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Chromatographic Fingerprinting of Sarasvata Churna–an Ayurvedic Polyherbal Formulation for Epilepsy

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ABSTRACT

Sarasvata churna is an Ayurvedic formulation prescribed for Epilepsy and other brain-related disorders. The objective of this study is to explore bioactive principles present in Sarasvata churna using GCMS and HPTLC techniques and establishing their correlation in Epilepsy treatment. The volatile oil obtained by hydrodistillation was used for the GCMS analysis and methanolic extracts were used for HPTLC studies. HPTLC fingerprints for the formulation and method for simultaneous qualitative analysis of biomarkers in the formulation was developed using HPTLC. The GCMS report shows the presence of α-Pinene, Propanal, 2-Caren-10-al, 3-Caren-10-al, Caryophyllene, Epiglobulol, Asarones, α-selinene, α-sesquiphellandrene, o-cymene, copaene etc. HPTLC fingerprints for methanolic extracts of Sarasvata churna was established with good separation and resolution in solvent system Toluene:Ethylacetate: Methanol (7.5:2.5:0.5 v/v/v). The HPTLC method for simultaneous analysis of marker compounds in the formulation was developed in solvent system Ethylacetate:Glycial acetic acid:Toluene:Water (4:1:1:0.5 v/v/v/v) with Rf 0.77 for Piperine, 0.07 for Bacosides and Rf 0.57 for Withnolides in the reference compounds and methanolic extracts of Sarasvata churna. Chromatographic patterns of reference compounds were easily detectable in the tracks of Sarasvata churna methanolic extracts. The chromatographic standards obtained from this study will help in chemical standardization of Sarasvata churna and enhance the use Sarasvata churna for management of Epilepsy.

INTRODUCTION

Polyherbal formulations are being prescribed for the management of illnesses for thousands of years. Traditional systems of medicines like Ayurveda, Unani, Siddha, and Homeopathy contain a large number of medicines which contains herbs for prevention and treatment of a variety of diseases. A large pool of human population on earth uses these traditional systems of medicines because of their time-tested efficacy, easy availability, low cost, holistic beliefs and fewer side effects.

In last three decades, there have been tremendous increases in consumption of herbal medicines due to high cost, toxicity, and failure of modern systems of medicines. Ancient Ayurvedic text Sarangdhar Samhita emphasizes the use of Polyherbalism in the long-term management of diseases (Kaushik *et al.*, 2017a). Polyherbal formulations contain a large number of bioactive principles contributed by the ingredient herbs in very low concentrations which makes the formulation to act on a disorder by multiple mechanisms of actions along with low toxicity and least side effects. Maximum therapeutic benefits can be achieved only when these formulations are prepared using authentic herbs and used as per the indications mentioned in the ancient texts.

Sarasvata churna is one such polyherbal formulation that is prescribed by an Ayurvedic system of medicines for the management of brain-related disorders and epilepsy. Sarasvata churna contains herbs like *Bacopa monnieri* (Brahmi), *Convolvulous pluricaulis* (Shankhpushpi), *Acorus calamus* (Vach), *Cissampelos pareira* (Patha), *Piper longum* (Peepli), *Piper nigrum* (Kalimirch), *Sassurea lappa* (Kustha), *Withania somnifera* (Ashwagandha), Rock salt (Lavana), *Carum roxburghianum* (Ajmod), *Carum carvi* (Kala Jeera), *Cuminum*

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cyminum (Safed Jeera) and *Zingiber Officinalis* (Sonth). Withanolides (Kulkarni *et al*., 2008; Candelario *et al*., 2015) of *Withania somnifera*, Bacosides (Paturu *et al*., 2013; Tripathi *et al*., 1996) of *Bacopa monneire* Asarones of *Acorus calamus* (Kaushik *et al*., 2017; Jayaraman *et al*., 2010) Piperine of *Piper nigrum* and *Piper longum* (Mishra *et al*., 2015; Syed *et al*., 2015), Sesquiterpenes and Gingerols (Hussein *et al*., 2015) from *Zingiber officinalis*, Pinene, Thymol, Caryophyllene (Oliveira *et al*., 2016), Sesquiphellandrene, Copaene, Cymene (Talita *et al*., 2015) and many other volatile principles from *Carum carvi*, *Carum roxburghianum*, *Cuminum cyminum* (Janahmadi *et al*., 2006; Sayyah *et al*., 2002) and *Sassurea lappa* posses significant antioxidant, antiepileptic and neuroprotective potentials (Kaushik *et al.,* 2017b). Physicochemical analysis of Sarasvata churna revealed the presence of alkaloids, glycosides, tannins, flavonoids, terpenoids, proteins, carbohydrates, fats and oils.

GC-MS (Liebler *et al*., 1996; Monisha *et al*., 2015; Wright *et al*., 2017; Annalakshmi *et al*., 2013) and HPTLC (Shakeel *et al*., 2015; Pandita *et al*., 2016; Garg and Bhutani, 2008) analysis are the backbones of herbal drug research industries. These analytical techniques help in accurate detection of phytoconstituents present in the herbal drugs even in minute quantities which otherwise very difficult to detect with traditional methods of isolation of phytoconstituents in pure form and then their manual structure elucidation. These techniques are combinations of modern instruments. The use of these techniques in herbal drug analysis now a day's become popular and most of the herbal pharmacopoeias are including these analytical techniques for accurate identification and standardization of herbal drugs. These techniques give a clear view of quality and quantity of phytoconstituents present in an herbal drug or formulation.

This study is aimed to develop chromatographic standards and fingerprints for the formulation so that the developed standards can be used by fast-growing herbal drug industries and other research organizations for proper standardization of Sarasvata churna along with other standardization parameters. Literature review shows that this formulation remains untouched till date and no major chromatographic studies using modern techniques have been performed on Sarasvata churna. Some ingredients of Sarasvata churna contain volatile oils in major concentrations which in turns forms major shares in the Sarasvata churna. The components of the volatile oils from Sarasvata churna as whole posses neuroprotective, antioxidant and antiepileptic potentials. Also, there are some other non-volatile herbs containing phytoconstituents having a neuroprotective activity that cannot be isolated using hydro-distillation. So it becomes necessary to isolate both volatile oil using hydro-distillation and other components in methanolic extracts so as to establish maximum chemistry of Sarasvata churna as also observed from phytochemical screening.

The developed HPTLC and GC-MS chromatographic fingerprinting will serve as a future reference for students, academicians and research fellows along with industries involved in production and development of Ayurvedic and herbal formulations.

MATERIAL AND METHODS

The Sarasvata churna have been prepared as per the formula prescribed by Ayurvedic Formulary of India in the institutional Pharmacognosy laboratory using herbs purchased from Aryavastu Bhandar, Dehradoon, Uttrakhand and herbal garden of Ram-Eesh Institute of Vocational and Technical Education, Greater Noida (Anonymous, 2000). The ingredient herbs are authenticated by Raw Material and Herbarium Department, NISCAIR, New Delhi with authentication certificate report number NISCAIR/RHMD/Consult/2016/2993-20 and NISCAIR/RHMD/Consult/2016/3026-53.

Extraction of volatile oil for GCMS

50 g of freshly prepared and shade dried powder of Sarasvata churna was subjected to hydro-distillation with 350 ml water in a round bottom flask and Clevenger apparatus (Borosil) for 2 hours. The volatile oil thus collected from the Clevenger to which added a pinch of anhydrous Sodium sulphate in order to absorb moisture from oil. The oil is then stored at a cool temperature and used for GCMS analysis. Oil yield has been calculated relative to the dry weight of the powder.

Sample Preparation for GCMS analysis

0.1 ml of extracted volatile oil from Clevenger's apparatus was diluted 10 ml with n-hexane (Ultra pure HPLC grade). 1 ml of the dilution was taken and transferred to autosampler with n-hexane as blank.

GCMS operating specifications

A program was made and 1 microlitre of the sample was injected to obtain GCMS spectra. The integrated assembly of GC-MS (Thermo Scientific 1300 TRACE and Thermo Scientific TSQ Quantum) with RXi 5-Sil MS (30 m × 0.25 mm with 0.25 μ) column was used for analysis. The column flow of 1 ml/min was maintained. Split ratio: 1:25, Injection volume 1 ml, Solvent cut time of 2.5 min. The column temperature was programmed as follows: 60° for 2 min rising at a rate of 5°C/min till 250° and hold for 5 min. Inlet temperature 250°C and Ion source temp. 280°C. Mass Scanning from 3-40 min at a scan speed of 2000 with mass range m/z 40.0 to 550.0.

The component identification was made by comparison using computer library NIST 11 and Willy database connected to the GC-MS.

HPTLC FINGERPRINTING OF SARASVATA CHURNA

Preparation of Test and Reference standard solutions for HPTLC analysis

Formulation: 1 g powder of Sarasvata churna was dissolved in 10 ml methanol and sonicated for 10 minutes. After sonication, the extract was centrifuged or filtered and the filtrate was used as test solution.

Methanolic extract: 1 g of powder of Sarasvata churna was dissolved in 10 ml methanol in a conical flask and left aside for maceration for 7 days with occasional 2-3 times stirring/shaking daily. On the 7th day, the extract was filtered and concentrated in a water bath and used for HPTLC analysis.

Piperine: 1 mg of Piperine standard (Sigma Aldrich Inc.) was dissolved in 1 ml methanol.

Withnolides and Bacosides: 1 mg of Withnolides (50.01% by HPLC) and Bacosides (51.23% by HPLC) (Sanat Products Ltd.) were dissolved in 1 ml methanol and used for analysis.

Chromatographic conditions

Stationary phase: Merck, Aluminum TLC plates with adsorbent Silica gel 60 F_{254}.

Application: CAMAG ATS-4 automatic sampler and applicator was used. Nitrogen gas was used as spray gas, Methanol is used as a solvent for test solution with application speed of 150 nl/s. For fingerprint development 2, 4 and 6 µl of formulation extract (on Track 1, 2 and 3) and methanolic extract (on Track 4, 5 and 6) were used. For fingerprint development with reference compound Bacosides (Track 5 and 6) and Piperine (Track 7 and 8) in 5 and 10 µl volume were used whereas Withnolides (Track 9 and 10) in 10 and 20 µl volume was applied on TLC plate respectively.

Solvent system

For Fingerprinting without reference compounds

Toluene:Ethylacetate: Methanol (7.5:2.5:0.5 v/v/v) with twin trough development chamber containing 5/5 ml solvent system with saturation time of 20 minutes.

For Fingerprinting with reference compounds

Ethylacetate:Glycial acetic acid:Toluene:Water (4:1:1:0.5 v/v/v/v) with twin trough development chamber containing 10/10 ml solvent system with saturation time of 20 minutes.

The TLC plates were developed in the CAMAG twin trough development chamber up to a height of 70 mm from the spotting line. Dried in the air and derivatized using Natural Product reagent and Anisaldehyde Sulphuric acid reagent and observed at short and long wavelengths of ultraviolet light.

The developed plates were air dried and scanned. A spectrodensitometer (Scanner 4, CAMAG) equipped with visionCATS planar chromatography manager software with automatic detector was used for the densitometric measurements and data processing. Absorbance/emission scanned at a scan speed of 20 mm/sec. Spots of fraction were scanned from 200 to 800 nm so as to record their UV-VIS spectrum and to obtain wavelengths of maximum absorption.

Natural Product Reagent

Reagent preparation: 1 g of 2-aminoethyl diphenylborinate dissolved in 200 ml of ethylacetate.

Treatment: Developed plate is heated for 3 minutes at 110°C before application of derivatizing reagent.

Anisaldehyde Sulphuric acid reagent

Reagent preparation: 170 ml of methanol was placed in a 200 ml glass bottle and cooled in ice cube water bath to which slowly and carefully added 10 ml acetic acid and 10 ml sulphuric acid and mixed well. The mixture was allowed to cool to room temperature and then 1 ml of Anisaldehyde was added.

Treatment: Developed plate was dipped in the reagent for 2 seconds and then the plate was heated for 3 minutes.

RESULTS AND DISCUSSION

The percentage yield of volatile oil from hydro-distillation of Sarasvata churna was found to be 2.40% v/w.

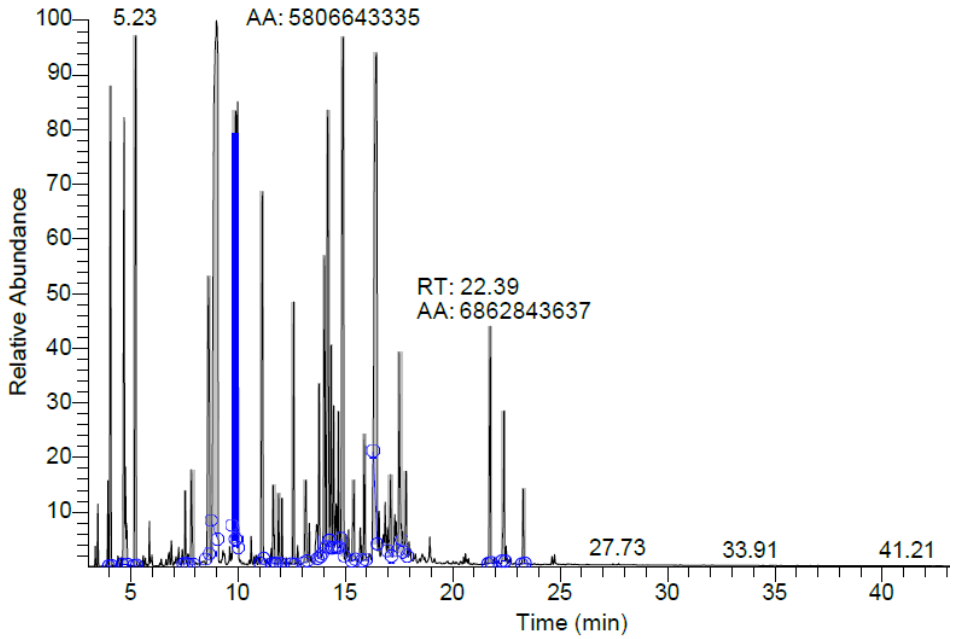

Fig. 1: GC-MS Chromatogram of Sarasvata churna volatile oil.

The relative percentage composition of each component of volatile oil was calculated by comparing its average peak area to the total area. Interpretation of GC-MS chromatogram was done by the computer-based software XCALIBUR using the library database of National Institute of Standard and Technology (NIST 2011) and Wiley. The spectrum of the separated components was compared with the spectrum of NIST library database in order to confirm identity. The GC-MS chromatogram of the volatile oil is presented in Figure 1 which shows the presence of 30 prominent compounds which are described along with their respective retention time, molecular mass, molecular formula and their respective percentage in Table 1. The chemical structures of major components of the

Sarasvata churna volatile oil were presented in Figure 2. There are scientific evidences that show that the components of Sarasvata churna volatile oil posses significant antioxidant, anticonvulsant and other neuroprotective activity which further strengthens the scientific basis of this ancient antiepileptic formulation. Many components of the volatile oil like β-Caryophyllene (Oliveira *et al*., 2016), β-Asarones (Kaushik *et al*., 2017b; Jayaraman *et al*., 2010), Copaene, α-Sesquiphellandrene and gingerols (Hussein *et al*., 2015) α-pinene, p-cymene (Talita *et al*., 2015), γ-terpinene etc were present in significant concentrations which contributes to antiepileptic potential of Sarasvata churna.

Table 1: Sarasvata churna volatile oil composition.

S. No.	Compound name	R.T	Area %	Molecular formula	Molecular Mass
1.	α-Pinene	4.06	4.13	$C_{10}H_{16}$	136
2.	o-cymene	4.69	4.84	$C_{10}H_{14}$	134
3.	γ-terpinene	5.23	5.38	$C_{10}H_{16}$	136
4.	p-Menth-1-en-4-ol	7.54	0.50	$C_{10}H_{18}O$	154
5.	4-(1-methylethyl) 1,3-Cyclohexadiene-1-methanol	7.83	0.85	$C_{10}H_{16}O$	152
6.	3,3-dimethylcyclohexylidene) Acetaldehyde	8.62	3.94	$C_{10}H_{16}O$	152
7.	2-methyl-3-phenyl Propanal	9.00	18.29	$C_{10}H_{12}O$	148
8.	2-Caren-10-al	9.89	8.87	$C_{10}H_{14}O$	150
9.	3-Caren-10-al	9.98	5.66	$C_{10}H_{14}O$	150
10.	3-Allyl-2-methoxyphenol	11.14	4.02	$C_{10}H_{12}O_2$	164
11.	Copaene	11.63	0.72	$C_{15}H_{24}$	204
12.	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl, [1S(1à,2á,4á)]	11.89	0.50	$C_{15}H_{24}$	204
13.	Caryophyllene	12.59	1.96	$C_{15}H_{24}$	204
14.	1,2-dimethoxy-4-(2-propenyl) Benzene	13.16	0.91	$C_{11}H_{14}O_2$	178
15.	2-methyl-6-p-tolyl-2-Heptene	13.77	1.38	$C_{15}H_{22}$	202
16.	Humulane-1,6-dien-3-ol	14.02	2.90	$C_{15}H_{26}O$	222
17.	α-Selinene	14.18	4.99	$C_{15}H_{24}$	204
18.	6-methyl-2-(4-methyl-3-cyclohexen-1-yl)-, (S)-(-)-1,5-Heptadiene	14.32	1.56	$C_{15}H_{24}$	204
19.	2-methoxy-4-(2-propenyl) acetate Phenol	14.45	1.18	$C_{12}H_{14}O_3$	206
20.	α-sesquiphellandrene	14.68	0.92	$C_{15}H_{24}$	204
21.	Epiglobulol	14.89	7.09	$C_{15}H_{26}O$	222
22.	γ-Asarone	15.38	0.79	$C_{12}H_{16}O_3$	208
23.	Caryophyllene oxide	15.88	1.01	$C_{15}H_{24}O$	220
24.	β-Asarone	16.45	10.09	$C_{12}H_{16}O_3$	208
25.	1-α-Cadin-4-en-10-ol	17.10	0.75	$C_{15}H_{26}O$	222
26.	α-Asarone	17.51	1.90	$C_{12}H_{16}O_3$	208
27.	Guai-1(10)-en-11-ol	17.82	0.74	$C_{15}H_{26}O$	222
28.	Eudesma-5,11-(13)-dien-8,12-olide	21.74	2.14	$C_{15}H_{20}O_2$	232
29.	Eudesma-5,11-(13)-dien-8,12-olide	22.39	1.39	$C_{15}H_{20}O_2$	232
30.	8,8-Dimethyl-2H, 8H-pyrano[3,2-g] Chromen-2-one	23.28	0.59	$C_{14}H_{12}O_3$	228

HPTLC fingerprinting of the polyherbal formulations is one of the important parameters for accurate and scientific standardization. HPTLC fingerprints of Sarasvata churna were developed using standard procedure. The TLC fingerprinting of methanolic extracts shows well-separated bands with good resolution in the solvent system Toluene:Ethylacetate:Methanol (7.5:2.5:0.5 v/v/v) as depicted in the Plate A, B, and C of Figure 3. More clear, separated and colored bands were observed after derivatizing the plates with Natural Product Reagent and Sulphuric acid Anisaldehyde reagent as clearly observed in plate D and plate E of Figure 4 respectively which also indicated the presence of 12 prominent bands in the plate D and 07 in plate E. The spot color and Rf values of major bands on plate D and plate E were given in Table 2 and Table 3 respectively. The fingerprinting method thus developed was easy and reproducible.

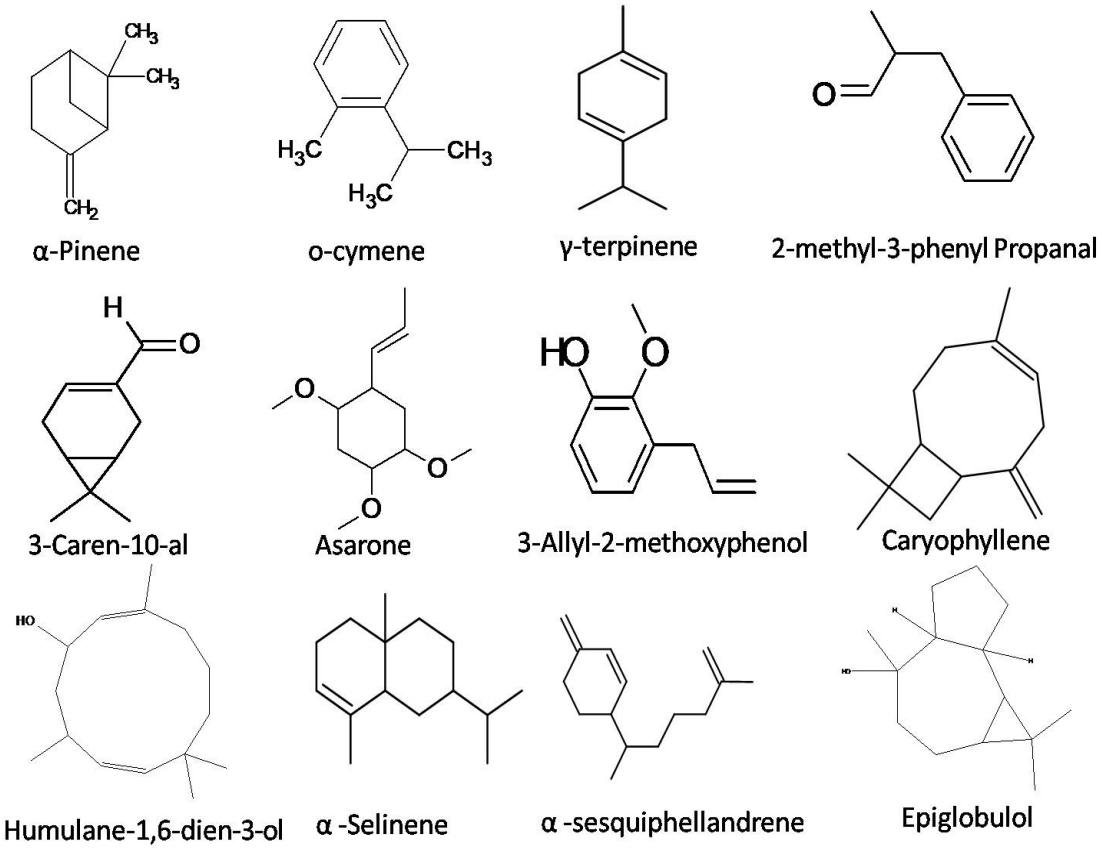

Fig. 2: Chemical structures of major components of Sarasvata churna volatile oil.

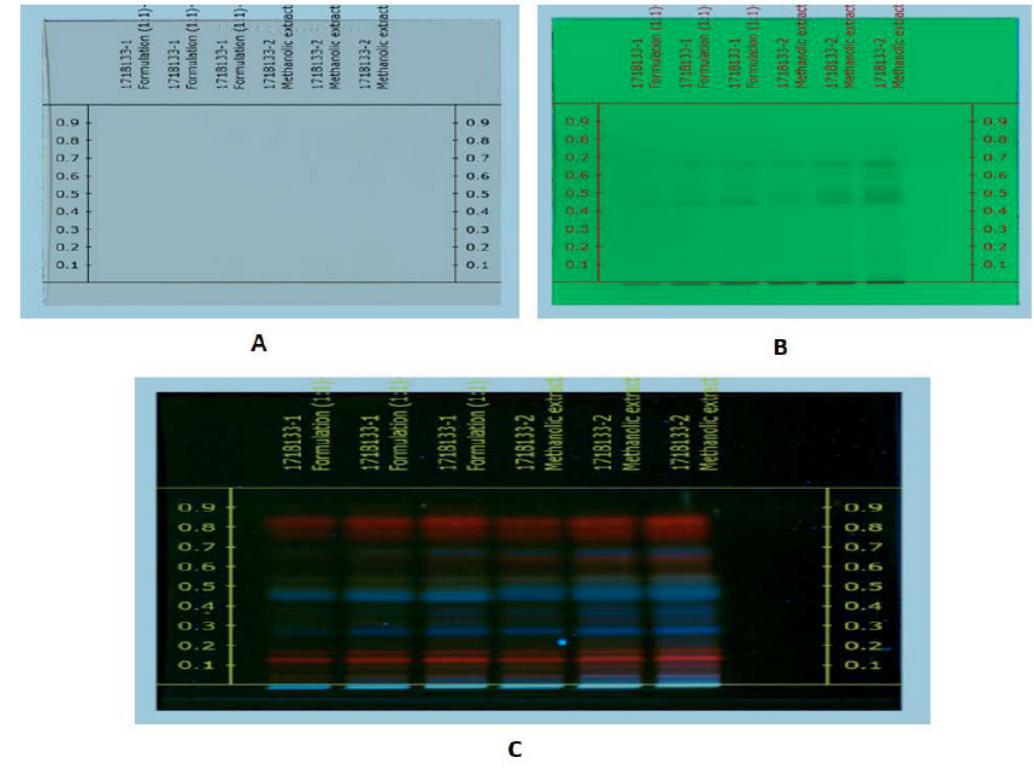

Fig. 3: TLC Fingerprints of Sarasvata churna methanolic extracts in Normal light (A), Ultraviolet 254 nm (B) and 366 nm (C) without Derivatization.

HPTLC Fingerprints of Sarasvata Churna simultaneously with reference compounds were also developed in solvent system Ethylacetate:Glycial acetic acid:Toluene:Water (4:1:1:0.5 v/v/v/v). The chromatogram thus developed is presented in plate F, G, and H in Figure 5. The figure clearly shows the presence of the chromatographic pattern of reference compounds in the test samples as seen in the formulation and methanolic extract track. Furthermore clear and separate colored bands were developed after derivatizing the plates with Sulphuric acid Anisaldehyde reagent as seen in Figure 6. Bacosides on Track 4 and 5 with Rf value of 0.07, Piperine on Track 7 and 8 with Rf value 0.77 and Withanolides on Track 9 and 10 with Rf value 0.57 of the major band along with additional fine bands can be easily traced in the Track 1, 2, 3, and 4 of Sarasvata churna as seen in the derivatized plate I and J. Peak display chromatogram of the Sarasvata churna as indicated in Figure 7 confirms the presence of well identifiable peaks for Bacosides, Piperine and Withanolides at Rf values of 0.07, 0.77, and 0.57, respectively. The matching of a chromatographic pattern of reference compounds in the formulation's chromatographic pattern confirms their presence in the formulation thereby establishing the chemical standardization method for the formulation using HPTLC. This method for developing fingerprints of Sarasvata churna with maximum separation and clear bands was simple, easy, reliable and reproducible. All the reference compounds like Bacosides (Paturu *et al*., 2013; Tripathi *et al*., 1996), Piperine (Mishra *et al*., 2015; Syed *et al*., 2015) and Withanolides (Kulkarni *et al*., 2008; Candelario *et al*., 2015) itself possess significant antioxidant, anticonvulsant and neuroprotective activity. Their presence in the Sarasvata churna potentiates the claim of this ancient Ayurvedic formulation to be useful in the management of epilepsy and other brain-related disorders.

Table 2: Observations and Rf values in Plate D on Track 5 & 6.

S. No.	Spot Color	Rf Value
1.	White	0.01
2.	Creamy white	0.07
3.	Light red	0.11
4.	Red	0.15
5.	Red	0.17
6.	Blue	0.29
7.	Blue	0.49
8.	Blue	0.55
9.	Reddish-blue	0.64
10.	Blue	0.69
11.	Dark red	0.77
12.	Pink	0.84

Table 3: Observations and Rf Values in Plate E on Track 6.

S. No.	Spot Color	Rf Value
1.	Black	0.14
2.	Navy blue	0.45
3.	Light blue	0.49
4.	Black	0.56
5.	Light brown	0.68
6.	Black	0.77
7.	Light purple	0.91

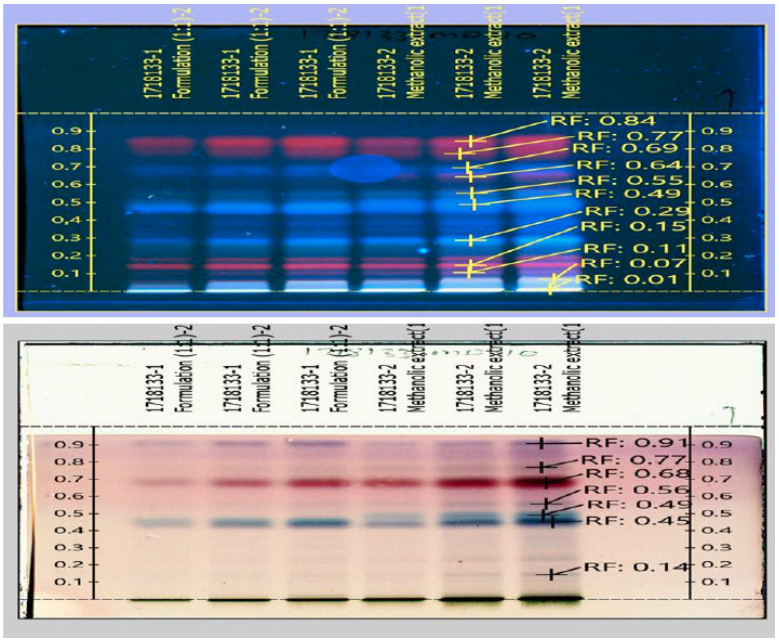

Fig. 4: Derivatized plate in Natural Product Reagent in Ultraviolet light 366 nm (D) and derivatized plate in Sulphuric acid Anisaldehyde reagent in Normal light (E) with Rf values of isolated spots.

CONCLUSION

The GC-MS and HPTLC fingerprints thus obtained will be useful in the standardization of Sarasvata churna and other polyherbal formulations containing similar ingredients. The method of HPTLC analysis developed for simultaneous analysis of biomarkers in the Sarasvata churna will help in establishing the

identity, purity, quality, safety, and efficacy of Sarasvata churna. These standards can be used by various herbal drug industries and laboratories engaged in research and production of herbal formulations to control the quality of their products and help in maintaining and assuring batch to batch consistency so that maximum therapeutic efficacy can be achieved.

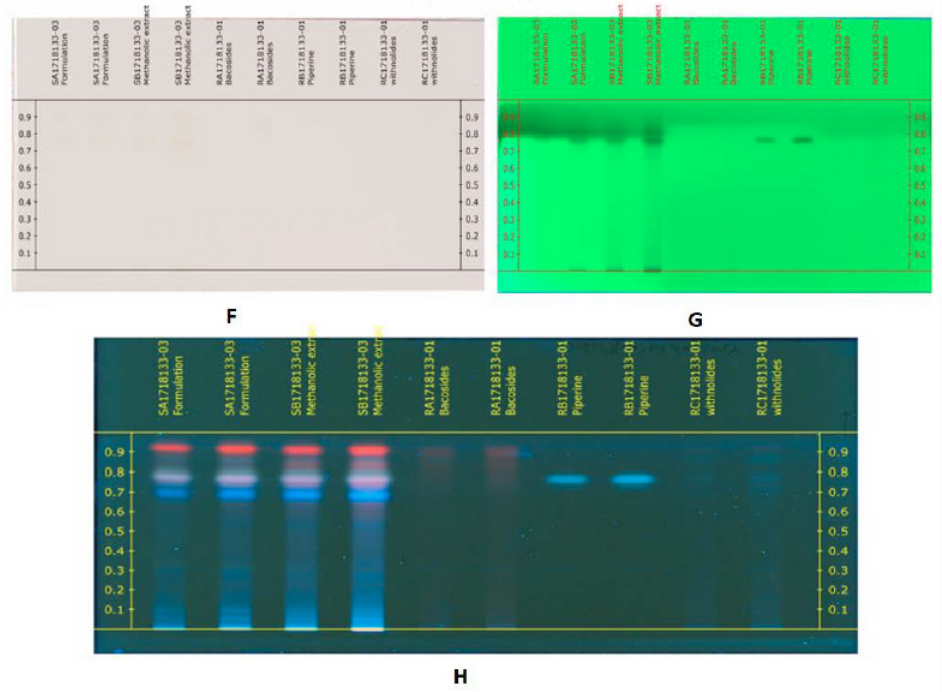

Fig. 5: TLC Fingerprints of Sarasvata churna methanolic extracts and reference compounds Bacosides, Piperine and Withanolides in Normal light (F), Ultraviolet 254 nm (G) and 366 nm (H) without Derivatization.

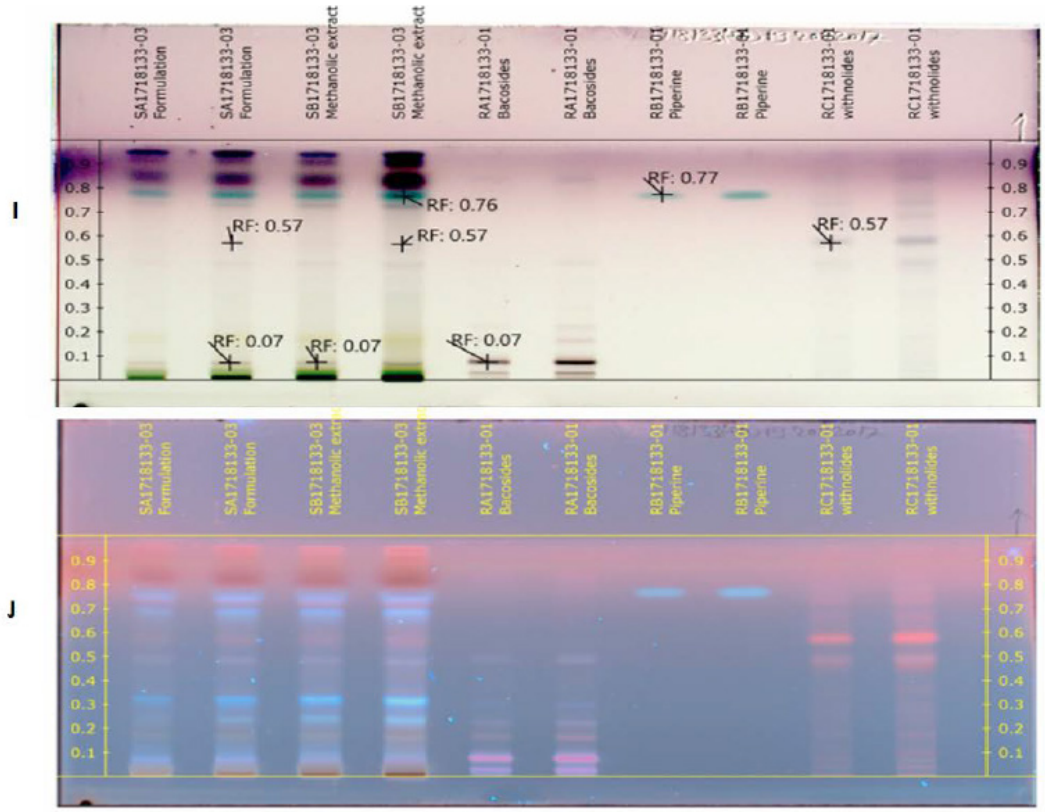

Fig. 6: TLC Fingerprints of Sarasvata churna methanolic extracts and reference compounds Bacosides, Piperine and Withanolides in Normal light (I) and Ultraviolet light at 366 nm (J) after Derivatization with Sulphuric acid Anisaldehyde reagent with Rf values.

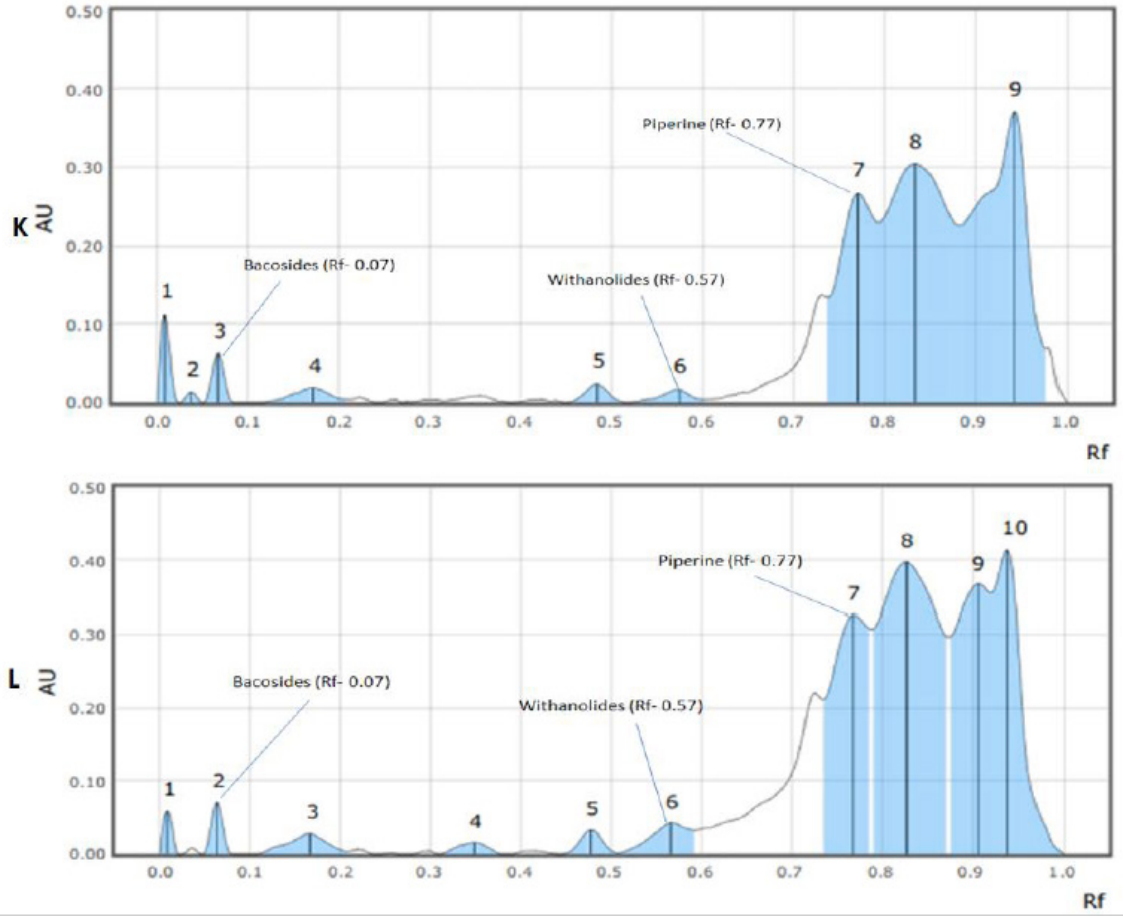

Fig. 7: Chromatogram of HPTLC analysis of Sarasvata churna formulation and methanolic extract with peak display for reference compounds with corresponding Rf values.

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CONFLICT OF INTEREST

Authors have no conflict of interest.

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