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CHEMO PROFILING OF METHANOLIC EXTRACT OF *PHYLLANTHUS EMBLICA* LINN FROM FIVE DIFFERENT LOCALITIES OF VINDHYA REGION IN INDIA

Shashi Kumar D.*[1], Deepak M.[1], Azaz K.[2] and Megha J.[2]

[1]Department of Biotechnology, AKS University, Panna Road, Sherganj, Satna, Madhya Pradesh 485001, India

[2]Pinnacle Biomedical Research Institute (PBRI), Near, Bharat Scout and Guides Campus, Shanti Marg, Shyamla Hills Road, Bhopal, Madhya Pradesh 462003, India

Correspondence Author : Shashi Kumar Dubey

Department of Biotechnology, AKS University, Panna Road, Sherganj, Satna,

Madhya Pradesh 485001, India

Email: shashidubey86@gmail.com

Abstract

Phyllanthus emblica (*P. emblica* Euphorbiaceae) known as Indian gooseberry is a very richest source of vitamin C found in abundant amount in deciduous forest of Madhya Pradesh. It is common all over tropical and sub-tropical India. Chemo-profiling of herbal drugs represent a comprehensive qualitative approach for the purpose of species authentication, evaluation of quality and ensuring the consistency and stability of drugs and their related products. The objective of the present investigation was to determine the presence of various phytochemicals in a methanolic leaves and fruit extract of *P. emblica* from 5 different localities of Vindhya region in India, followed by a high performance thin layer chromatography analysis for standardization of the extract using gallic acid as standards. Qualitative analysis of various phytochemical constituents were determined by the well-known test protocol available in the literature. TLC were performed for preliminary identification of constituent in solvent system toluene: ethyl acetate: formic acid: methanol (5:5:0.5:0.2), gallic acid was used as standard for phenolic compound. Phenolic compound was estimated in methanolic extract of *P. emblica* (leaves and fruits) by high performance thin layer chromatography (HPTLC). Pre-coated Silica gel 60 F 254 (E. Merk) TLC plates were used as stationary phase and toluene: ethyl acetate: formic acid: methanol (5:5:0.5:0.2) was used as mobile phase. Detection and quantification were performed by densitometry at λ 254 nm. The linear range was 200ng to 600ng. This HPTLC method was found to reproducible, accurate and precise.

***Keywords*:** *Phyllanthus emblica,* Chemo-profiling, Qualitative analysis, HPTLC

Introduction

Natural products, such as plants extract, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity (Cosa *et al.,* 2006). According to the World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. The use of herbal medicines in Asia represents a long history of human interactions with the environment. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases (Duraipandiyan *et al.,* 2006). Due to the development of adverse effects and microbial resistance to the chemically synthesized drugs, men turned to ethno-pharmacognosy. They found literally thousands of phytochemicals from plants as safe and broadly effective alternatives with less adverse effect. Many beneficial biological activity such as anticancer, antimicrobial, antioxidant, antidiarrheal, analgesic and wound healing activity were reported. In many cases the people claim the good benefit of certain natural or herbal products. However, clinical trials are necessary to demonstrate the effectiveness of a bioactive compound to verify this traditional claim. Clinical trials directed towards understanding the pharmacokinetics, bioavailability, efficacy, safety and drug interactions of newly developed bioactive compounds and their formulations (extracts) require a careful evaluation. Clinical trials are carefully planned to safeguard the health of the participants as well as answer specific research questions by evaluating for both immediate and long-term side effects and their outcomes are measured before the drug is widely applied to patients. According to the WHO, nearly 20,000 medicinal plants exist in 91 countries including 12 mega biodiversity countries. The premier steps to utilize the biologically active compound from plant resources are extraction, pharmacological screening, isolation and characterization of bioactive compound, toxicological evaluation and clinical evaluation (Sasidharan *et al.,* 2011).

Chromatographic fingerprinting has been in use for a long time for single chemical entity drug substances. Chemical and chromatographic techniques may also be used to aid in identification of herbal medicine or extract and in assessment of their potency and stability (Bala & Saini, 2013). High performance thin layer chromatography (HPTLC) has recently emerged as a preferred analytical tool for fingerprinting and quantification of marker compounds in herbal drugs because of its suitability for high throughput screening sensitivity and reliability in quantification of analytes at nanogram level (Lazarowych & Pekos, 1998; Vundac *et al.,* 2005; Michael *et al.,* 2012). Emblica (*P. emblica* L.) as a euphorbiaceous plant is widely distributed in subtropical and tropical areas of India, China, Indonesia and Malaysia. *P. emblica* fruit is well accepted by consumers for its special taste. It has abundant amounts of vitamin C and superoxide dismutase (Verma & Gupta, 2004) and is used in many traditional medicinal systems, such as Ayurvedic medicine, Chinese herbal medicine and Tibetan medicine (Zhang *et al.,* 2000) *P. emblica* is known as amalaka in Sanskrit, amla in Hindi, olay in Punjabi , aamla in Gujarati, nellikkai in Tamil, Amloki in Bengali (Zhu *et al.,* 2013). The fruit of this plant is almost spherical, quite smooth, 18-25

mm wide and 15-20 mm long, light greenish yellow in color, with 6 vertical streaks, and hard in appearance (Krishnaveni & Mirunalini, 2011). Traditionally, the fruit is beneficial as an anti-inflammatory, antipyretic, astringent, diuretic, laxative, stomachic, liver and hair tonic (Perianayagam *et al.,* 2004). This fruit is a great source of numerous phytoconstituents such as alkaloids, flavonoids, terpenoids, tannins, and pectin (Krishnaveni & Mirunalini, 2010). The important pharmacological actions of this fruit are antioxidant, analgesic, anti-inflammatory, neuroprotective, antitussive, anti-atherogenic, adaptogenic, cardioprotective, immunomodulatory, gastroprotective, antiviral, antiemetic, anthelmintic, nephroprotective, and anticancer activities (Dasaroju & Gottumukkala, 2014; Ihantola-Vormisto *et al.,* 1997; Dhale & Mogle, 2011; Raghu & Ravindra, 2010).

Material and Methods

Plant materials

Fresh, young, disease free leaves and fruits of *P. emblica* were collected from 5 different localities of Vindhya region in India. The identification and authentication of plant was done by Dr. Saba Naaz, Botanist, from the Department of Botany, Saifia College of Science, Bhopal. A voucher specimen number 175/Saif/Sci/Clg/Bpl was kept in Department of Botany, Saifia College of Science, Bhopal for future reference.

Chemical reagents

All the chemicals used in this study were obtained from Hi-Media Laboratories Pvt. Ltd. (Mumbai, India), Sigma Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India). All the chemicals used in this study were of analytical grade.

Extraction

Collected plant material washed under running tap water and kept in shade for drying. Dried plant materials were then powdered using blender and further observed for colour, odour, and texture then placed in packed labelled air tight container for further use. Plant material was extracted by continuous hot percolation method using Soxhlet apparatus. Powdered material of *P. emblica* was placed in thimble of soxhlet apparatus. Soxhlation was performed at 60°C using petroleum ether as non-polar solvent. Exhausted plant material (marc) was dried and afterward re-extracted with methanol solvent. For each solvent, soxhlation was continued till no visual colour change was observed in siphon tube and completion of extraction was confirmed by absence of any residual solvent, when evaporated. Obtained extracts was evaporated using rotary vacuum evaporator (Buchi type) at 40°C. Dried extract was weighed and percentage yield for each extract was determined.

Qualitative phytochemical analysis of plant extract

The *P. emblica* plant extract obtained was subjected to the preliminary phytochemical analysis following standard methods. The extract was screened to identify the presence or absence of various active principles like phenolic compounds, carbohydrates, flavonoids, glycosides, saponins, alkaloids, fats or fixed oils, protein and amino acid and tannins.

Thin layer chromatography (TLC)

The preliminary phytochemical investigation revealed the presence of tannins, flavonoids and phenolic compounds. The extracts of leaves and fruits of *P. emblica* were subjected to thin layer chromatography to detect the various constituents present in it.

Adsorbent: Silica gel GF 254 (activated)

Thickness: 0.2 mm Plate

Size: 12x18cm

Activation temp: 110°C for 1hr

Volume of spot: 20µl

Solvent system: Toluene: ethyl acetate: formic acid: methanol (5:5:0.5:0.2). The spots were observed in UV chamber [21, 22].

High performance thin layer chromatography (HPTLC)

Preparation of standard solution: The reference standard solution of galic acid was prepared in methanol in concentration range of 100 ng to 700 ng.

Chromatographic conditions: The following chromatographic conditions were used to quantify the gallic acid:

Stationary phase: Silica gel 60 F 254 (E. Merck) pre-coated TLC plates

Mobile Phase: Toluene: ethyl acetate: formic acid: methanol (5:5:0.5:0.2)

Sample volume: 2 µl

Temperature: 60°C

Migration Distance: 70 mm

Detection wavelength: 254nm

Linearity was performed by applying standard solution at different concentrations ranging from 100 ng to 700 ng on 20×20 cm HPTLC plates, pre-coated with silica gel 60 F 254 (E. Merck) in the form of sharp 8 mm bands. The plates were developed in a solvent system of toluene: ethyl acetate: formic acid: methanol (5:5:0.5:0.2), up to a distance of 70mm, at 60°C. The detector response for galic acid was measured for each band at wavelength of 254 nm, using Camag TLC Scanner and win CAT software. The peak areas of gallic acid were recorded for each concentration. The linearity curve of gallic acid was obtained by plotting a graph of peak area of gallic acid vs applied concentrations of gallic acid (ng).

Results and Discussions

Five individual populations of *P. emblica* were collected from different areas (Table 1) and subjected to qualitative and quantitative analysis. Qualitative phytochemical testing of species of *P. emblica* was done to study the presence or absence of various phytochemical constituents using standard tests methods. The Phytoconstituents were identified by chemical tests, which showed the presence of various constituents. The results of the species of the *P. emblica* are shown in Table 2-6. Extracts showed the presence of various chemical constituents mainly alkaloids, flavonoids, tannins and phenolics compounds. Thin layer chromatography of extracts of *P. emblica* revealed violet green spot under UV (Rf = 0.42) which are almost

comparable to that of standard gallic acid (Rf = 0.40) and showed the presence of phenolic content in plant extract. The method utilizes silica gel 60F 254 HPTLC plates as stationary phase and toluene: ethyl acetate: formic acid: methanol (5:5:0.5:0.2) as mobile phase which gives good separation of gallic acid. The identity of the band of gallic acid in the sample extract was confirmed by overlaying the UV absorption spectra of samples with that of reference standard which showed λmax at 254nm. Phytochemical investigations (qualitative chemical analysis, TLC and HPTLC) were carried out with methanolic extract of *P. emblica*. Qualitative chemical analysis and TLC determination showed the presence of several phyto-constituents like phenolic compounds, flavonoids, tannins and the presence of phenolic compound were confirmed by HPTLC Figure 1. The maximum amount of gallic acid content was found in S1F and fewer amounts were found to be in S1L. The order of gallic acid content in different species of *P. emblica* was found to be as fallow: S1F> S2F> S3F> S3L> S4F> S5L> S2L> S5F> S4L> S1L Table 7 & 8.

Table 1 : *P. emblica* plant species collected from different localities

S. No.	Sample Code	Area of study	Geographical location
1.	S1	Sirmour area (wardha ghat), Rewa	24.85°N 81.38°E
2.	S2	Semariya,Rewa	24°47′42″N 81°9′8″E
3.	S3	Rampur Naikin, Sidhi	24°20′23″N 81°28′29″E
4.	S4	Hanumana, Rewa	24°46′30″N 82°5′24″E
5.	S5	Mukundpur, Rewa	24.4218° N, 81.2436° E

Table 2 : Phytochemical evaluations of S1 samples of same species

S. No.	Experiment	Result of species S1	
		Fruit	Leaves
Test for Carbohydrates			
1.	Molisch's Test	-ve	+ve
2.	Fehling's Test	-ve	-ve
3.	Benedict's Test	-ve	-ve
4.	Barfoed's Test	-ve	+ve
Test for Alkaloids			
1.	Dragendorff's Test	+ve	+ve
2.	Wagner's Test	+ve	+ve
3.	Mayer's Test	+ve	+ve
4.	Hager's Test	+ve	+ve
Test for Triterpenoids and Steroids			
1.	Libermann-Burchard Test	+ve	+ve
2.	Salkowski Test:	+ve	-ve
Test for Saponins			
1.	Froth Test	-ve	-ve
Test for Tannin and Phenolic Compounds			
1.	Ferric Chloride Test	+ve	+ve
2.	Gelatin Test	+ve	+ve
3.	Lead Acetate Test	+ve	+ve
Test for Flavonoids			
1.	Shinoda's Test	+ve	+ve
Test for Glycosides			
1.	Borntragers Test	-ve	-ve
2.	Keller Killiani Test	-ve	-ve
Test for Protein & Amino acids			
1.	Biuret's Test	-ve	-ve
2.	Ninhydrin Test	-ve	-ve

Table 3 : Phytochemical evaluations of S2 samples of same species

S. No.	Experiment	Result of species S2	
		Fruit	Leaves
Test for Carbohydrates			
	Molisch's Test	-ve	+ve
	Fehling's Test	-ve	-ve
	Benedict's Test	-ve	-ve
	Barfoed's Test	-ve	+ve
Test for Alkaloids			
	Dragendorff's Test	+ve	+ve
	Wagner's Test	+ve	-ve
	Mayer's Test	-ve	+ve

	Hager's Test	+ve	+ve
Test for Triterpenoids and Steroids			
	Libermann-Burchard Test	+ve	+ve
	Salkowski Test:	+ve	-ve
Test for Saponins			
	Froth Test	-ve	-ve
Test for Tannin and Phenolic Compounds			
	Ferric Chloride Test	+ve	+ve
	Gelatin Test	+ve	-ve
	Lead Acetate Test	+ve	+ve
Test for Flavonoids			
	Shinoda's Test	+ve	+ve
Test for Glycosides			
	Borntragers Test	-ve	-ve
	Keller Killiani Test	-ve	-ve
Test for Protein & Amino acids			
	Biuret's Test	-ve	-ve
	Ninhydrin Test	-ve	+ve

Table 4 : Phytochemical evaluations of S3 samples of same species

S. No.	Experiment	Result of species S3	
		Fruit	Leaves
Test for Carbohydrates			
1.	Molisch's Test	-ve	-ve
2.	Fehling's Test	-ve	-ve
3.	Benedict's Test	-ve	-ve
4.	Barfoed's Test	-ve	-ve
Test for Alkaloids			
1.	Dragendorff's Test	+ve	+ve
2.	Wagner's Test	+ve	+ve
3.	Mayer's Test	+ve	+ve
4.	Hager's Test	+ve	-ve
Test for Triterpenoids and Steroids			
1.	Libermann-Burchard Test	+ve	+ve
2.	Salkowski Test:	+ve	+ve
Test for Saponins			
1.	Froth Test	-ve	-ve
Test for Tannin and Phenolic Compounds			
1.	Ferric Chloride Test	+ve	+ve
2.	Gelatin Test	+ve	-ve
3.	Lead Acetate Test	+ve	+ve
Test for Flavonoids			
1.	Shinoda's Test	+ve	+ve
Test for Glycosides			
1.	Borntragers Test	-ve	-ve
2.	Keller Killiani Test	-ve	-ve
Test for Protein & Amino acids			
1.	Biuret's Test	-ve	-ve
2.	Ninhydrin Test	-ve	+ve

Table 5 : Phytochemical evaluations of S4 samples of same species

S. No.	Experiment	Result of species S4	
		Fruit	Leaves
Test for Carbohydrates			
1.	Molisch's Test	-ve	+ve
2.	Fehling's Test	-ve	-ve
3.	Benedict's Test	-ve	-ve
4.	Barfoed's Test	-ve	-ve
Test for Alkaloids			
1.	Dragendorff's Test	+ve	+ve
2.	Wagner's Test	+ve	+ve

3.	Mayer's Test	+ve	+ve
4.	Hager's Test	+ve	+ve
Test for Triterpenoids and Steroids			
1.	Libermann-Burchard Test	+ve	-ve
2.	Salkowski Test:	+ve	+ve
Test for Saponins			
1.	Froth Test	-ve	-ve
Test for Tannin and Phenolic Compounds			
1.	Ferric Chloride Test	+ve	-ve
2.	Gelatin Test	+ve	+ve
3.	Lead Acetate Test	+ve	-ve
Test for Flavonoids			
1.	Shinoda's Test	+ve	-ve
Test for Glycosides			
1.	Borntragers Test	-ve	+ve
2.	Keller Killiani Test	-ve	+ve
Test for Protein & Amino acids			
1.	Biuret's Test	+ve	-ve
2.	Ninhydrin Test	-ve	-ve

Table 6 : Phytochemical evaluations of S5 samples of same species

S. No.	Experiment	Result of species S5	
		Fruit	Leaves
Test for Carbohydrates			
	Molisch's Test	-ve	-ve
	Fehling's Test	-ve	-ve
	Benedict's Test	-ve	-ve
	Barfoed's Test	-ve	+ve
Test for Alkaloids			
	Dragendorff's Test	+ve	+ve
	Wagner's Test	+ve	-ve
	Mayer's Test	+ve	+ve
	Hager's Test	+ve	+ve
Test for Triterpenoids and Steroids			
	Libermann-Burchard Test	+ve	+ve
	Salkowski Test:	+ve	-ve
Test for Saponins			
	Froth Test	+ve	+ve
Test for Tannin and Phenolic Compounds			
	Ferric Chloride Test	+ve	-ve
	Gelatin Test	+ve	+ve
	Lead Acetate Test	+ve	-ve
Test for Flavonoids			
	Shinoda's Test	+ve	-ve
Test for Glycosides			
	Borntragers Test	-ve	-ve
	Keller Killiani Test	-ve	-ve
Test for Protein & Amino acids			
	Biuret's Test	-ve	+ve
	Ninhydrin Test	-ve	-ve

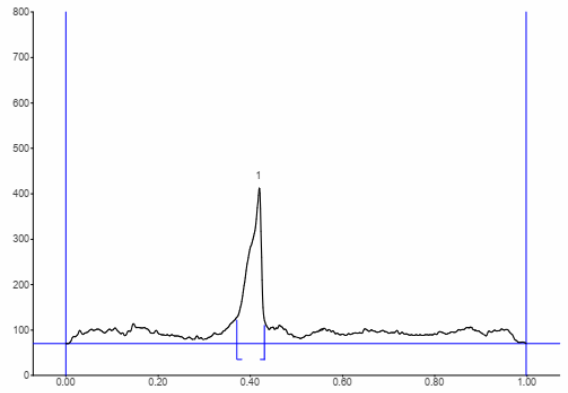
+ve = Present; -ve = absent

Table 7 : HPTLC profile of standard gallic acid

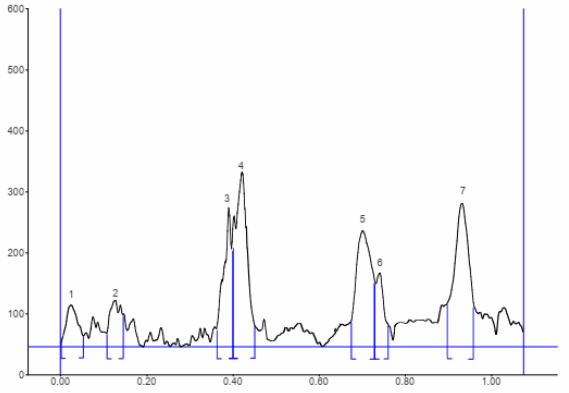
S. No.	Rf value	Concentration	Peak height	Peak area
1	0.44	200ng	151.11	1624.41
2	0.46	400ng	274.08	3340.72
3	0.46	600ng	151.21	1455.77

Table 8 : HPTLC profile of methanolic extract of different sample of *P. emblica*

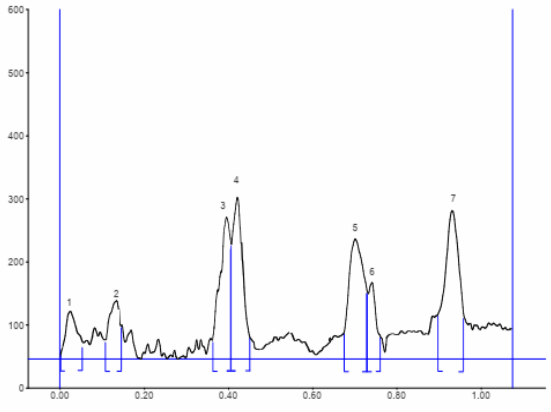
S. name	Rf value	Quantitative yield
S1F	0.44	269.72ng
S2F	0.46	265.98ng
S3F	0.46	263.78ng
S4F	0.45	260.34ng
S5F	0.45	260.10ng
S1L	0.42	257.34ng
S2L	0.45	260.21ng
S3L	0.44	261.45ng
S4L	0.45	259.00ng
S5L	0.46	260.34ng



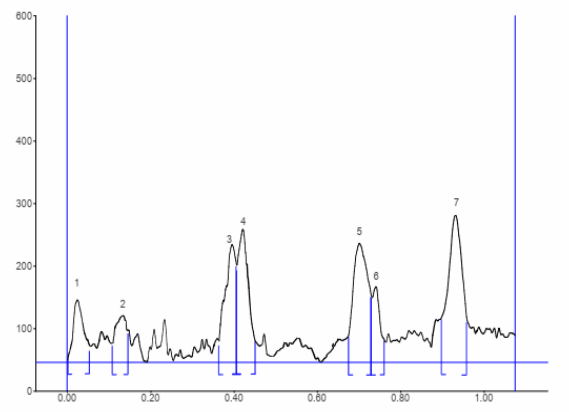
Gallic Acid Stranded



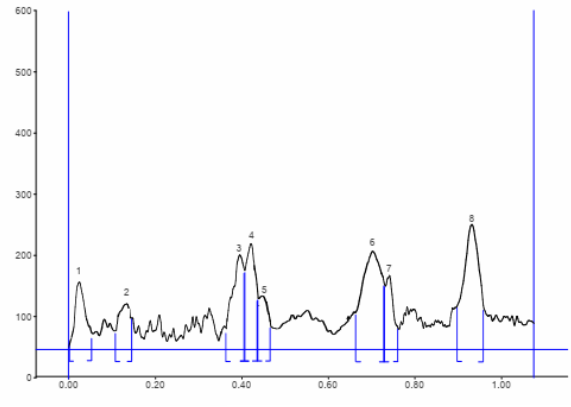
S1F



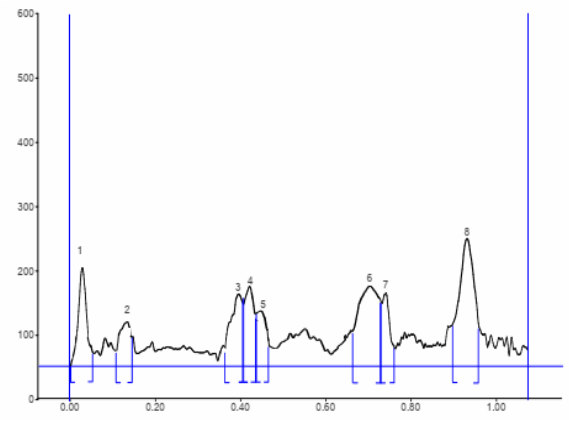
S2F



S3F



S4F



S5F

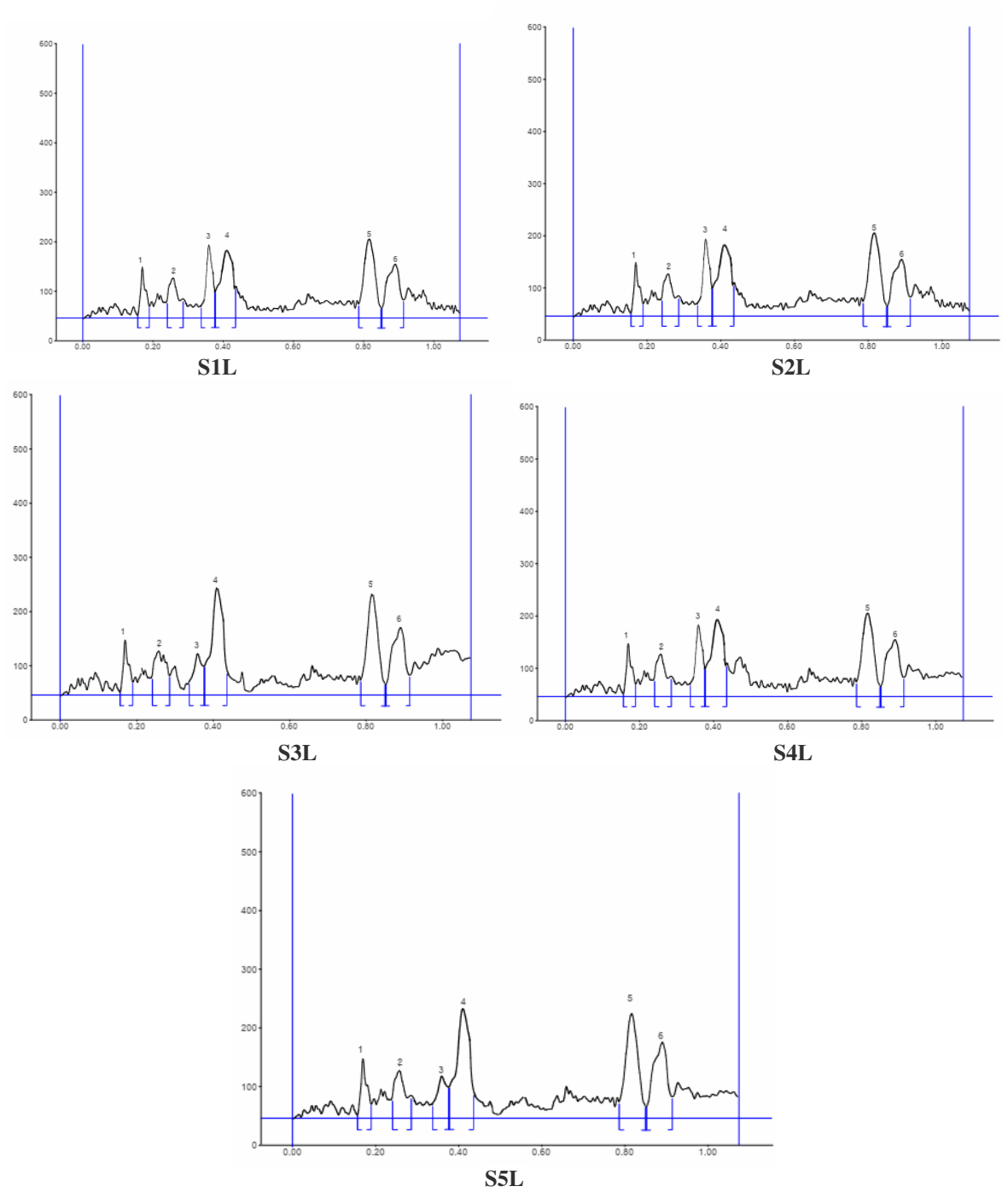


Fig. 1 : HPTLC chromatogram of gallic acid and different sample of *P. emblica*

Conclusion

The phenolics contents in methanolic extracts of emblica fruits and leaves from 5 different localities of Vindhya region in India were analyzed. The maximum amount of gallic acid content was found in S1F and fewer amounts were found to be in S1L. The order of gallic acid content in different species of *P. emblica* was found to be as fallow: S1F> S2F> S3F> S3L> S4F> S5L> S2L> S5F> S4L> S1L. The above results indicated that region could lead to significant differences both in the content of bioactive compounds and their bioactivities. Emblica fruit can be a source of plant antioxidants, with a potential use in food, cosmetics and pharmaceutical fields. The phenolics might be the major active component responsible for the strong antioxidant activity. The proposed HPTLC method was found to be precise, specific, accurate and robust and can be used for identification and quantitative determination of herbal extract and its formulations. HPTLC method is especially suitable for the fingerprinting and high throughput analysis of botanical samples and herbal formulations. However, a more detailed investigation between the individual phenolic compounds present in emblica fruit and the antioxidant activities needs to be carried out.

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