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Research Article

PHYTO-PHARMACEUTICAL ASSAY OF SHILAJATU RASAYANA - A NOVEL COMPOUND IN THE MANAGEMENT OF DIABETIC POLYNEUROPATHY

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Abstract

Diabetes Mellitus is one of the major lifestyle disorders of this century as declared by WHO. Diabetic Sensorimotor Peripheral Polyneuropathy is manifested as a microvascular complication of Diabetes Mellitus. There is no direct reference in Ayurvedic classics regarding the nomenclature of this. But analyses of the lakshana samprapti of the disease, exhibits manifestation of vataja symptoms as individually or as a resultant of anya or anyonya avarana. At this point the wise application of rasayana and vatahara drugs is best. The given combination serves the purpose of both, thereby terminating the disease samprapti. With an objective to provide considerable relief to this burning health problem, the application of shilajatu rasayana compound was proposed. This compound was analyzed and standardized through basic phyto-pharmaceutical parameters. In an attempt to acquire pure shilajatu (Black Bitumen / *Asphaltum punjabianum*), efforts were made to obtain Nepal shilajatu which is generally spoken as the best. The quantitative analysis of humic substances in shilajatu rasayana compound was carried out with reference to Fulvic acid (as standard) by UV spectrophotometer and Gravimetric Methods; which was specially designed here. Lignin decomposition logic has been attempted using UV & chromatographic assay. Chromatography was performed as per Certified Reference Material. Results showed the shilajatu rasayana compound contained high purity and greater percentage of fulvic acid along with the other ingredients used for bhavana. The data may be used as a reference parameter for purity analysis of processed shilajatu containing drugs.

Key words: Diabetic Polyneuropathy; Shilajatu Rasayana; Ayurveda; Phyto-pharmaceutical assay.

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INTRODUCTION

Diabetes is one among the three major lifestyle disorders of this century as declared by World Health Organisation. Diabetic Sensorimotor Peripheral Polyneuropathy (DSPN) being a common microvascular complication of diabetes affects 16% of patients with diabetes.[1] The metabolic and microvessel alterations to vasa nervorum (small blood vessels that supply the nerves) are as a result of chronic hyperglycemia exposure and cardiovascular risk covariates are the major culprits.[1] Even though there is no direct reference in Ayurvedic classics regarding the nomenclature of this. But analyses of the lakshana samprapti of the disease, exhibits manifestation of vatic symptoms as individually or as a resultant of anya or anyonya avarana. At this point the wise application of rasayana and vatahara drugs is best. The shilajatu rasayana compound (SRC) serves the purpose of both, thereby terminating the disease samprapti, also helps in nourishing the cells with glucose by the catalytic action of shilajatu. In Ayurveda, shilajatu (Black Bitumen / *Asphaltum punjabianum*) and rasayana drugs are clinically found effective in any sort of pathology were multifaceted symptom exhibits; either as a result of vata alone or by its avarana.

This is an effort to counteract the pathology complex by proposing this novel compound in capsule form which will ease oral administration and also to establish its quality parameters too. A research on DSPN was previously done with Dashamoola Rasayana Compound (DRC).[2] The basis of this formula was from Vagbhata, with slight modifications as per senior physicians.[3] This drug was further modified by adding shilajatu to prepare SRC, thus establish the added effect of shilajatu in patients of DSPN. The present study aims at assessing the guinuinity of the drug and purity assessment of procured shilajatu.

AIMS AND OBJECTIVES

To evaluate Pharmacognostical and Phytochemical analysis of Shilajatu Rasayana Compound a novel drug in management of DSPN.

MATERIALS AND METHODS

The study involved following operating procedures:

- Collection, Identification and authentification of raw drugs.
- Preparation of drug at Pharmacy.
- Phytochemical analysis of compound drug.

A. Collection, identification and authentification of raw drugs

The raw drugs for the study were procured from the pharmacy of I.P.G.T. & R.A., Gujarat Ayurved University, Jamnagar. The shoditha shilajatu was procured from Nepal. The ingredients and the parts used are given in Table 1.

The raw drugs were identified and authenticated by the department of Pharmacognosy, I.P.G.T. & R.A., Gujarat Ayurved University, Jamnagar. The identification was carried out based on the morphological features, organoleptic features and powder microscopy of individual drugs. The API (Ayurvedic Pharmacopeia of India) standards were used for authentification.[4] The ingredients of SRC were indentified pharmacognostically.

In the present work suddha shilajatu was procured from Nepal itself to maintain the quality standard of drug. As Shilajatu is said as the miracle of Himalayas, found mainly in the pristine mountains of Nepal altitudes between 2500-5000 m.[5] And Shilajatu found in Nepal is considered as the best and is also acclaimed for a superfluity of highly potential

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medicinal herbs.[6] Shilajatu in its raw form contains free radicals and may also contain mycotoxins and fungal toxins. The processing needs to remove the free radicals, Polymeric Quinone radicals, toxins, mycotoxins and inactive ingredients. Only the standardized extract gives the desired benefits of shilajatu.[7] For the present work, readymade sample of shilajatu which had been processed with triphala qwatha (Decoction of *Terminalia chebula, Terminalia bellerica, Emblica officinalis*) and cow's milk was obtained. The powder microscopy of SRC was also done.

B. Preparation of the drug

The ingredients enlisted from 1 to 4 (Table 1) were made into a fine powder and sieved through mesh no. #80. The ingredients are mixed well in equal quantity in mass mixing machine till a homogenous mixture was obtained. This mixture was transferred in electric mortar and pestle and shodhita shilajatu (readymade material from the Nepal market was added to the ingredients) which was in black semi liquid form was added. The above mixture was given two bhavana (trituration) with Dashamoola kwatha, which was prepared as per classical kwatha kalpana procedure.[8] The obtained drug was in the form of thick blackish brown paste, which was dried in shade. After proper drying of the mixture, it was finely powdered and filled into capsules of 500 mg each.

C. Phytochemical assay of SRC

SRC was analyzed by using qualitative and quantitative parameters at Pharmaceutical chemistry laboratory of I.P.G.T. & R.A., Gujarat Ayurved University, Jamnagar.

Parameters like loss on drying, ash value, acid insoluble ash, water soluble extractive, and methanol soluble extractive and qualitative tests for different functional groups were assessed.

High Performance Thin Layer Chromatographic study (HPTLC)

HPTLC was carried after Thin layer chromatography (TLC). HPTLC has developed from classical TLC, offers greater separation efficiency, greater sensitivity and reproducibility, accurate quantification and automation. Methanol extract of SRC prior to HPTLC solvent system was fixed through TLC. Then extract of SRC and Fulvic acid (standard) were spotted on pre-coated silica gel GF 60_{254} alluminium base plate as 5 mm bands, 5 mm apart and 1 cm from the edge of the plates, by means of Camag Linomate V sample applicator fitted with a 100 µL Hamilton syringe. 10 ml of 25% ammonium Hydroxide: n-propanol (7:3 v/v) was used as a mobile phase. The development distance was 6.4 cm (development time 30 min). After development, densitometric scanning was performed with a camag TLC scanner III in reflectance absorbance mode at 254 nm and 366 nm under control of win CATS software (V 1.2.1 camag). The slit dimensions were 6 mm× 0.45 mm and the scanning speed was 20 mm s-1.

UV Spectrophotometry

There is no any accepted method to quantify fulvic acid, which is practical and cost effective. Hence it was aimed to evaluate and develop a spectro-photometric fulvic acid quantification protocol for future laboratory analysis of shilajatu containing drugs. UV spectrophotometry was carried out to evaluate the quantity of fulvic acid present in the SRC compared to the reference standard fulvic acid.

The alkaline extract of SRC was prepared, after suitable dilution of one gram of SRC with 50ml of 0.5 Normal NaOH (Sodium hydroxide). It was kept overnight. Next day the supernatant was collected, and was scanned through 200–800 nm in a Shimadzu UV-visible double beam recording

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Table 1: Showing the ingredients of Shilajatu rasayana compound

Sl.No.	Drug	Botanical name (family)	Parts used
1	Shilajatu	Black Bitumen / *Asphaltum punjabianum*	Exudates
2	Gokshura	*Tribulus terrestris* Linn. (Zygophyllacease)	Fruits
3	Amalaki	*Emblica officinalis* Gaertn. (Euphorbiaceae)	Dried fruit
3	Amruta	*Tinospora cordifolia* (Willd.) Miers. (Menispermaceae)	Stem
5	Ashwagandha	*Withania somnifera* Dunal. (Solanaceae)	Root
6	Bilva	*Aegle marmelos* Carr. (Rutaceae)	
7	Agnimantha	*Clerodendrum phlomidis* Linn. (Verbenaceae)	
8	Shyonaka	*Oroxylum indicum* Vent. (Bignoniaceae)	Stem bark
9	Patala	*Stereospermum suaveolens* DC. (Bignoniaceae)	
10	Gambhari	*Gmelina arborea* Roxb. (Verbenaceae)	
11	Shalaparni	*Desmodium gangeticum* DC. (Fabaceae)	
12	Prushniparni	*Uraria picta* Desv. (Fabaceae)	
13	Brihati	*Solanum indicum* Linn. (Solanaceae)	
14	Kantakari	*Solanum xanthocarpum* Schrad. &Wendl , (Solanaceae)	
15	Gokshura	*Tribulus terrestris* Linn. (Zygophyllaceae)	Fruits

spectrophotometer (UV-160A) and the absorbance in spectra were recorded based on reference to standard range. Three standard wavelengths 260 nm, 280 nm, 472 nm were selected. The UV-visible spectrum of the alkaline extract of SRC was recorded.

Shilajatu has been found to consist of a complex mixture of organic humic substances and plant and microbial metabolites occurring in the rock rhizospheres of its natural habitat.[9] Humic substances are formed by the microbial degradation of dead plant matter, such as lignin. These can be divided into three main fractions: humic acids, fulvic acids, and humin.[10] The active principle of shilajatu is fulvic acid.[9] Fulvic acid seems to have that unique capacity to dilate and permeate the thick cell walls so as to transmit the minerals into the cells, thereby overcome tiredness, lethargy, and chronic fatigue.[9] Based on the ratio of fulvic acid (FA) with humic acid (HA), purity of shilajatu can be ascertained. As shilajatu is an organic compound, and naturally the purity of organic compound can be assessed based on the FA: HA.[11]

An index was generated to quantify fulvic acid in a sample with both humic acid (HA) and fulvic acid (FA). If FA is more than HA, then the sample is good; or vice versa. The reactive moiety is equal to FA. There is no absorption in the visible region. The following absorbance ratios was used for calculation.[12]

Q2/6(HA) = Absorbance Ratio of 260/664, denotes the relation between non-humified and strongly humified material. No decay started.

Q4/6(FA) = Absorbance Ratio 472/664, is often called the humification index. Typical values of the Q4/6 ratio for humified material are usually <5.

Q2/4(lignin & others) = Absorbance Ratio 280/472, reflects the proportion between the lignins and other materials at the beginning of humification, and the content of materials at the beginning of transformation.

Gravimetric method

The acid precipitation method has been widely accepted for the separation and subsequent quantification of humic acid.[13] The quantification of humic substances is important because humic materials have a relatively high content of free radicals which play important roles in polymerization and redox reactions. This affects the mobility of

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metals (both those with nutrient value and those that are of concern because they are pollutants.[14] The humic acid precipitates at pH < 2 and thus can be quantified by gravimetric measurements. A comparison of extract methods of HA analysis favors HA precipitation from alkaline solution by addition of concentrated Hcl followed by washing of the precipitate with water and oven drying at 110^{o} C were carried out.[15] The humic and fulvic acids which were extracted into a strongly basic aqueous solution of sodium hydroxide, was made acidic by adding normal hydrochloric acid (6NHCl) by adjusting the pH up to 2 and kept overnight, precipitate obtained was filtered and the precipitate obtained was oven dried at 110^{o} C. Humic acids are precipitated from this solution, leaving the fulvic acids in solution. This measure implies to the weight of HA in SRC. This measure should be low to say if good sample. This is the operational distinction between humic and fulvic acids.

RESULTS AND DISCUSSION

Organoleptic parameters

The characters of the sample are tabulated in Table 2.

Microscopy of finished product (SRC)

Under microscopy suddha shilajatu contained oil globules, crystalline material and prismatic crystals**. (**Figure 1)

Microscopy of SRC showed only the main ingredient characteristics. The microscopy study of SRC shows the border pitted vessel of Amrutha (*Tinospora cordifolia*), Cork in surface view of Amrutha, Fibres of Amrutha, Sclerenchyma of Amrutha, Starch Grains of Amrutha, Fibres of Amalaki (*Emblica officinalis*), Crystalline material of Shilajatu, Oil Globules of Shilajatu, Pitted vessels of Ashwaganda (*Withania somnifera*), Schleroied of Ashwaganda, Group of stone cells of Gokshura (*Tribulus terrestris*), Prismatic Crystal of Gokshura, Starch with Hylem of Gokshura, Stone cell of Gokshura, Stratified fibres of Gokshura, Trichome of Gokshura. Images of powder microscopy of SRC are shown in Figure 2.

Physico-chemical parameters

The results of physico-chemical parameters of SRC are placed in Table 3. Presence of more moisture content in the sample may create preservation problem. Hence loss on drying was selected as a parameter. The water and methanol soluble extractive were 37.44% and 18.1% respectively. The pH showed 5 on litmus pointing towards acidic.

Qualitative tests

The water extract of the sample when analyzed for different functional groups showed presence of Proteins, Tannins, Flavinoids, and Reducing sugars. Details of this are placed in Table 4.

Thin Layer Chromatography (TLC) of SRC (Figure 3)

HPTLC of SRC, Figure 4. Visual observation under densitometer showed 2 spots. However the chromatogram shows two prominent spots at hR_f at 30, 80 in SRC, one prominent spot at hR_f 80 in fulvic acid standard in short wave UV 254 nm.

UV Spectrophotometry

Results observed under UV visible spectrum shows two absorption peaks at 472 nm and 664 nm, negative peaks at 280 nm, 260 nm. (Table 5) As per index SRC contained good amount of FA in comparison to Fulvic acid (standard). (Table 6)

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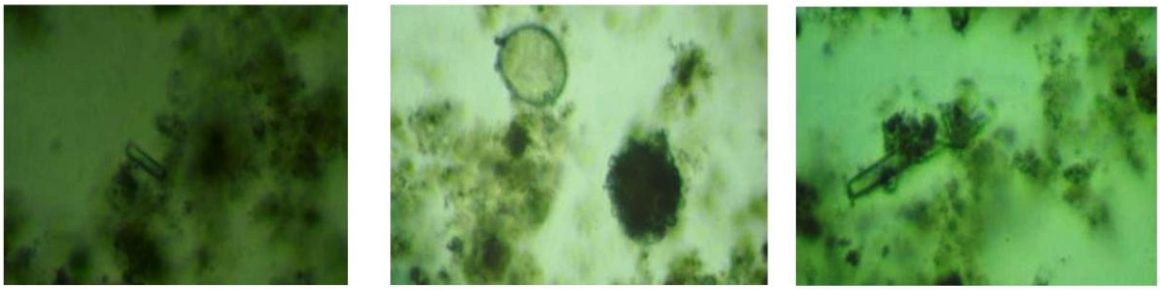
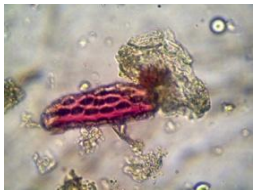
Figure 1: Microscopy of shudda Shilajatu

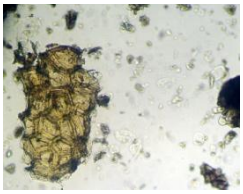


Crystals in group | Oil globule | Prismatic crystal

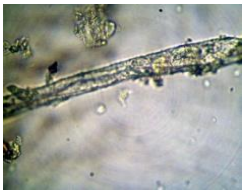
Figure 2: Powder microscopy of SRC



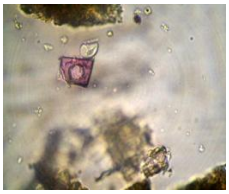
Border pitted vessel of Amrutha



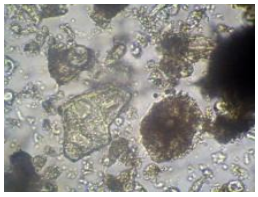
Cork in surface view of Amrutha



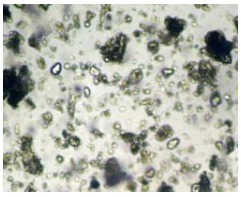
Fibres of Amrutha



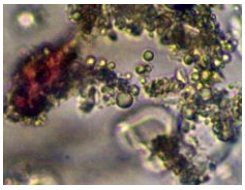
Sclerenchyma of Amrutha



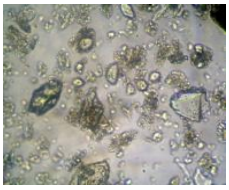
Sclerenchyma of Amrutha



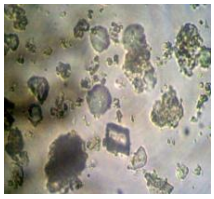
Starch Grains of Amrutha



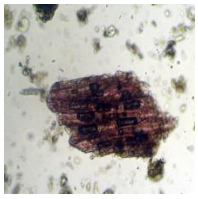
Oil Globules of Shilajatu



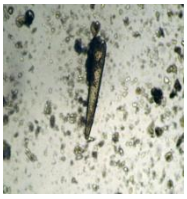
Crystalline material of Shilajatu,



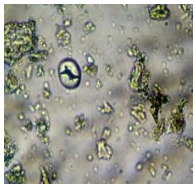
Prismatic crystal of Gokshura



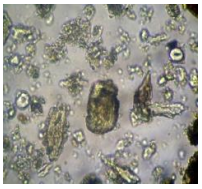
Stratified fibres of Gokshura



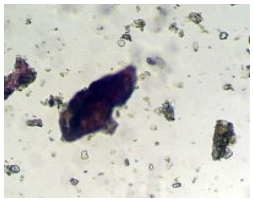
Trichome of Gokshura



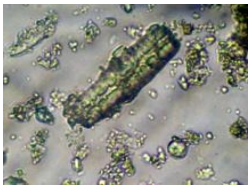
Starch with Hylem of Gokshura



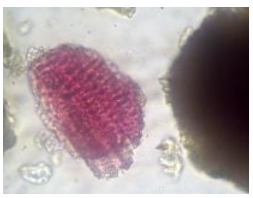
Stone cell of Gokshura



Group of stone cells of Gokshura



Schleroied of Ashwaganda



Pitted vessels of Ashwaganda

Fibres of Amalaki

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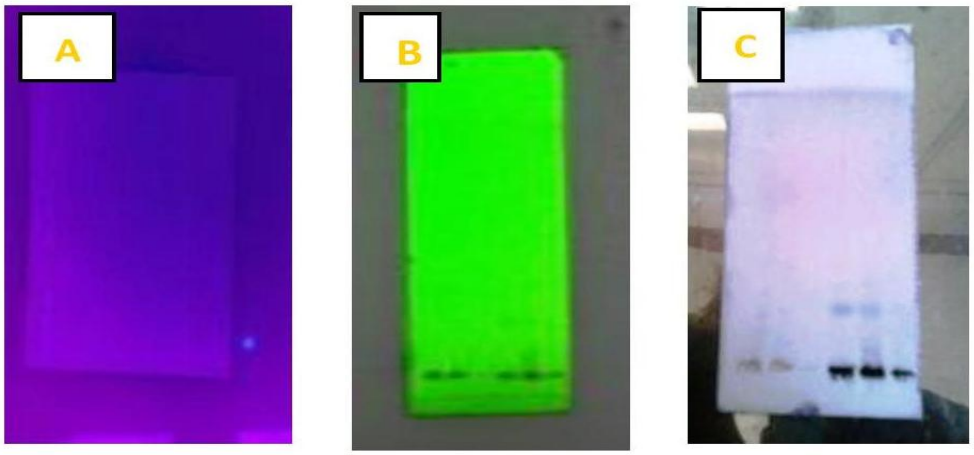
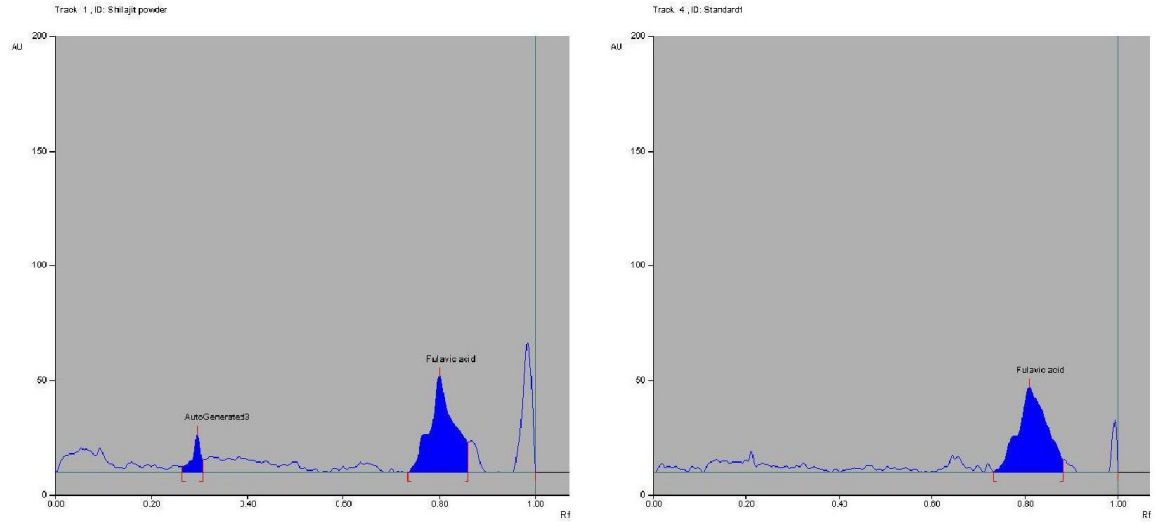
Figure 3: Thin Layer Chromatography of SRC



A - 260 nm B - 472 nm C - After spraying with Anisaldehyde Sulphuric acid

Figure 4: Densitometer curve of methanol extract of SRC and standard fulvic acid at 254 nm and 366 nm



Shijajatu Rasayana Compound Standard – Fulvic acid

Table 2: Organoleptic parameteres of Shilajatu rasayana compound (SRC)

Sl.No	Parameters	Sample – SRC
1	Colour	Greyish brown
2	Odour	Smell of cows urine
3	Taste	Sweet, Astringent
4	Consistency	Fine powder

Table 3: Physico-chemical parameters

Sl.No.	Parameters	Sample- SRC
1	Loss on drying	4.49%w/w
2	Ash value	11.23%
3	Acid insoluble ash	0.23%
4	Water soluble extractive	37.44%
5	Methanol soluble extractive	18.1%

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Table 4: Qualitative parameters for SRC for different functional groups

Sl. No	Functional groups	Name of the test/reagent	Results
1	Alkaloids	Wagner's Reagent	Negative
2	Tannin	Lead acetate	Positive
3	Saponin Glycosides	Foam test	Negative
4	Protein	Biuret test	Positive
5	Steroids	Libermann/Burchard's test	Negative
6	Flavinoids	NaOH test	Positive
7	Reducing sugars	Fehling's test	Positive

Table 5: Quantitative estimation of Humic Substances in SRC and Fulvic acid by Gravimetric Method

Ratio	SRC	FA
$Q_{2/6}$= HA	-2.130	-10.703
$Q_{4/6}$= FA	7.577	1.666
$Q_{2/4}$=lignin and others	-0.208	-5.288

Table 6: Showing the absorbance at different wave lengths

Sample	280nm	472nm	664nm	260nm
SRC	-0.194	0.932	0.123	-0.262
Fulvic acid	-0.238	0.045	0.027	-0.289

By gravimetric method, SRC measured 0.625 g and fulvic acid measured 0.094 g in 1 g of sample. This high amount compared to standard may be due to the procedure of preparation of drug. As the drugs were kept for two days processing which might have switched humification.

CONCLUSION

The use of shilajatu in Diabetes Mellitus patients is in practice since ancient time when all treatment options fails; resort to shilajatu administration is advised. Shilajatu has a unique beneficial effect in neurodegenerative disorders as per the word meaning its "conqueror of mountains and destroyer of weakness". The shilajatu was processed with rasayana drugs and fortified by dashamoola qwatha for better clinical efficacy. The finished drug was administered in capsule form. It is reported that the formulation meets the minimum standards as reported in API at the preliminary level. Qualitative tests for functional groups showed presence of proteins, tannins, flavinoids, and reducing sugars. Results of UV spectrophotometry and gravimetry showed the shilajatu rasayana compound contained high purity and greater percentage of fulvic acid compared to fulvic acid (standard). As fulvic acid is the active principle in shilajatu, its presence in high percentage shows the action potential and purity. This UV spectrophotometry for quantification of fulvic acid using Q2 ratio, can be used as a reference standard for further experimental protocol - for purity analysis of processed shilajatu containing drugs.

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