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Research Article

HPTLC FINGERPRINTING ANALYSIS OF SHILAJIT: AN AYURVEDIC HERBO-MINERAL DRUG

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ABSTRACT

Introduction: Shilajit (Black bitumen: *Asphaltum punjabinum*) is an important herbo-mineral drug in Ayurvedic system of medicine. It is a blackish brown exudate which oozes out from sedimentary mountain rocks. Due to its high cost, increased demand and difficulty in getting pure forms, adulteration of shilajit is common in raw drug industry. So quality control parameters are necessary to scrutinize genuine shilajit. **Objectives:** The present work aims to develop simple quality control parameters of shilajit in terms of physico- chemical properties and HPTLC (High Performance Thin Layer Chromatography) fingerprinting. **Materials & Methods:** Microscopic evaluation, flame test and organoleptic evaluation of shilajit were done to confirm the identity. Physico-chemical parameters such as specific gravity, pH, refractive index, loss on drying, total ash, acid-insoluble ash, solubility, fluorescence analysis and qualitative analysis were carried out. Moreover, HPTLC fingerprint was developed using Camag HPTLC instrument and detected its presence in a pharmaceutical preparation. **Results:** Physico-chemical parameters of shilajit were established and HPTLC chromatogram was developed with hydro-alcoholic extract of shilajit using the mobile phase Toluene: Ethyl acetate. This HPTLC method was also proved successful in confirming its presence in Chandraprabha gudika. **Conclusion:** The present study on identification, physico-chemical evaluation and HPTLC fingerprinting of shilajit provides useful information regarding quality control parameters and identifying parameters to substantiate and authenticate the drug and could be used for comparison of market samples to ensure its genuineness. A simple, specific and accurate HPTLC method was validated for its fingerprint analysis and detection in a pharmaceutical preparation, Chandraprabha gudika.

Keywords: Shilajit, *Asphaltum punjabinum*, HPTLC, Physico-chemical parameters.

INTRODUCTION

Shilajit (Black bitumen: *Asphaltum punjabinum*) is an important herbo-mineral drug in Ayurvedic system of medicine. Early Ayurvedic literatures, Caraka Samhita and Susruta Samhita describe it as a cure for all diseases as well as a 'Rasayana' (rejuvenating and anti-ageing)[1,2]. Rasaretna samuchaya, an Ayurvedic text on metals and minerals has been included shilajit as one among 'maharasa drugs' (a group of alchemical mineral agents having rejuvenating property)[3] Therapeutic uses focus on diabetes, diseases of urinary tract, edema, skin diseases, hemorrhoids, liver diseases, sexual dysfunction, geriatric problems, gastro-intestinal infections, epilepsy, renal calculi and chronic respiratory diseases[1,2]. Most of the biological effects of Shilajit and its bioactive compounds are scientifically proven by pharmacological screening. Some of them are anti-ulcerogenic property, mast cell protecting effect in bronchial asthma, anti-diabetic effect, learning and memory enhancing effects, cholinergic inhibiting property, anti-stress and adaptogenic effects, anti-anemic property, blood platelet anti-aggregating effect, anti-craving property, immunomodulatory effect, effects on male infertility, development and protection of embryo, female sexual dysfunction and libido enhancement[4].

Shilajit is a pale brown to blackish brown exudate of variable consistencies which oozes out from sedimentary mountain rocks in the peak summer months at altitudes between 1000-5000m and it is widely distributed in India[3,4]. It is a paleo-humus mixture with marine invertebrate and plant fossils being the major contributors and is formed by the decomposition of soil microorganisms. Shilajit is one such complex mixture of humic substance used widely for its strengthening and rejuvenating qualities. Chemically, shilajit is composed of three primary chemical units; non-humic organic compounds- DBP (Dibenzo-α pyrones) and DCP (dibenzo-α pyrone chromoproteins) and metallo-humates like fulvic acid and humic acid. It also contains benzoic acid, hippuric acid, their salts and gums, albuminoids, traces of resins and fatty acids; proteins like, glycine, proline, hydroxyproline, threonine and metal salts. DCPs and DBPs are the two unique compounds in shilajit which are potent Immunomodulators and antioxidants. Humic substances are used in medical science with substantial benefits in improving health status[4]. The major beneficial effects of shilajit are as simulator of bioenergetics like ATP and creatine synthesis, immune-modulator, Captivators of ROS (Reactive Oxygen Species) and RNS (Reactive Nitrogen Species), scavengers and chelators of loose metal ions[4].

Pharmaceutical products of shilajit are subjected to some processing, but the purity among samples varies, thereby therapeutic efficacy also differs. Due to its high cost, increased demand, scanty availability and difficulty in getting pure form, commercial samples of shilajit are often adulterated with clay particles, Quercus gum, resins and cow's urine. Hence purification and quality analysis of Shilajit are an imperative necessity before it is recommended for therapeutic use. A large spectrum of modern scientific methods of isolation, purification and standardization of this strongly potent organic-mineral drug has been developed already, but most of these methods, including ESR (Electron Spin Resonance) spectra and GC-MS (Gas chromatography- Mass Spectrometry) need an expensive and sophisticated instrumentation with extensive care[4]. So, there is a

need for development of simple quality control parameters which could help pharmaceutical manufacturers to scrutinize good sample.

Shilajit is also used to prepare pharmaceutical preparations in Ayurvedic system of medicine such as Siva gulika[5], Chandraprabha gudika, Kanmada bhasma and Prabhakara vati[6]. Among these, Chandraprabha gudika is commonly prescribed by Ayurvedic Physicians mainly for disorders of Urinary system[6]. Considering the importance of ensuring the quality of medicines, scientific reports assessing the quality of Ayurvedic formulation, Chandraprabha gudika are not available in the current scientific literature. Hence this study aims to establish simple quality control parameters of shilajit in terms of physico chemical analysis and HPTLC fingerprinting and qualitative detection of shilajit in a pharmaceutical preparation; Chandraprabha gudika by high performance thin layer chromatography method.

MATERIALS & METHODS

Material

Genuine sample of shilajit was collected from Ludhiana, Punjab (Figure 2A). A voucher specimen was retained in Drug museum of Drug Standardization unit, Govt. Ayurveda College, Thiruvananthapuram. Chandraprabha gudika was purchased from The Pharmaceutical Corporation (IM) Kerala Ltd (Batch No: P161817).

Identification and Authentication

Identification and authentication of shilajit was done using organoleptic features and flame test as per Rasa Retna samuchaya[3].

Microscopical evaluation: A smear of shilajit was prepared and observed under 10x and 40x magnifications. Microphotographs were made by using Olympus Microscope (Model CX 41; Tokyo, Japan) with CCD camera 2 mega pixels.

Chemicals and Reagents

All the chemicals and solvents used in study were procured from Merck, India and were of HPLC/ chromatographic grade. Precoated silica gel $60F_{254}$ TLC plates were purchased from Merck, Darmstedt (Germany).

Physico-chemical analysis

Solubility, Total ash, Acid insoluble ash, Loss on drying, pH, Specific gravity and Refractive index were determined using standard methods as per API[7] (Ayurveda pharmacopoeia of India) guidelines and was carried out at Drug Standardization unit, Govt. Ayurveda College, Thiruvananthapuram.

Preliminary qualitative phytochemical analysis

Qualitative tests for the presence of alkaloids, tannin, saponin, protein, steroids, flavonoid, phenol, and sugar were carried out[8].

Fluorescence analysis: The fluorescence property of shilajit extracts taken in different solvent systems was analyzed under UV light (254nm and 366nm)[9].

Heavy Metal Analysis: Atomic absorption Spectroscopy was used to determine the heavy metal elements and some nonmetal elements in atomic state. Determination of Lead, Copper, Zinc and Nickel in ppm levels in shilajit samples were determined using Atomic absorption Spectrophotometer (Software Solar AA, 11.01 version, Thermo-scientific iCE 3500 series, United States).

HPTLC fingerprinting (High Performance Thin Layer Chromatography)

One gram of Shilajit was weighed and ammonia, aqueous, 50% methanolic extracts were prepared. HPTLC was done (CAMAG, Switzerland) using $60F_{254}$ TLC plate, keeping in TLC twin trough developing chamber (after saturation with solvent vapor) with respective mobile phase Toluene: Ethyl acetate in the ratio of 4:6 (after sprinkling three drops of Acetic acid) up to 70 mm. The developed plate was dried and kept in Photo-documentation chamber (CAMAG REPROSTAR 3). The plate was fixed in scanner stage (CAMAG TLC SCANNER 3) and scanning was done at 254 nm and 366nm. The software used was WinCATS 1.3.4 version.

Qualitative detection of shilajit in Chandraprabha gudika

Chandraprabha gudika was finely powdered in a porcelain mortar. One gram of powder was weighed, and methanol extract was prepared by refluxing at 80^0c and HPTLC was done by spotting shilajit and Chandraprabha gudika. The method was validated using the following parameters; specificity, precision, repeatability, intermediate precision, and robustness[10].

Table No.1 Organoleptic features of Shilajit

Organoleptic features	Results
Color	Tarry black
Odor	Smell of cow's urine
Taste	Slightly bitter, pungent and salty
Consistency	Waxy, Semisolid and thread forming
Transparency	Opaque
Luster	Shiny
Pliability	Melts in hands becoming sticky

Table No.2 Physico-chemical parameters of shilajit.

Physico-chemical parameters	Results
Total ash	6.22%
Acid insoluble ash	Nil
Refractive index	1.648
pH (10% solution)	4.75
Specific Gravity (1% solution)	1.003
Water soluble extractive value	96.5%
Hydro-methanolic soluble extractive value(water: alcohol in 1:1 ratio)	74%
Alcohol soluble extractive value	2%
Loss on drying	11-15%
Solubility in (A)Water (B)Alcohol (C)Acetic acid, HCl, H_2SO_4	 Fairly soluble Insoluble Slightly Soluble

Table No.3 Results of qualitative chemical evaluation of shilajit.

Chemical constituents	Name of the test or Reagents	Results
Alkaloids	Dragendroff's reagent	Absent
Tannin	Lead acetate	Present
Saponin	Foam test	Traces present
Protein	Xanthoprotein test	Present
Steroids	Libermann Burchard's test	Absent
Flavonoid	Shinoda test	Present
Phenol	Ferric chloride test	Present
Sugar	Benedict's reagent	Present

Table No.4 Fluorescence analysis of shilajit in different solvents

Solvent	Under UV (256nm)	Under UV (366nm) (Color & Intensity)
Aqueous	Mild yellow	Yellowish green
Con.HNO_3	Mild yellow	Yellow
Acetic acid	Mild yellow	Yellowish green
HCl	Bluish yellow	Blue
Con. H_2SO_4	Mild blue	Bluish yellow
NaOH	Bluish yellow	Blue

Table No.5 Heavy metal analysis of shilajit

Heavy metals	Shilajit (ppm)
Cadmium	0.0026
Lead	0.2967
Copper	0.1975
Iron	5.3567
Zinc	0.2661
Nickel	0.0138

Table No.6 Peak table of the tracks

Ammonia extract of Shilajit: 6 peaks		Aqueous extract of Shilajit: 8 peaks		50 % methanolic extract of Shilajit: 9 peaks	
Rf values	Area (AU)	Rf values	Area (AU)	Rf values	Area (AU)
0.32	22862.4	0.15	1352.7	0.18	6439.6
0.46	3711.7	0.22	588.7	0.26	947.2
0.49	2294.1	0.26	217.3	0.37	2303.8
0.53	17295.1	0.35	4170.3	0.42	1015.1
0.64	38856.9	0.52	6201.2	0.51	1646.5
0.84	10407.4	0.58	12471.9	0.56	2331.1
		0.76	31612.6	0.62	10700.8
		0.84	23850.3	0.81	20759.4
				0.87	18259.9

Table no.7 Peak table

Shilajit; Rf values	Chandraprabha gudika; Rf values
0.12	0.19
0.22	0.43
0.36	0.57
0.40	0.69
0.43	0.73
0.51	0.77
0.57	0.84
0.70	0.92
0.84	0.96

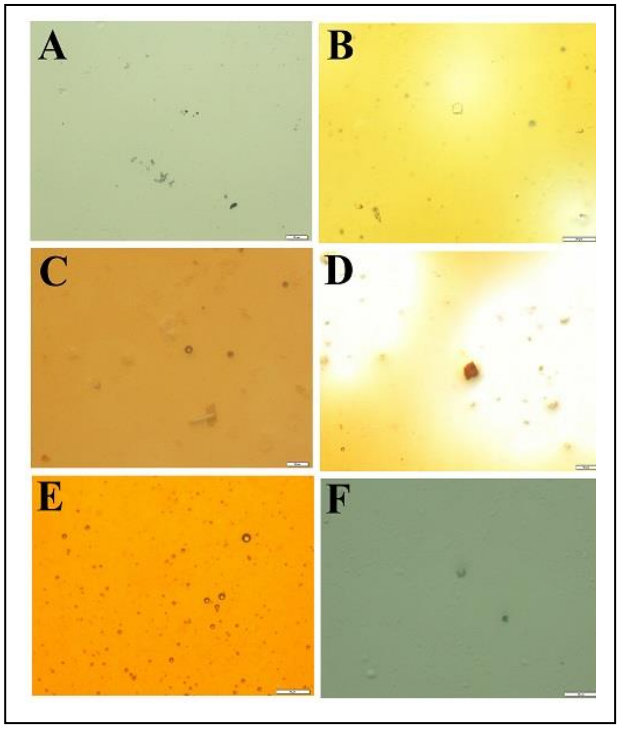

Figure.1 Microscopy of Shilajit (A)Aggregates of crystals ,10x (B) Crystals, 40x (C) Prismatic crystals of calcium oxalate and oil globules, 10x (D)Tannin stains, 10x (E), (F) Oil globules, 40x.

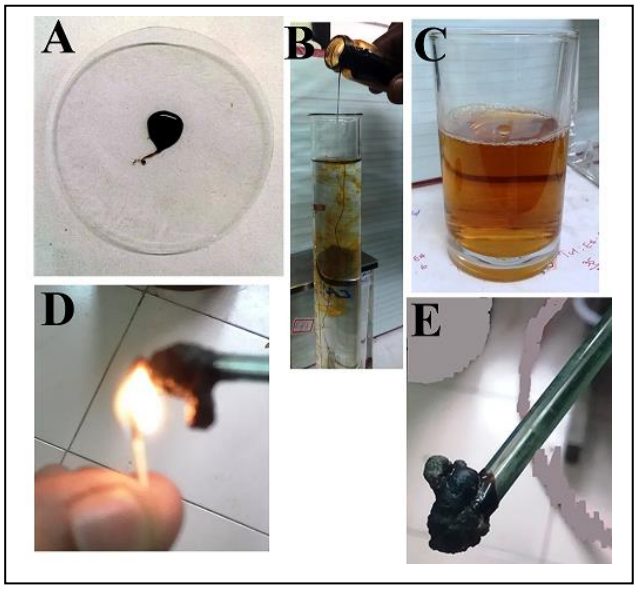

Figure 2. (A)Shilajit in semisolid form. (B) shilajit forming threads when poured into water. (C) Shilajit mixed with water, dissolves completely forming golden brown color liquid. (D)Shilajit burns with non-smoky flame. (E) Shilajit charred into a blackish residue.

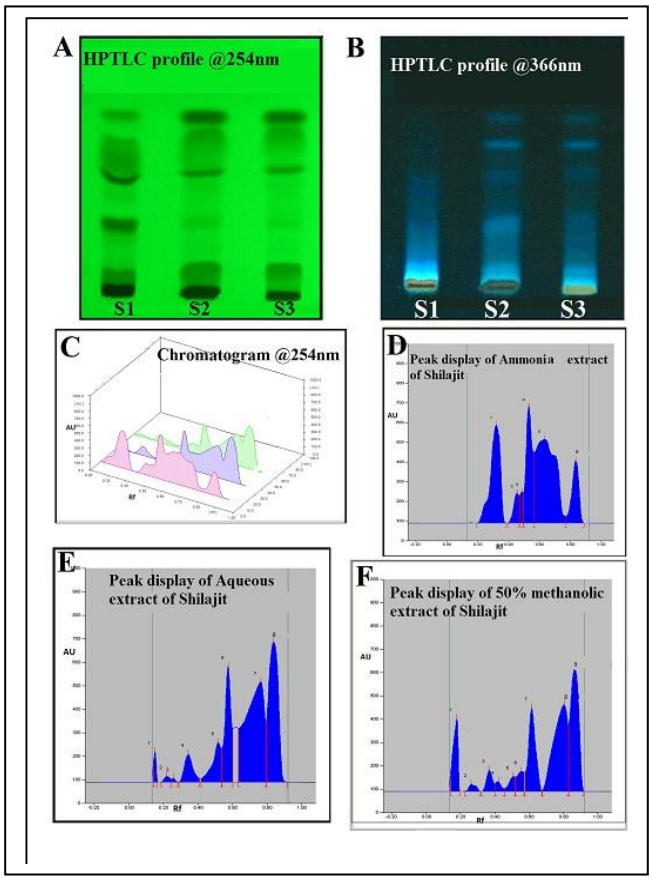

Figure.3. HPTLC profile (A) Photo documentation: HPTLC fingerprint of Ammonia, Aqueous and 50% methanolic extracts of shilajit @ 254nm. (B) HPTLC fingerprint comparison of Ammonia, Aqueous and 50% methanolic extracts of shilajit @366nm. (C) 3D display of HPTLC chromatogram @254nm. (D) Peak densitogram of Ammonia extract of Shilajit (E) Peak densitogram of aqueous extract of Shilajit. (F) Peak densitogram of 50% methanolic extract of Shilajit.
S1: Ammonia extract of shilajit; S2: Aqueous extract of shilajit; S3: 50% methanolic extract of shilajit.

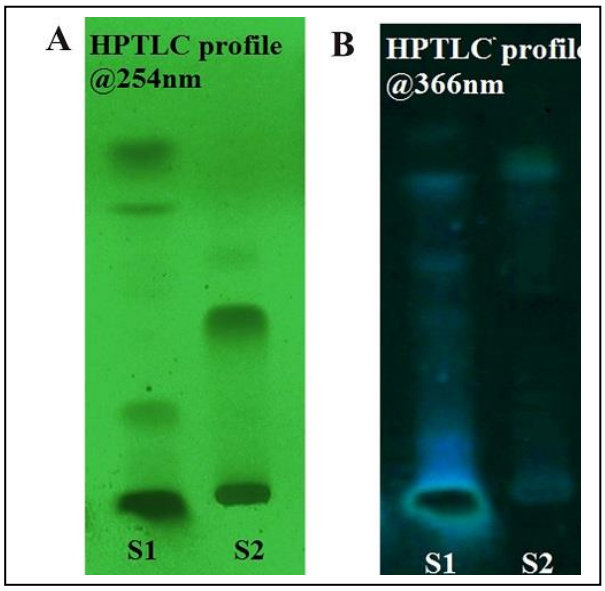

Figure.4. HPTLC fingerprint of shilajit and Chandraprabha gudika; S1: Shilajit extract, S2: Chandraprabha gudika extract. (A) at 254nm, (B) at 2366nm

RESULTS

Microscopical evaluation

Microscopy of shilajit revealed the presence of high amount of crystalline material**,** aggregates of crystals, tannin, prismatic crystals of calcium oxalate and oil globules. (Figure.1)

Organoleptic evaluation

The organoleptic features of Shilajit were evaluated and is shown in Table 1 and Figure 2.

Flame test

Shilajit was taken in a glass rod and shown to a flame[3]. It burnt with non-smoky flame and finally charred into a blackish residue. (Fig.2D & 2E)

Physico-chemical analysis

The results of physico-chemical analysis are shown in Table 2.

Preliminary qualitative phytohemical examination

The 50% methanolic extract of Shilajit was analyzed for the presence of different chemical constituents. Results of qualitative tests are shown in Table 3.

Fluorescence analysis

Specimens were recorded as fluorescent with color and intensity under UV light 256nm and 366nm and are presented in Table No.4.

Heavy Metal Analysis

The heavy metals lead, Copper, Zink and Nickel were found within permissible limits in all the samples. The results are shown in Table 5.

HPTLC

HPTLC fingerprints of shilajit in ammonia, water, and 50% methanol extracts were developed and compared using the solvent system Toluene: Ethyl acetate (4:6). Best separation with nine peaks was observed in 50% methanol extract. Two compounds were separated with almost same Rf values (0.53 and 0.84) in ammonia and aqueous extracts and four compounds were separated with nearly same Rf values (0.26, 0.35, 0.52 and 0.58) in aqueous and methanolic extracts. One compound having Rf value 0.53 was observed common in all the three extracts. This indicate that two compounds separated in ammonia and aqueous extracts may be similar and four compounds found common in aqueous and methanolic extracts may also be similar. The data of the analysis are as shown in Fig3 and Table 6.

Qualitative detection of Shilajit in Chandraprabha gudika

HPTLC fingerprints of hydromethanolic extracts of shilajit and Chandraprabha gudika were developed using the solvent system Toluene: Ethyl acetate (4:6). Similar compounds having Rf values 0.43, 0.57, 0.69 and 0.84 were present in shilajit and Chandraprabha gudika which confirms the presence of shilajit in Chandraprabha gudika. The results are shown in Figure 4 and Table 7.

DISCUSSION

Shilajit is a herbo-mineral drug which is used in Ayurvedic system of medicines especially as a Rasayana (Rejuvenator[1,2]. Moreover, it is the key ingredient of many Ayurvedic therapeutic formulations such as Siva gulika[5], Chandraprabha vati, kanmada bhasma and Prabhakara vati[6]. According to WHO guidelines of research and evaluation of traditional medicine, scientific research is needed to evaluate the safety and efficacy of traditional medicine[12]. The quality control of crude drugs and their bio constituents is of paramount importance in ensuring its therapeutic efficacy. Lack of quality control parameters will lead to sub-standard and adulterated drugs in commercial market[13].

Common adulterants of shilajit are resin, gum, cow's urine, coal, charcoal, fertilizers, heavy metals, burnt potato and ozokeritu (similar to humic substances without any medicinal property)[4]. Hence an accurate analytical method is essentially required for identification and authentication of shilajit before manufacturing pharmaceutical preparations.

Some sophisticated methods like ESR (Electron Spin Resonance) spectra and GC-MS (Gas chromatography- Mass Spectrometry) are developed for standardization of Shilajit[4]. But simple, cost-effective and reliable methods should be developed to ensure the identity and purity of crude drug in raw drug industry. The present study aims to establish identification and quality control parameters of shilajit which will help pharmaceutical manufacturers to prevent its adulteration in raw drug industry. Rasa retna samuchaya, an Ayurvedic medical literature on mineral and metallic drugs clearly describes the organoleptic features and flame test for proper identification of shilajit[3]. Besides microscopy was done which revealed the presence of crystalline structures, prismatic crystals of Calcium oxalate and oil globules (Figure.1). These organoleptic features (Table.1, Figure2), flame test and microscopy were sufficient to confirm the identity of shilajit.

The physico-chemical parameters such as total ash, acid insoluble ash, solubility, extractive values, pH, specific gravity, refractive index, loss on drying and qualitative chemical evaluation were carried out to ensure purity of Shilajit. Total ash value represents the presence of inorganic salts like calcium oxalate crystals found naturally in the drug and inorganic matter derived from external sources like sand[14]. Total ash value of shilajit was 6.22% because of the presence of mineral matters and metal salts comprising of Calcium, Sodium, Potassium, Iron, Copper, Manganese, Magnesium and Zinc[4]. The presence of impurities in the drug can be detected by evaluating acid insoluble ash. Hence the ash obtained was treated with acid so that most of the natural ash dissolves in acid leaving silica like impurities. The acid insoluble ash of shilajit was nil due to the absence of silicaceous matter (Table No.2).

Shilajit was acidic in nature with pH value 4.75 and its specific gravity was 1.003. The presence of adulterant in a drug can be detected by solubility studies[9]. Pure shilajit is 98-100% soluble in water (Fig.2C) meanwhile only non-humic organic compounds of 8-20% are soluble in common organic solvents[4]. Shilajit completely dissolved in water yielding an extractive value of 96.5% which points out to the absence of physical impurities. The presence of coal like impurities could be detected by water solubility study. 50% methanolic extract was taken for qualitative evaluation and HPTLC which yielded an extractive value of 74%. It is slightly soluble in acidic solutions like Acetic acid, HCl and H_2SO_4.

Qualitative chemical examination revealed the presence of tannin, saponin, protein, flavonoid, phenol and sugar. Amino acids mainly arginine and lysine and simple proteins like histone and protamine were present in Shilajit as per Ghoshal[4].

The use of fluorescence analysis is very useful adjunct to microscopic evaluation, as it can be applied as rapid and easy test to verify certain identifications of the botanicals. The fluorescence property of the drug taken in different solvent systems were analyzed under UV light (at 254nm and 366nm) as shown in Table 4.

Herbal preparations and botanical extracts are complex matrixes due to the presence of multiple components. With the advancement, the modern high-performance TLC method is an efficient instrumental way to evaluate the herbal drugs and extracts qualitatively as well as quantitatively[13].It is a rapid and reliable method frequently used for identification and authentication in quality control of herbal medicines[9]. Hence, HPTLC profiling of shilajit was developed for further authentication and identification of chemical bio active compounds present in it and it will help to detect the adulterated commercial samples.

Analytical methods for the detection of shilajit in pharmaceutical preparations were not reported yet. In this study, HPTLC fingerprint is used to confirm the presence of shilajit in pharmaceutical preparation, Chandraprabha gulika.. Further, the HPTLC method is validated for specificity, precision, repeatability, intermediate precision, and robustness as per ICH guidelines.

CONCLUSION

The present study on identification, physico-chemical parameters and HPTLC fingerprinting of shilajit provides useful information for quality control analysis which will help to confirm its identity and purity in crude form in raw drug industry. A simple, specific, accurate and rapid high-performance thin layer chromatographic method was validated for the fingerprint analysis and detection of shilajit in a pharmaceutical preparation, Chandraprabha gulika. These can be considered as identifying parameters to substantiate and authenticate the drug and could be used for comparison of market samples to ensure genuineness of shilajit. From the study, it is evident that HPTLC method can be used in the analysis of adulteration in single as well as combined products of shilajit.

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