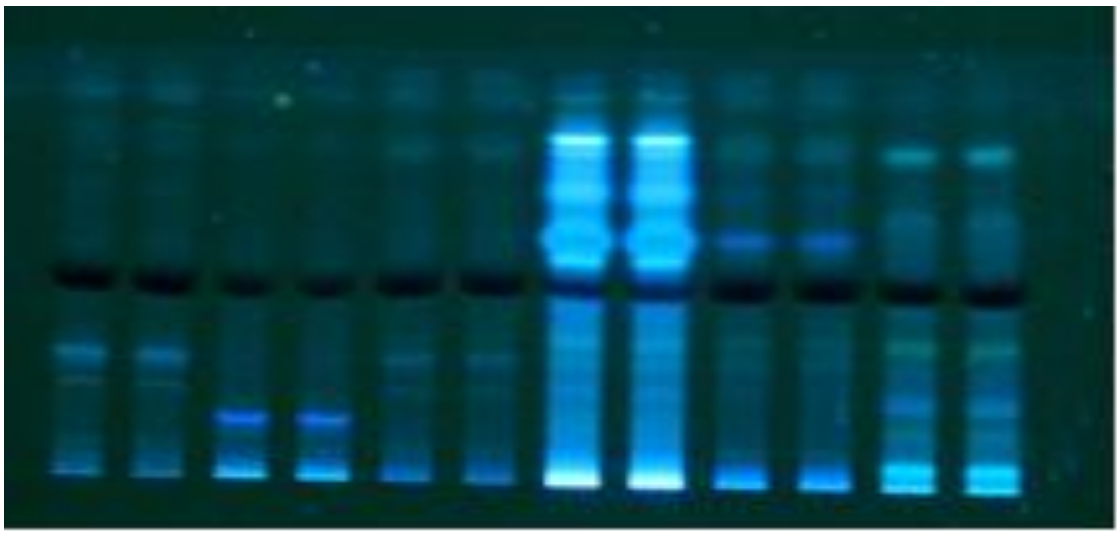


Figure 1: HPTLC Chromatogram of the samples compared with the std
Mobile Phase: Chloroform: Toluene: Ethyl acetate: Diethylamine(3.5: 3.5: 2: 0.5)



1)IHR, 2)KERALA, 3)HIMALAYA, 4)AJMEER, 5)HEBSUR, 6)FATEHPUR SIKRI
Figure 2: HPTLC Chromatogram of the samples compared with the std
Mobile Phase: Ethyl acetate : Methanol : Water : Glacial acetic acid(77:15:8:5)
Detection :At 366nm.

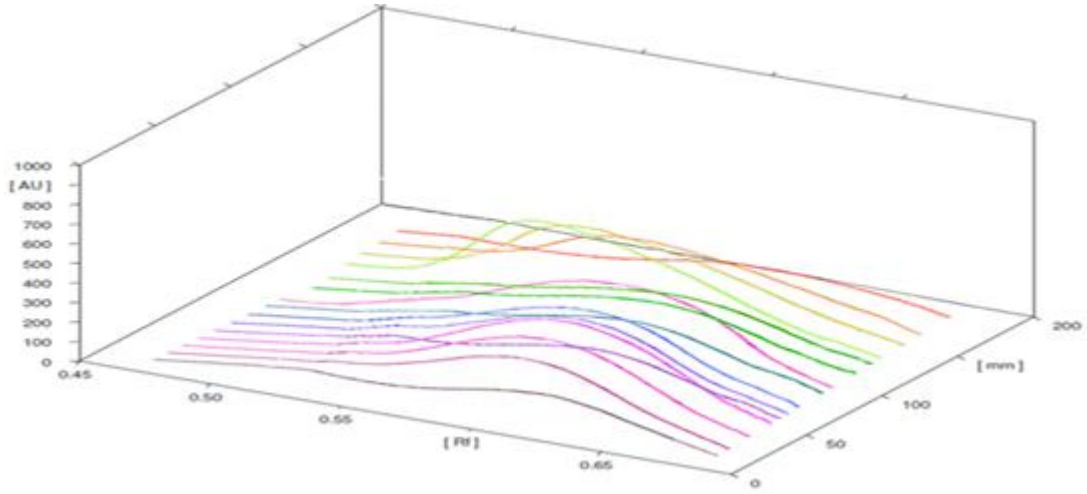


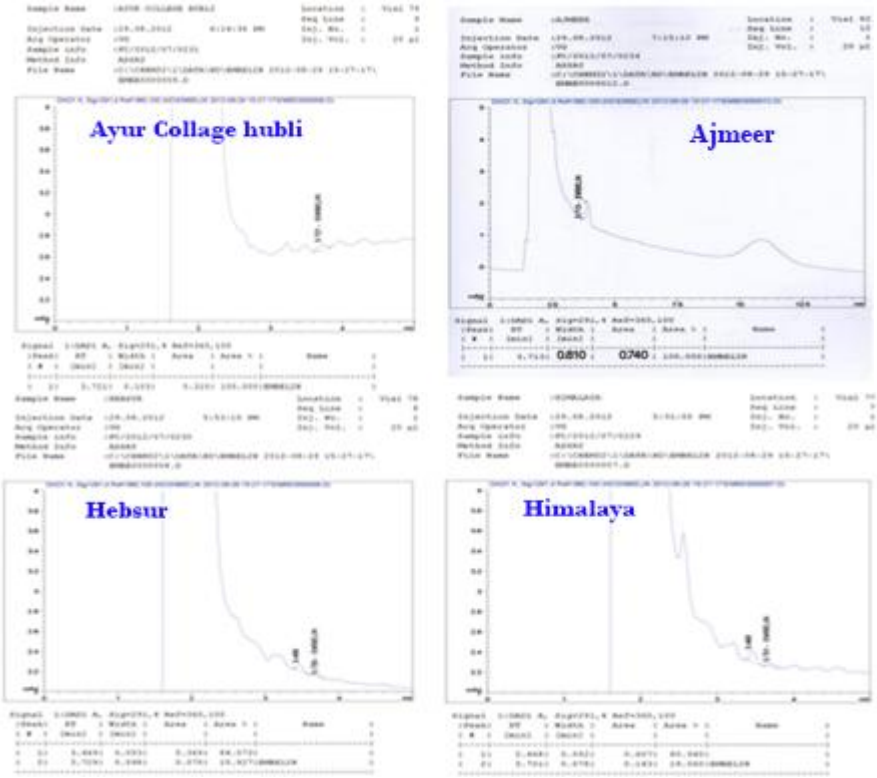
Fig-5: HPTLC Chromatogram of the samples compared with the std
Mobile Phase : Toluene: Ethylacetate: Formic acid (60:35:15)
Figure 3: HPTLC Chromatograph of the samples compared with the std

HPLC analysis of the extracts

The HPLC analysis of all the samples was carried out and were compared with the standard drug Embelin. Results showed that the methanolic extract was found to contain maximum amount of emeblin content when compared to other extracts. This was followed by ethyl acetate extract. Minimum amount of Embelin was found to be in the acetone extract. Results are tabulated in **Table-3** and chromatograms are shown in **Fig-4**.

Table 3: Data showing the Embelin content in different varieties

Serial no	State	Embelin content..mcg/ml
01	Himalaya	0.818mcg/ml.
02	Hebsur	0.816mcg/ml.
03	Ayurhubli collage	0.822mcg/ml.
04	Kerala	0.816mcg/ml.
05	IIHR	0.823mcg/ml.
06	Ajmeer	2.634mcg/ml.
07	FathepurSikri	0.817mcg/ml.
08	Standard-1	20.927 mcg/ml.
09	Standard-2	84.474 mcg/ml



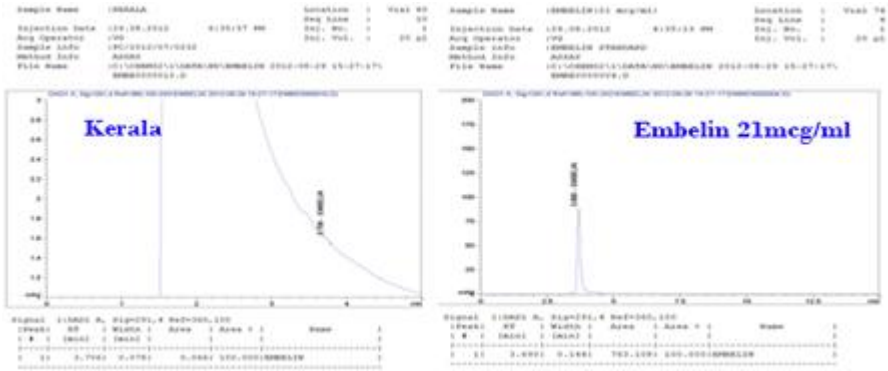


Figure 4: Chromatographs depicting the peaks of various samples and standard

Phytochemical screening of the extracts

Phyto-chemical investigation of the extracts of *Embelia ribes* of Ajmeer variety revealed presence of Alkaloids, Resin, Flavanoids, steroidal sapogennins, glycosides, Lipids, Tannins, Sterols &Triterpenes , volatile oil, Coumarins and Quinones. The results of phytochemical screening are tabulated in **Table-4.**

Table 4: Results of phytochemical analysis of methanolic extract of *E ribes* (Ajmer variety)

Chemical test	Observation	Inference
Test for volatile oils	Red colour developed	Volatile oil present
Tests for resins 1) $FeCl_3$ test 2) HCl test	 Greenish blue colour pink colour	 Resins present
Tests for flavonoids 1) $FeCl_3$ test 2) Flavonoid test 3) Shinoda test	 Dark brown colour yellow colour red colour	 flavonoids present ,, ,,
Tests for glycosides 1) Borntragers test 2) Haemolysis test 3) Foam test 4) Keller killiani test 5) Baljet test 6) Legal test 7) Raymond test	 Pink colour Red colour Formation of foam Junction-reddish-brown colour Results in orange colour Results in red colour Results in blue colour	 Anthraquinones present Saponins present Saponins present cardiac glycosides present ,, ,, ,,
Tests forsteroids-triterpenoids 1) Libermann b 2) Salkowski 3) Antimony--- 4) Trichloro--- 5) Tetranitro---- 6) Zimerrmann--	 Reddish brown colour ,, ,, Blueish colour ,, ,,	 steroids &triterpenoids present ,, ,, ,, ,,
Tests for Alkaloids,	Yellow precipitate	Alkaloids present
Testsforsteroidalsapogennins	Blueish precipitate	steroidal sapogennins present
Tests for Tannins	Yellowish brown precipitate	Tannins present
Tests for Quinones	Yellowish brown precipitate	Quinones present
Tests for coumarins	Yellowish brown colour	coumarins present
Tests for CHO	Reddish brown precipitate	CHO present

4. Conclusion

It was concluded that HPTLC and HPLC method were developed for fingerprinting and estimation of Embelin. The various extracts of berries of embelia were subjected to fingerprinting and embelin was quantified. Maximum amount of embelin was found to be present in the methanolic extract of Ajmer variety. The methanolic extracts of *E ribes* of Ajmer variety were evaluated for their nature of chemical constituents and identified.

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