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Evaluation of organic and inorganic constituents of naturally occurring shilajit: augmentation of fulvic and humic acid and its fingerprinting

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Abstract
Shilajit is a natural substance, used in traditional medicine as a powerful restorative and health tonic. It is formed by the decomposition of plants and mineral by the action of pressure and microorganisms. Present study deals with the Inorganic and Organic chemical constituents of Shilajit rock or exudate, its efficient extraction procedures to enrich Fulvic and Humic acid content and TLC method of monitoring. The study shows that Organic acids are the main constituents contributing about 62 % along with 30 % inorganic minerals. Study also provides method to enrich Fulvic acids up to 80 %. The extract contains Potassium, Calcium, Magnesium as Inorganics. Apart from Fulvic and Humic acids, Oxalic, Benzoic, and Acetic acid are also present in extract. Major amino acids found in extract include Threonine, Glycine, Aspartic, Glutamic, and Arginine. Present finding of Shilajit and its extracts open the possibility for therapeutic study of this medicine.

Keywords: *Asphaltum punjabianum*, Shilajit, Fulvic acid, humic acid, Thin Layer Chromatography.

Introduction
Asphaltum punjabianum. L (Shilajit) is an organic-mineral rich exudate that has been used for centuries in traditional Ayurveda and Sidda [1] system of medicine. It has been known by different names depending on where it is found such as: Mumijo, Mumie, Myemu (Russia), Saljit, Shilajit, Silajita, Silajatu (India), Kao-Tun (Birma), Arakul Dshabal (Kyrgyzstan), Memiai-Faqurul-yahud (Persia), Hajar-ul-musa (Arabic), Asphalt, Jew Pitch, Bitumen (English), Mumie, Salhumin (German) [2, 3]. The word Shilajit is composed of two parts "Shila" means rock and "jit" means having won. So, its literary meaning is " conqueror of mountains". Its Sanskrit meaning is " Conqueror of mountains and destroyer of weakness. As per the literature it was believed to be formed by the slow decomposition (humification) of plant material under specific geological conditions thousands of years ago. It's generally found at 1000 to 5000 meters above sea level, on cave wall or as a rock exudates under specific temperature variations, sunlight exposure and precipitation levels [4, 5]. It naturally occurs as tar-like, solid, or elastic substance, or as a blackish to brown colour irregularly shaped stone with smooth surface with characteristic odour [6] (Figure 1). Its therapeutic and remedial properties have made it interest for modern pharmacological researchers.

Detailed studies were conducted by modern day researchers to know the exact chemical composition of Shilajit, but still the mystery is unsolved. Many researchers across the world like Kong *et al*. in 1987 [7], Ghosal *et al*. in 1988 and 1991 [8, 9], has confirmed that Humus is the main constituent of Shilajit contributed to about 60 to 70 % of its total. Humus is a complex bioactive constituent of all microorganism, plant and animal exist in nature. Due to death, and decay of these natural products, soil, peat, and humus were formed. Hence, Humus is a structurally complex Organic macro molecule formed by the decomposition of natural organisms. Chemically, Shilajit is divided into three components based on their solubility: fulvic acids (acid-soluble), humic acids (alkali-soluble), and humin (insoluble residue). Apart from Fulvic and Humic acid, other major constituents of Shilajit are minerals, amino acids, vitamins, primary and secondary metabolites.

Traditionally, Shilajit has been used to maintain general fitness for boosting energy levels and support mental health. Bioactive compounds present in Shilajit are known to have antioxidant, anti-inflammatory, and adaptogenic properties [10]. Organic compounds of Shilajit, such as Fulvic acid, Humic acid, amino acids are reported to be used for neurological therapy,

especially for Alzheimer's disease [11]. Aqueous extract of Shilajit is largely employed in therapeutic applications as a food additive to strengthen the immune system and promote muscle development. It is generally advised for numerous medical conditions, like Genitourinary diseases, Diabetes, Angina, Jaundice, Digestive disorders, chronic Bronchitis, Anemia, Menorrhagia and Osteoporosis [12].

Modern scientific studies have proved that Shilajit can be used for the prevention of chronic inflammation, and as anti-aging. During invitro study on rat it was known for relaxation of Corpus Cavernosum [13]. In another study treatment with Shilajit for consecutive 90 days revealed that it has significantly increased total testosterone, free testosterone and dehydroepiandrosterone. Gonadotropic hormones (LH and FSH) levels were also maintained by use of Shilajit [14]. Thus, Shilajit is proving to be an effective supplement to enhance health and well-being due to its chemical constituents of inorganic (mineral content) and Organic bioactive compounds [15, 16]. Present study describes the broad-spectrum chemical composition, process of enriching bioactive organic compound (Fulvic acid) by extraction technique, and distribution of these compounds along with amino acids, organic acids and minerals in an enriched extract.

Materials and Methods

Sodium hydroxide AR-Grade, Hydrochloric Acid 37% purity, and Whatman filter paper No.-41 were purchased from local market of Bangalore, India.

Shilajit was obtained from Zivisha Herbal and Organic Pvt Ltd. Kosmi, Madhya Pradesh - 460001, India. The material was dried under sunlight for 5 to 7 hours, to remove excess moisture during excavation and transportation till it became brittle and hard. The dried mineral was powered by using an industrial mixer, and sieved through 20 mesh sieve (850 µm size). This sieved powder is used throughout the studies.

Determination of water content (Loss on Drying [LOD])

Weigh around 2 to 2.5 g of Shilajit sample in an LOD bottle. Placed the bottle in a hot air oven for 3 hours at 105 ºC. Remove the bottle and placed it in desiccators and allow to cool at room temperature. Record the weight of the bottle with the dried sample. Repeat the experiment and measure the weight different and report in the form of percentage variation. Repeat the drying experiment two to three times until a constant weight is achieved [17].

Determination of Inorganic (Total Ash) content

Weigh around 2 to 2.5 g of sample in a silica crucible. Place the crucible in a furnace for 3 hours at 675 ± 25ºC. Remove the crucible and place it in desiccators and allow it cool to room temperature. Record the weight of the crucible with the charred sample. Measure the weight different and report in form of percentage variation. Repeat the experiment two to three times until a constant weight is achieved (United State Pharmacopeia 2024).

Determination of Fulvic Acid and Humic Acid

Through detailed literature and chemical study, it was found that Humic acids are soluble in alkaline conditions, while Fulvic acids maintain solubility at high and lower pH. Which means, Humic acid (CAS no. 1415-93-6) can be obtained as a precipitate when alkaline extracts of Humus substance are acidified, whereas Fulvic acid (CAS no. 479-66-3) remains dissolved during this process. The solubility of Fulvic acids is influenced by their hydrophilic nature resulting from a high presence of acidic functional groups, while the associations within humic acids are hydrophobic, leading to stability at neutral pH and aggregation at acidic pH. These agglomeration and solubility define that both Humic acid and Fulvic acids are not singular, easily defined compounds, but rather complex aggregates composed of multiple compounds. Proposed general pseudo structures of Fulvic acids is shown in Figure 1 [18].

Considering these criterions, an inhouse method was developed to quantify Humic acids and Fulvic acids in Shilajit. About 5 to 10 grams of Shilajit sample was transferred into a conical flask. 100 mL of 0.1 N Sodium hydroxide solution was added and warm the mixture on a water bath at 80 ºC for 45 minutes. Decant the supernatant into beaker. Repeat the extraction process with 50 mL of same solution twice, or until the extract becomes colorless. Combine the extracts and filter the solution using Whatman filter paper No. 41, collect the filtrate. After alkylation and extraction, the filtrate was acidified with 1 N Hydrochloric acid until the pH reaches 2. This led to precipitation of Humic acids which was then allowed to settle for 30 minutes. The solution was then filtered through pre-weighed Whatman filter paper No. 41 and residue was dried in an oven at 70 to 80 ºC until constant weight is achieved. Content of Humic acids was calculated in percentage considering residue obtained and initial weight of Shilajit.

For Fulvic acids estimation, the filtrates after humic acid extraction were bought to neutral (pH 7) by adding 20 % Sodium hydroxide solution. Solution was boiled for one hour on water bath and pH was maintained. The solution was then concentrated to about 30mL and acidify to pH 2 with 1.5 N Hydrochloric acid. The solution was transferred to a pre-weighed silica crucible and evaporated first to dryness at on water bath and then at 105 ºC in hot air over. Crucible was cooled and measured the dry weight. Calculate the Inorganic (Ash) content by charring and heating the crucible in a muffle furnace at 500 to 515ºC for 5 to 8 hours. Crucible was cooled and weigh the inorganic ash presents in the sample. The content of Fulvic acids was calculated in percentage of initial Shilajit sample considering the dry residue and subtracting the inorganics.

Determination of Amino acids, Organic acids and Inorganic compounds

The testing was performed by third party lab. [Eureka Analytical Services Pvt Ltd, Banaswadi, Bengaluru, Karanataka 560043].

Extraction of Shilajit to enrich Fulvic acids

Considering the fact that Fulvic acids is a water-soluble compound, the grinded material of Shilajit were placed in round-bottom flask, and 1:4 volume of water was added with respect to raw material, pH 3 to 5 was maintained by adding 1 N Hydrochloric acid. The material was constantly stirried for 4 hours at room temperature to facilitate the extraction process. The flask was heated at 65 ºC for 4 hours on water bath. The resulting solution was removed, cooled at room temperature and filtered with Whatman filter paper. The process was repeated three more times till the extract is colourless to ensure complete extraction. The filtrate was combined and concentrated under reduce pressure in rotavapor. Thick black colored resin was obtained. The resin was dried at 70 ºC in vacuum oven to obtain free flowing powder (Figure 1).

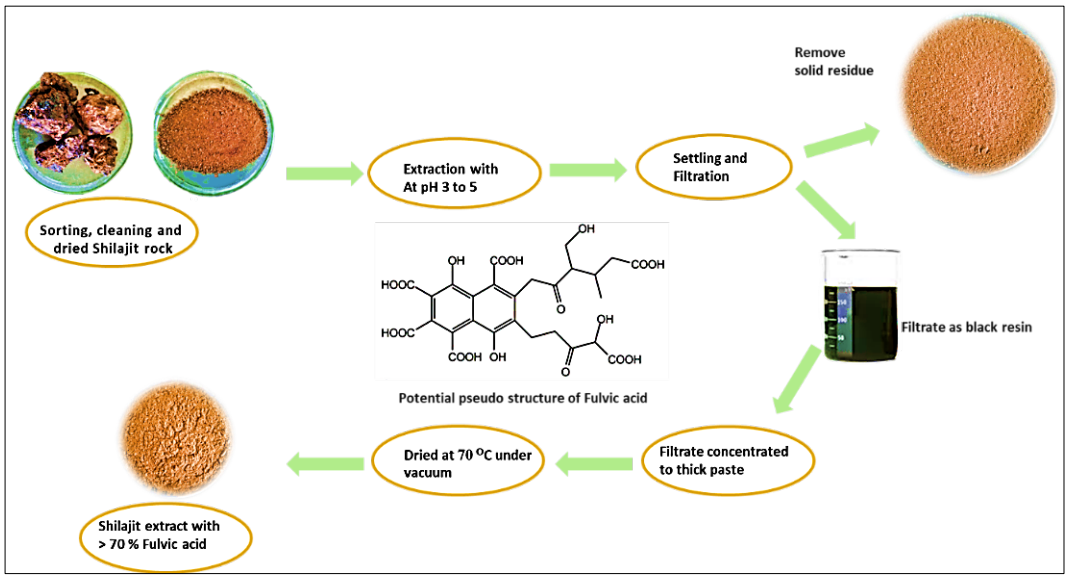

Fig 1: Demonstrate the method for enriching the Fulvic acid. The process indicates the Shilajit rock after sorting, cleaning, drying, and milling is converted into a fine powder. Powder was subjected to acidic water extraction at pH 3 to 5 followed by settling and filtration. This will remove the solid waste, and black coloured resin is obtained. The resin was concentrated and dried at 70 ^{O}C under vacuum. The dried powder obtained shows Fulvic acid greater than 70 %. The Potential pseudo structure of Fulvic acid constituents (carbon, hydrogen, and oxygen) containing functional groups (hydroxyl, carboxyl, carbonyl, and phenolic).

In order to evaluate the efficiency and repeatability of the process, 4 different batches (Batch # RM/25/0001, 0002, 0003 and 0004) having different concentration of bioactive compounds were used during the study. Prior to proceeding with the extraction process, the raw material was analyzed to evaluate its major components. This parameter helps decide the quality and suitability of raw material for extraction process as shown in [Table 1]. After the extraction and enrichment, the resultant powder extracts (Batch # BT/25/0005, 0008, 0164, 0166) were analyzed for similar parameters to evaluate the enrichment of Fulvic acids and other compounds [Table 1].

Thin Layer Chromatography

Weigh around 2 to 2.5 g of Shilajit raw material and dissolve in 50 mL methanol. Shake and sonicate at 60 to 70°C for 30 minutes. Filter the solution and concentrate 50 mL to 10 mL. Same sample preparation procedure was followed for enriched extracts but equivalent amount of sample was dissolved in methanol. About 10 µL of band were spotted on TLC silica plate (silica gel 60 F_{254} (cat # 1.05554.007, Merck, Mumbai, India). The plates were developed in solvent systems, Toluene/Ethyl acetate/Glacial acetic acid (7:3:0.3 v/v/v).

Results and Discussion: Shilajit, an organometallic blackish brown gum exudate obtained from steep rock of different part of the world like, India, Afghanistan, Australia, Russia at an altitude of 1000-5000m. It generally contains two class of compounds, first organic compounds which is mainly Humic substances and secondly inorganic minerals and metals. Organic compounds contributing 60 to 70 % of Shilajit are mainly composed of Humic substances along with other amino acids, organic acids. Humic substances are complex organic matters having molecular weight ranging from few hundred (Fulvic acids) to several thousand (Humic acid), and end up to several million (Polymeric Humin).

Organic composition of Shilajit primarily contains two classes of compounds, namely, Humic substances and Non-Humic metabolites. Humic Substances are the major organic constituents contributing about 80-85%, and have molecular weights ranging from several thousand for Humic acids, and up to several million for polymeric Humins, to only a few hundred for its Fulvic acid component. These substances also are found in Soils and Sediments distributed over the earth's surface, occurring in almost all terrestrial and aquatic environments. Humic Substances are produced by the interactions of plants, algae, and mosses (bryophtes), with microorganisms, by a process known as humification. Humification of latex- and resin bearing plants is primarily responsible for the production of the water-soluble humic substances.

Table 1: Chemical composition of Shilajit Raw material and Enriched Shilajit Extract

Raw material	RM/25/0001	RM/25/0002	RM/25/0003	RM/25/0004	AVG	Std. Dev. (σ)
Ash (Inorganic)	32.60	35.34	24.93	28.18	30.26	4.62
LOD	6.74	7.41	7.52	5.74	6.85	0.82
Organic	60.66	57.25	67.55	66.08	62.89	4.78
Fulvic acid	36.29	36.67	29.94	21.50	31.10	7.11
Humic acid	0.47	0.25	3.68	0.92	1.33	1.59
Sum of Fulvic & Humic acid	36.76	36.92	33.62	22.42	32.43	6.84
Other Organic acids	23.90	20.33	33.93	43.66	30.46	10.52
Enriched Extract	BT/25/0005	BT/25/0008	BT/24/0164	BT/24/0166	AVG	Std. Dev. (σ)
Ash (Inorganic)	20.705	21.24	18.45	19.20	19.90	1.30
LOD	4.33	3.71	3.51	2.14	3.42	0.92
Organic	74.965	75.05	78.05	78.67	76.68	1.95
Fulvic acid	73.81	74.47	71.28	75.34	73.73	1.75
Humic acid	0.47	1.83	1.55	1.83	1.42	0.65
Sum of Fulvic & Humic acid	74.28	76.30	72.83	77.17	75.15	1.96
Percentage yield with respect to starting material	45	48	40	30	40.75	7.89
Recovery efficiency with respect to Fulvic acid	91.53	97.48	95.23	105.13	97.34	5.74

In the present study, to simplify and identify the chemical composition of Shilajit, first the raw material is analysed for three parameters, Inorganic content (by determining the Ash), Water content (by measuring the LOD), and subtracting it by 100 to measure the Organic composition. As shown in Table 1 and Figure 2 A, for quadruple trials, on an average the Inorganic content is about 30.26 % (with standard deviation (σ) 5.40), LOD is 6.85 (with σ = 0.42) and Organic content were 62.89 % (σ = 5.25). The Fulvic and Humic acids were found to be 31.10 % (σ = 3.78) and 1.33 % (σ = 1.92) respectively. Study confirms that apart from Fulvic and Humic acids, other Organic acids are also present in Shilajit which contribute about 30.46 % (σ = 7.05).

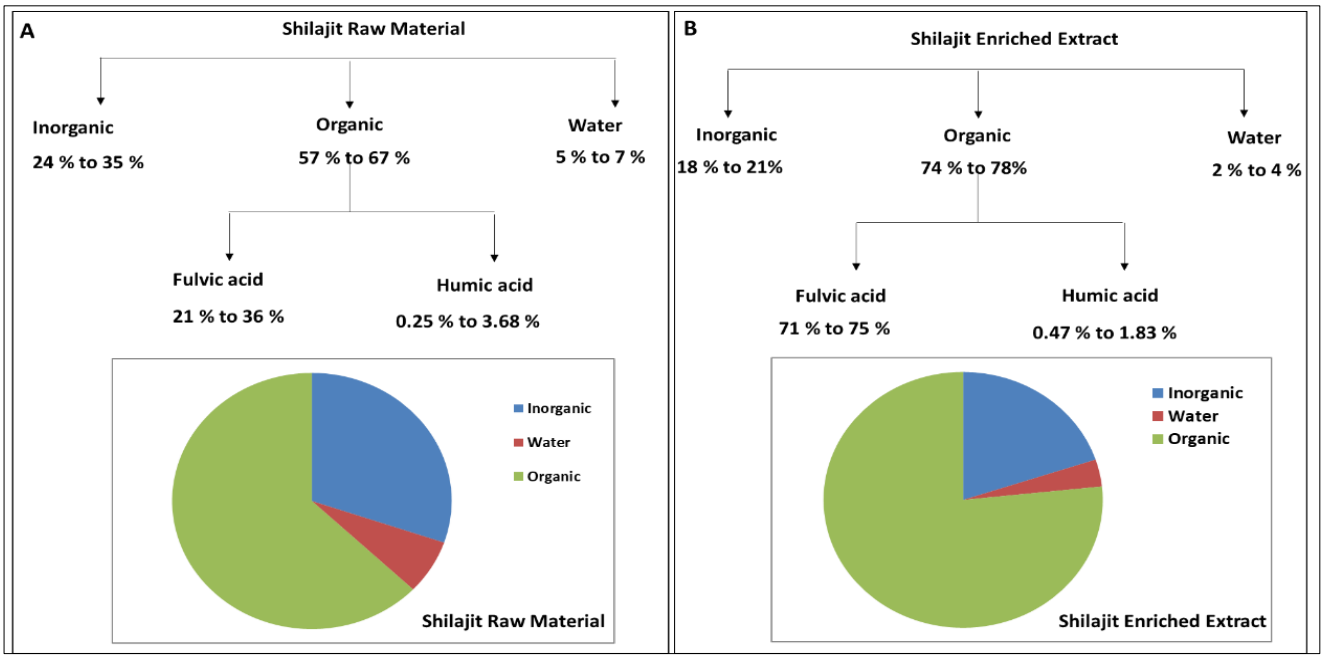

Fig 2: Shows the distribution of Inorganic, Organic (Fulvic acid and Humic acid) and water content in Shilajit Raw Material and Extract. Section A shows that Inorganic content is ranging from 24 % to 35 %., Organic 57 % to 67 % and water 5 % to 7 % in raw material. Further the identified acids (Fulvic and Humic) are in the range of 21 % to 36 % and 0.25 % to 3.68 % respectively. The pie graph shows the distribution of Inorganic, Organic and water content in Shilajit raw material. Similarly, section B shows range and distribution of Inorganic, Organic (Fulvic and Humic acid), and water in Shilajit Enriched extract.

To enrich the Fulvic acid, two major routes can be used. First is to remove the Inorganic constituents (which contribute about 30 %) so that Organic constituent is straight away increased by 50 % (30 % of 60 %) i.e. 90 %. Second approach is to increase Organic content by solvent extraction. As per our knowledge and literature study there is no commercially viable and cost-effective technique available which straight away remove the Inorganic constituents and maintain the integrity of Organic constituent.

To overcome this challenge second approach of solvent extraction technique was used to increase the Organic content targeting Fulvic acids. Fulvic acid being highly polar in nature and its better solubility in acid pH, various trials were performed for its extraction under different pH condition ranging from low to neutral pH (pure demineralize water). In conclusion it was found that when Shilajit rock powder extracted with 4 to 5 volumes of water (respect to Shilajit raw material and 3 to 4 cycles) at pH 3 to 5 maximum yield were obtained.

Moreover, soaking for Shilajit raw material for 8 to 10 hours in acidic water also give better yield but with constant stirring the outcome of yield and Fulvic acid content is significantly higher in 4 hours and in 3 times extraction. The final processing technique mentioned above were used to extract Organic acids targeting Fulvic acids. On analysing the dried powder extract the Inorganic content reduced from 30.26 % to 19.90 % (σ = 1.48) ie. about 34 % and Organic acid increased from 62.89 % to 76.68 % (σ = 1.75) i.e. about 22 %. But Fulvic acid content were found to be increased significantly from 31.10 % to 73.73 % (σ = 1. 68) i.e. almost 137 %. There was no major change in Humic acids content. The detailed chemical composition is shown in Table 1, Figure 2 A and B.

An efficient and reproducible Thin Layer Chromatography (TLC) was developed inhouse to monitor each stage of extraction procedure. Several permutations and combination were tried to develop the fingerprint which can competently monitor the various stage of extraction. Finally, The TLC fingerprint with mobile phase Toluene/Ethyl acetate/Glacial acetic acid (7:3:0.3 v/v/v) shows two characteristic bands at Rf about 0.50 and 0.75. The TLC fingerprint of starting raw material (track 1 and 4), intermediate resin (track 2 and 5), and enriched Shilajit extract (track 3 and 6) is as shown in Figure 3.

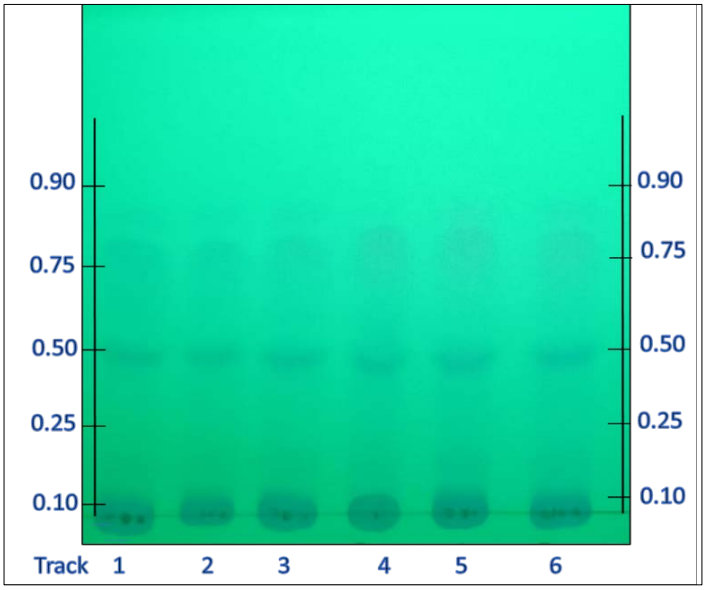

Fig 3: Thin Layer Chromatography for monitoring the extraction procedure. TLC fingerprint developed on aluminum plate coated with silica and using mobile phase Toluene/Ethyl acetate/Glacial acetic acid (7:3:0.3 v/v/v) shows characteristic bands at Rf 0.5 and 0.75. Track 1 and 4 is starting Shilajit raw material, 2 and 5 is intermediate resin, 3 and 6 is enriched extract.

Yield is an important parameter that indicates the outcome of any process. It defines as the ratio of the amount of the desired product (analyte) extracted from a sample to the total amount of that product initially present in the sample. However, the efficiency of any process is determined by how efficient your extraction process is to recover bioactive compounds by maintaining the minimal loss both in terms of weight and craving molecule. Considering this, the average percentage yield obtained was 40.75 % (σ = 7.89) with respect to starting raw material and the average recovery efficiency with respect to Fulvic acids was 97.34 % (σ = 5.76) as shown in Table 1.

For the commercial acceptance and validating its applicability at large scales the optimized process of extraction should maintain negligible loss of bioactive compounds, both in term of purity and yield. The comparison of four batches data with recovery percentage of Fulvic acids ranging from 91 % to 105 % further validated the consistency and reproducibility of the extraction and analytical process.

To understand the other chemical composition of Shilajit extract, detailed evaluation of the presence of other Organic, Inorganic and Amino acids was assessed in one of the enriched batches of Shilajit extract (#: BT/24/0164). Other Organic acids like Oxalic acid, Acetic acid, and Benzoic acid were found to be 0.63 g/100g, 0.10 g/100g and 0.83 g/100g respectively. Lactic acid, Malic acid, Propanoic acid, and Citric acid, were also evaluated but were not detected (detection level 0.01 g/100g) as shown in Table 2 and Figure 4 A.

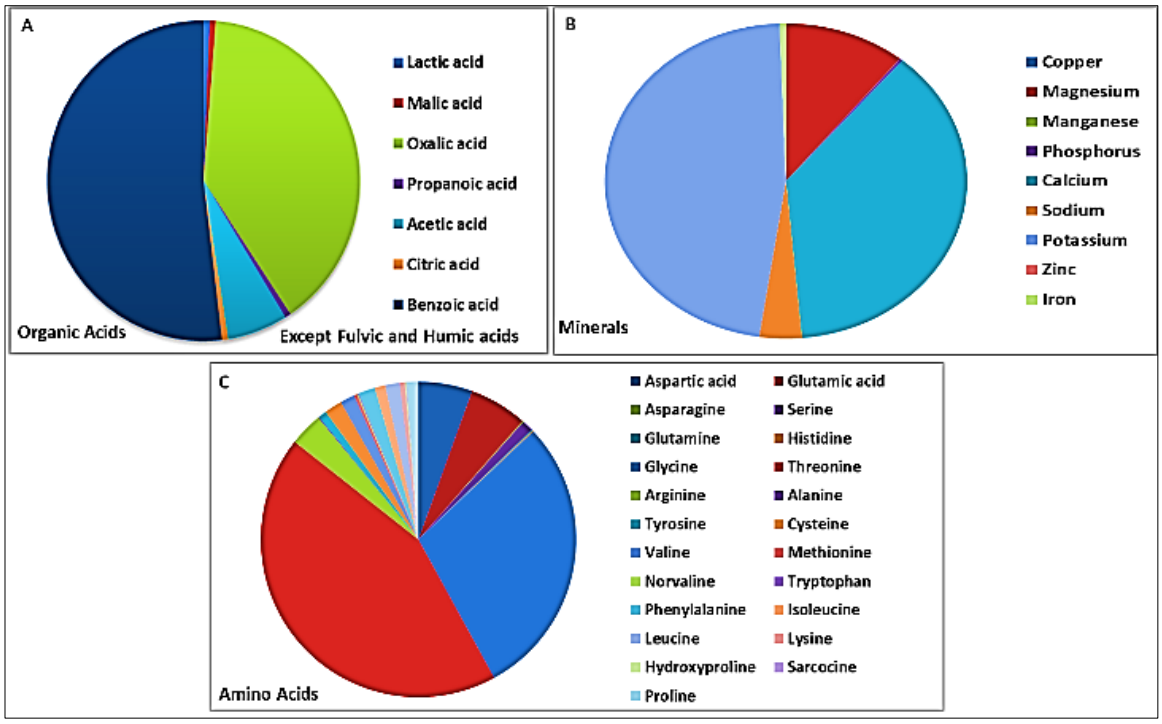

Fig 4: Shows the distribution of Organic acids (except Fulvic and Humic acid), Minerals, and Amino acids in Shilajit enriched extract drawn from table 2. Section A confirm that Benzoic acid is the major acid followed by Oxalic acid, and Acetic acid. Similarly, section B confirms that

Potassium is the major minerals in Shilajit followed by Calcium and Magnesium. Section C shown that enriched Shilajit extract contains Threonine as major Amino acids, followed by Gycine, Aspartic acid, Glutamic acid etc.

Similarly, the amount of major Inorganic compounds or mineral present in enriched Shilajit extract are Potassium 7.77 g/100g, Calcium 6.19 g/100g, and Magnesium 1.78 g/100g. Other minerals like Copper, Manganese, Phosphorus, Sodium, Zinc, Iron are present in the range of 0.001 g/100g to 0.094 g/100g as shown in Table 2. The graphical distribution of Organic acids (Except Fulvic and Humic acids) and Inorganic minerals, in form of pie graph, are shown in Figure 4 A and B respectively.

Table 2: Organic, Inorganic and Amino acids present on Enriched Shilajit Extract

Constituents	Compound	Content in g/100g
Organic acids	Lactic acid	≤0.01
	Malic acid	≤0.01
	Oxalic acid	0.63
	Propanoic acid	≤0.01
	Acetic acid	0.10
	Citric acid	≤0.01
	Benzoic acid	0.83
	Total Organic acids	1.56
Inorganic	Copper	0.003
	Magnesium	1.787
	Manganese	0.003
	Phosphorus	0.044
	Calcium	6.189
	Sodium	0.620
	Potassium	7.775
	Zinc	0.001
	Iron	0.094
	Total Inorganic	16.52
Amino acids	Aspartic acid	0.52
	Glutamic acid	0.56
	Asparagine	≤0.01
	Serine	0.11
	Glutamine	≤0.01
	Histidine	≤0.01
	Glycine	2.73
	Threonine	4.10
	Arginine	0.32
	Alanine	≤0.01
	Tyrosine	0.08
	Cysteine	0.17
	Valine	0.14
	Methionine	0.03
	Norvaline	≤0.01
	Tryptophan	≤0.01
	Phenylalanine	0.16
	Isoleucine	0.10
	Leucine	0.14
	Lysine	0.05
	Hydroxyproline	≤0.01
	Sarcocine	≤0.01
	Proline	0.11
	Total Amino acids	9.32

This Inorganic mineral plays a significant role in shilajit's bioactivity and therapeutic potential. High mineral content, such as potassium, calcium aids muscle function, bone, and cellular activity. Magnesium plays an important role in enzymatic functions and metabolic processes. Iron is useful in synthesis of hemoglobin and the transportation of oxygen. Trace minerals such as zinc, copper, manganese and phosphorus contribute to various biological activities, like immune function and enzymatic reactions.

Shilajit is known to contain various amino acids especially exogenous, like Methionine, Leucine and Threonine, and also endogenous such as Histidine, Proline, Glycine, Tyrosine, Arginine and Aspartic acid (2). The present study investigated present of various essential and non-essential amino acids in enriched Shilajit extract as shown in Table 2 Figure 4 C. The major amino acids obtained are Threonine (4.10 g/100g), followed by Glycine (2.73 g/100g), Glutamic acid (0.56 g/100g), Aspartic acid (0.52 g/100g), Arginine (0.32 g/100g). Apart from these amino acids, other amino acids like Serine, Tyrosine, Cysteine, Valine, Methionine, Phenylalanine, Leucine, Lysine, and Proline are present in the range of 0.17 g/100g to 0.03 g/100g. Other amino acids like Asparagine, Glutamine, Histidine, Alanine, Norvaline, Tryptophan, Hydroxyproline, Sarcocine were not detected in enriched Shilajit extract as shown in Table 2 and Figure 4 C.

These amino acids play a significant role in therapeutic activity of Shilajit. Enriched Shilajit extract contain these amino acids may leads to various biological activities. Threonine plays an important role in protein synthesis and immune function. Glycine was known to contribute for collagen production and neurotransmission. Similarly other minor ammino acids found in lower levels supports in muscle growth and metabolic functions.

Conclusion

The study provides a comprehensive analysis and distribution of chemical composition of Shilajit. It's known that Shilajit obtained by the decomposition of plant thousands of years back are converted into Humus and Inorganic minerals. The major composition of Shilajit rock is Inorganic minerals and Organic compounds like Fulvic acids and Humic acids. The findings demonstrate the process of enriching Fulvic acids up to 70 to 80 %. This augmentation contributes a significant health benefit like enhancing the nutrient absorption, detoxification, antioxidant, and supporting the traditional use in Ayurvedic medicine.

It also shows the presence of mineral salts of significant importance, as such as Potassium, Calcium, Magnesium, Sodium and Iron that are the significant for physiological functions including enzyme activities and oxygen transportation. The presence of Amino acids helps in the supplement to facilitate protein synthesis, tissue repair, and physical health.

In addition, the presence of Benzoic acid along with other Organic acids indicate the antimicrobial, antioxidant, and anti-inflammatory properties of Shilajit. These properties make Shilajit a potential natural supplement in fighting with oxidative stress, chronic inflammation, and metabolic disorders. The finding opens the door for further study of these enriched complex molecules for biological, physiological, and psychological activity.

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Declarations

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- **Conflict of interest:** All other authors have declared no conflict of interest
- **Data availability:** Datasets used and analysed during the current study are available from the corresponding author upon reasonable request.
- **Ethics approval and consent to participate:** This article does not contain any studies involving animals and human participants performed by any of the authors.

Authors Contribution

Jyotish Srivastava: Conceptualization, Investigation, Supervision, Review and Editing; Sudha Chaturvedi: Resources, Project administration, Funding acquisition, Conceptualization; Aji Abraham: Developing TLC fingerprinting, Review and Editing; Vinay Hanote: Data curation, Formal analysis; RM Mohan: Writing, Review and Editing. All authors have read and agreed to the published version of the manuscript.

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