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NOVEL PARAMETERS TO DEFINE BIO-EFFICACY OF SHILAJIT RESIN AND ITS VALIDATION

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ABSTRACT: Shilajit, a herbo-mineral blackish brown resin, extracted from humic rocks, is attributed to several therapeutic claims in Ayurveda like antiaging, health-promoter, immune-booster, urogenital problems and bio-enhancer. The lack of strong parameters for its quality control and standardization; and the knowledge gap to explain its mechanism of action are the important factors behind its restricted use. Here, for the first time, we have used a High-Resolution Advance Mass Spectrophotometer (HRAMS) to visualize the metabolites of shilajit and further identified novel metabolites for its characterization. Seven brands of shilajit, available in the market (5 resins and 2 capsules), were purchased and used to validate our findings in terms of molecular weight, abundance, retention time and structural characteristics. We found 14 novel metabolites and six of them were acidic, eight were non-acidic. Among them, only 4 metabolites were common in all seven samples, which were (a) NP-020214, (b) 3-tert- Butyladipic acid, (c) Dodecanedioic acid and (d) Azelaic acid. However when only resins were compared, then additional nine common metabolites were identified as (1) 4-Acetamidobenzoic acid, (2) Phenylacetylglycine, (3) Benzamide,3-(Sulfooxy)benzenepropanoic acid, (4) 4-(2-Hydroxyethyl)phenyl hydrogen sulfate, (5) N-Benzylformamide, (6) p-Cresol, (7) 2- Hydroxyhippuric acid, (8) thymol sulfate and (9) Prostaglandin-B1. Thus, the first 4 metabolites may be considered as universal novel marker compounds in addition to existing compounds, for standardization purposes. However, the remaining 9 can only be used for pure resin preparations of shilajit. The bioinformatics-based analysis indicated that all 14 metabolites passed the drug-likeness, but only 7 of them could cross the Blood blood-brain barrier (BBB). Further, the presence of heavy metals and trace elements were analyzed by Induction Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) and the presence of no heavy metals (Pb, As, Cd and Hg) were detected in any sample, but all of them showed a high amount of essential trace elements but in variable amount. Aqueous solutions of shilajit showed high conductance and solubility (92-95%) at normal pH. Thus, here we could identify the additional 14 novel molecules, which could be used for characterization and quality control of shilajit.

INTRODUCTION: Shilajit, (asphaltum) is an amorphous organic material, with black lustre, burns with a bright flame and is soluble in water. Shilajit is derived from the microbial decomposition of herbs, mainly bryophytes, over thousands of years and gets deposited in the rocky-mountains, but its composition and bio-efficacy depend on its natural habitat, which contributes to its minerals, vitamins, fatty acids and essential trace elements. The Dibenzo- alpha pyrones, urolithin A, urolithin B and acetophenone and other organic molecules having different functional groups like carboxyl, phenolics, alcohol, ketones, aldehydes, amines, amides, polymeric quinones, humins, and Nitrogenous, Phosphorus and Sulphur

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containing heterocyclic metabolites, have been reported in shilajit, but mainly Urolithins and Hydroxy- acetophenones (HAP) were used as the marker compounds for its quality control. The Urolithins is mixture of urolithin-A and B, which are the metabolites of the ellagic acid and belong to the family of 6H-dibenzo-[b,d] pyran-6-one, a combination of coumarin and iso- coumarin, with different phenolic hydroxylation patterns.

The major organic constituents of shilajit are humic substances, a mixture of [1] humins, [2] humic acid and [3] fulvic acid (FA), which contributes to 93-95% of purified shilajit. Fulvic Acids (FA) are a group of organic compounds, of low molecular weight, highly oxygenated and highly soluble in water, at neutral pH. It is represented by a general formula of $C_{14}H_{12}O_8$, with a molecular weight of 308.24028 g/mol, and IUPAC name as 3,7,8-trihydroxy-3-methyl-10- oxo-4,10- dihydro-1H,3H-pyrano [4,3-b]chromene-9-carboxylic acid 123. Biologically, FA serves as a better carrier at intercellular and intracellular levels (cell-organelles), attributing to enhancing the bioavailability of accompanied drugs and nutrients.

MATERIAL AND METHODS: Here, we have used High-Resolution Advance Mass Spectro-photometer (HRAMS) to determine the metabolites and Induction Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) to determine the elements in Shilajit. The tools of Bioinformatics have been used to determine the drug-likeness and therapeutic claims of selected metabolites. The objective of this study is to identify some novel metabolites, which can be used for quality control and standardization of shilajit products and to explain the mechanism of action of its claimed therapeutic claims. The shilajit of 7 brands, available in the market, were purchased online. Among them, 5 samples were in resin form and 2 samples were in the capsule form. All of them were coded, as detailed in **Table 1.**

TABLE 1: CODING OF DIFFERENT SAMPLES OF SHILAJIT

S. no.	Sample Code	Name of the companies and doses form of Shilajit
1	SS01	Upakarma ayurveda (resin)
2	SS02	Sanskar (resin)
3	SS03	Baidyanath (resin)
4	SS04	Kapiva (resin)
5	SS05	Rasayanam (resin)
6	SS06	Dabur (capsules)
7	SS07	Zandu (capsules)

Solubility Assessment: Since it is reported that FA is highly soluble in normal distilled water and HA is sparingly soluble, so to ascertain the % of FA in the samples, we assessed their solubility in HPLC-grade distilled water.

pH Determination: 500 mg of each sample of shilajit was dissolved in 30 ml of HPLC- grade distilled water, to a final concentration of 16.66 mg/ml and pH of each solution was measured by using a glass electrode pH meter.

Conductivity Assessment: The conductivity of the above solutions was also measured. It is the indication of the presence of charged ions, whether +ve or -ve, which is attributed to the capacity of electric conduction through the solution.

Elemental Analysis by ICPAES: The elemental analysis of the Fe, Mn, Mg, Ni, P, S, Sr, Ti, Zn, Ag, Se, Cu, Cr, Co, Al, Pb, As, Cd and Hg were carried out by using ICPAES, at the central facility of IIT, Bombay. The results of each sample were prepared in terms of "ppm", which was compared among all the 7 samples, by Microsoft Excel software.

UV Analysis: The Spectrophotometer of model-Cary (60UV-Vis) Agilent, USA was used. The shilajit sample was dissolved in HPLC-grade water at different concentrations (w/v). The variation in OD (absorbance) for the calculated concentration of shilajit in the test solution was plotted. Since humic substances usually yield un-characterized spectra at UV and visible wavelengths, the E4/E6 ratio (ratio of the absorbance of the solution at 465 and 665 nm) has been chosen to determine the concentration of Fulvic Acid (FA) in 0.05 N NaHCO3 solution.

Experimental Details of Equipment, Software and Methodology of HRAMS: This analysis was

carried out in the central facility of BHU, under the Central Discovery Centre (CDC). The instrument detail: High-Resolution Accurate Mass Spectrometry System; Model: Q Exactive Plus; Make: Thermo Fischer Scientific. For Small Molecules, UHPLC Dionex Ultimate 3000 RS Series has been applied to isolate different metabolites. The solvent composition, used for the elution of these small molecules was a mixture of Solvent A: 100% Water + 0.1% Formic Acid; Solvent B: 100% Acetonitrile + 0.1% Formic Acid and Solvent C: 100% Methanol + 0.1% Formic Acid. The column Detail: Hypersil GOLD™ C18 Selectivity HPLC Column, Particle size 1.9 µm, Diameter 2.1 mm, Length 100 mm. The software used for documentation of these metabolites was a patented product of the manufacturer, i.e. "Thermo Compound Discoverer 3.3.2.31" by using default settings and Online databases. The workflow was designed as untargeted Metabolomics using Online Databases, mzLogic, and Molecular Networks to find and identify the differences between samples, based on the retention time alignment, unknown compound detection, and compound grouping across all samples. It predicts elemental compositions for all compounds, fills gaps across all samples, and hides chemical backgrounds (using Blank samples). Identify compounds using mzCloud (ddMS2) and ChemSpider (formula or exact mass). Maps compounds to biological pathways using Metabolika. Apply QC-based batch normalization if QC samples are available. Calculates differential analysis (t-test or ANOVA), determines p-values, adjusted p-values, ratios, fold change, CV, *etc*.). Generate Molecular Network to visualize compounds.

Analysis of Mean of AUC of Total Metabolites and their Comparison: The mean of AUC of total metabolites, reported in the HRAMS data, was calculated and compared among all samples. The mean AUC of Bioactive major metabolites of the shilajit of Upakarma Ayurveda were considered as a base to compare with other samples, to validate our findings, by using the Microsoft Excel software.

Analysis of Common Metabolites and Drug-Likeness: To achieve this objective, we filtered 30 major metabolites, having AUC > $2x10^9$, out of the total 5467 metabolites, reported in the HRAMS data sheet and their mean-AUC values were compared, among all the 7 samples. Among them, only common metabolites were further filtered out and taken forward for their drug-likeness and capacity to cross the blood-brain barrier (BBB). For this study, we explored the CID number of each metabolite, with the online server of PubChem, which was subjected to Swiss ADME online server.

RESULTS:

The Solubility of Shilajit Samples in Water: We found that the sample with code SS01 had the highest solubility, in the range of 92-95%. Other samples were less soluble, as measured in the form of residue on filter paper. This indicated the high ratio of FA in SS01. Samples SS06 and SS07 were not compared on this parameter, as they were in capsule form and had some excipients in them.

The pH of Shilajit Solutions: As given in **Table 2**, the SS01 had the highest pH of 8.08, in the alkaline range. Interestingly, the pH of other samples was either neutral or slightly acidic. The slightly alkaline pH of SS01 might be attributed to its better solubility in water, which indirectly indicates the high content of fulvic acid and some humic acids.

TABLE 2: COMPARISON OF PH AND CONDUCTIVITY OF DIFFERENT SHILAJIT SOLUTION (16.66mg/ml.)

S. no.	Sample	pH	Conductance mS/Cm
1	Water	7.3	0.0018
2	SS01	8.08	7.569
3	SS02	5.31	4.338
4	SS03	6.08	5.717
5	SS04	7.78	8.253
6	SS05	6.16	4.014
7	SS06	5.28	2.863
8	SS07	4.44	0.696

The Conductivity of Shilajit Solutions: The solution of SS01, indicated a conductivity of 7.57 mS/Cm, which was very high compared to the pure water (1.87 µ seconds/cm), considered as the

reference solution. This high conductivity of SS01 may be attributed to the higher numbers of ionized solutes in its solution, which may be responsible for its better potential as a drug carrier. The sample SS07 showed the lowest conductivity, in the range of 0.69 mS/Cm **Table 2.**

The Comparison of Heavy Metals and Trace Elements: Sample SS01, showed the presence of Fe, Mn, Mg, Ni, P, S, Sr, Ti, Zn and the absence of Ag, Se, Cu, Cr, Co, Al, Pb, As, Cd and Hg. Among them, S and Mg were the highest and Zinc was the lowest. Further, the Sr was found to be highest in the sample SS04, followed by SS01. Similarly, the Fe concentration was highest in SS04 followed by SS03 **Table 3**. This elemental analysis indicates that all the samples of shilajit, available in the market, are safe and sample SS01 has a highest amount of Mg and Sulphur.

TABLE 3: THE CONCENTRATION OF HEAVY AND TRACE ELEMENTS IN BY ICPAES MEASUREMENT

Elementsdetected	Comparison of ICP-AES data: Ours vs. Literature						
	Concentration in ppm in different samples of Shilajit						
	SS01	SS02	SS03	SS04	SS05	SS06	SS07
S	14260	7610	7887.237	17237.56	2502.045	3872.338	580.128
Mg	12700	7270	8498.628	10129.4	4650.847	6198.17	1476.709
Sr	327	127	124.199	398.267	24.697	109.031	16.026
P	132	188	230.101	276.132	1415.194	375.42	602.778
Fe	40	130	284.767	321.409	182.593	147.32	147.222
Mn	12	28	20.128	28.787	57.895	9.806	9.188
Ti	2	13	12.809	36.892	5.061	13.074	14.744
Ni	1	1	1.83	2.795	3.644	3.736	3.205
Zn	0.011	0.007	11.665	10.62	13.36	11.907	6.624
As	ND	5.3	ND	ND	ND	ND	ND
Al	ND	60	ND	ND	ND	ND	ND
Co	ND	ND	ND	ND	ND	ND	ND
Cr	ND	ND	ND	ND	ND	ND	ND
Cu	ND	ND	ND	ND	ND	ND	ND
Pb	ND	ND	ND	ND	ND	ND	ND
Se	ND	ND	ND	ND	ND	ND	ND
Cd	ND	ND	ND	ND	ND	ND	ND
Ag	ND	ND	ND	ND	ND	ND	ND

The Ultraviolet (UV) Spectroscopic Studies of Shilajit: The UV spectra of all samples showed a common pattern, with some variation in the AUC of major peaks. The absorbance and fluorescence graphs have been provided in **Fig. 1** and **2**. On the validation front, it was observed the AUC of SS02 was higher than SS01, which may be attributed to the higher content of aromatic metabolites in SS02.

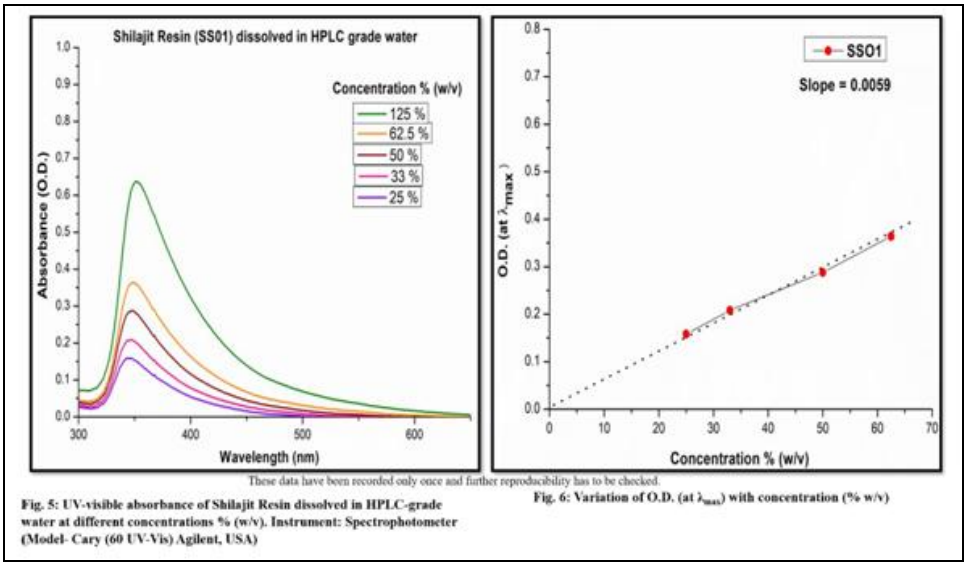


FIG. 1: UV SPECTRA OF SS01 (UPAKARMA AYURVEDA) AT DIFFERENT DILUTIONS

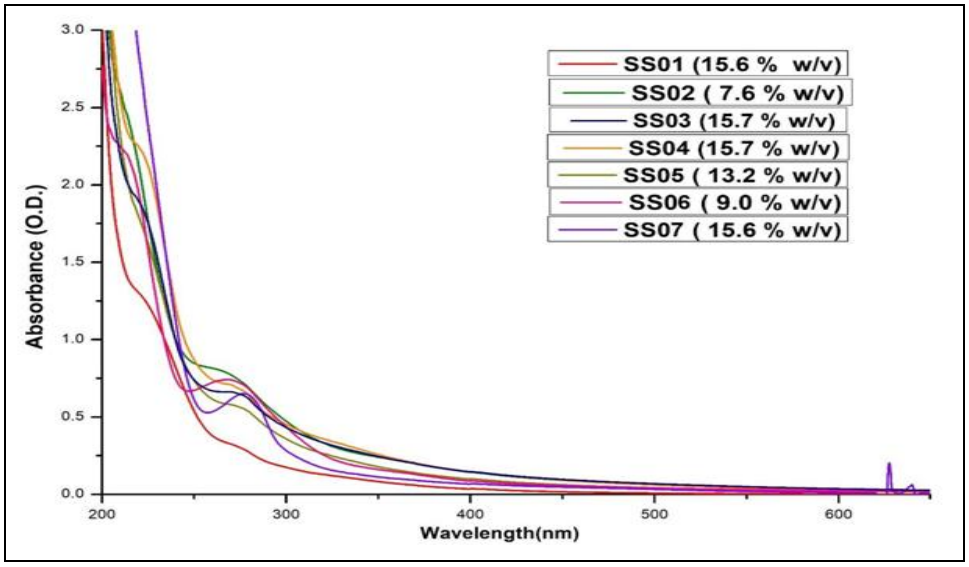


FIG. 2: UV SPECTRA OF ALL 7-SAMPLES OF SHILAJIT, OVERLAPPED ON EACH OTHER

Analysis of Shilajit with HRAMS: Since it has already been reported that the FA and HA contents of shilajit are a mixture of a variety of non-aromatic oxygenated hydrocarbons, we further analyzed these samples with HRAMS. The master data sheet of SS01 showed the presence of 5467 metabolites. On removal of duplicates, only 2149 metabolites were recorded and chosen for further studies.

Comparison of Cumulative Abundance (Area under Curve-AUC) and Total Number of Metabolites: The mean AUC of SS01 was the highest among all 7 samples **Table 4**, but the number of metabolites was not the highest. This may be attributed to metabolites with very low AUC values.

TABLE 4: COMPARISON OF THE NUMBER AND MEAN AUC OF ALL METABOLITES OF HRAMS DATA (number) and mean AUC± SD of metabolites

SS01	SS02	SS03	SS04	SS05	SS06	SS07
(5467)	(5977)	(7766)	(6402)	(6785)	(5954)	(3785)
0.12 ± 1.35	0.06 ± 0.50	0.04 ± 0.27	0.05 ± 0.49	0.08 ± 0.51	0.08 ± 0.57	0.04 ± 0.29

Selection of Common Metabolites and Their Comparison: Here, we have used an online platform of bioinformatics to compare the number of metabolites of SS01, which are common with individual shilajit samples. The results indicated the similarity of metabolites in the range of 400 to 452, with all the samples, except SS07, where 2152 metabolites were found common, though its mean AUC was about 1/3 of that of SS01. This high similarity could be attributed to a common source of ore for these companies for extraction of shilajit, but by using different methods, which affects the quality of extraction, (indicated by low AUC value). Further, we identified those metabolites of SS01, which are collectively common in all 7 samples, and we found only 129 common metabolites **Table 5.**

TABLE 5: COMPARISON OF THE NUMBER OF METABOLITES OF SS01 WITH THE OTHER 6 SAMPLES OF SHILAJIT, INDIVIDUALLY AND COLLECTIVELY

SS01 Only	SS01 vs SS02	SS01 vs SS03	SS01 vs SS04	SS01 vs SS05	SS01 vs SS06	SS01 vs SS07	SS01 vs All
2149	406	459	452	408	424	2152	129

Selection of 30 Major Metabolites of Shilajit Based on their Abundance: In another approach of analysis, we initially filtered 30 major metabolites in SS01, having AUC > 2x109 and compared their mean AUC in all the 7 samples. Its mean AUC was highest (9.97±16.56x109),

followed by SS04, SS06, SS05, SS02, SS03 and SS07. Interestingly, SS07 had the lowest mean-AUC value of 0.01±0.06 x109 **Table 6** though the common metabolites in these 2 samples were highest **Table 5.**

TABLE 6: MEAN AUC OF 30 MAJOR METABOLITES (AUC > 2X10^9) AMONG ALL THE 7 SHILAJIT SAMPLES

Mean ± SD (AUC) of 30metabolites	SS01	SS02	SS03	SS04	SS05	SS06	SS07
	9.97 ±16.56	2.34 ±4.80	1.63 ±3.88	3.79 ±8.06	2.14 ±5.74	2.25 ±7.17	0.01 ±0.06

Selection of Common Metabolites among 30 Major Metabolites: Here we identified only 14 metabolites of SS01, which were common in all 5 resin preparations and among them, 6 were acidic, and 8 were non-acidic **Table 7**. However, when these 14 metabolites were compared among all 7 samples, then only 4 metabolites were found common namely (1) NP-020214, (2) 3-tert-Butyladipic acid, (3) Dodecanedioic acid and (4) Azelaic acid. Thus, it could be suggested that while standardizing the resins of shilajit, all 14v metabolites may be considered as unique/novel, but when any shilajit preparation is being considered for testing, then only these 4 metabolites should be considered as marker compounds.

TABLE 7: COMPARISON OF ABUNDANCE OF COMMON METABOLITES (NOVEL AND ALREADY KNOWN) AMONG ALL 7 SHILAJIT

Sr. no.	Name	SS01	SS02	SS03	SS04	SS05	SS06	SS07
1	4-Acetamidobenzoic acid	85.75	0.61	0.12	0.06	29.45		
2	Phenylacetylglycine	32.91	4.6	0.66	2.51	10.01	0.7	
3	Benzamide	21.41	2.28	1.26	9.63	2.84	3.61	
4	3-(Sulfooxy)benzenepropanoicacid	8.89	1.52	2.1	2.66	2.53	3.2	
5	4-(2-Hydroxyethyl)phenylhydrogen sulfate	7.39	1.47	1.36	2.37	0.62	4.2	
6	N-Benzylformamide	6.86	0.28	0.12	0.56	0.86	1.1	
7	p-Cresol	4.75	20.48	16	32.78	1.65	39.51	
8	NP-020214	3.59	0.08	0.2	1.03	0.11	0.17	0
9	2-Hydroxyhippuric acid	3.47	2.11	0.8	0.67	1.54	0.11	
10	3-tert-Butyladipic acid	2.93	1.49	0.06	0.8	0.95	3.55	0
11	thymol sulfate	2.73	0.8	0.09	0.68	0.05	1.43	
12	Prostaglandin B1	2.67	0.05	0.95	2.12	0.61	0.14	
13	Dodecanedioic acid	2.54	1.17	1.16	2.11	0.16	3.62	0
14	Azelaic acid	2.23	0.24	0.34	2.17	1.81	3.25	0.03
15	Urolithin A	1.80	0	0	0	0	0	0
16	Urolithin B	0.34	0.12	0.35	0.02	0	0.01	0
17	Acetophenone	0.16	0.05	0.5	0.05	0.17	0.12	0.02

We further examined the presence of Urolithin A, Urolithin B and Acetophenone, which are currently being used as marker compounds for the standardization of shilajit. Interestingly, Urolithin A, was only found in SS01, but Urolithin B and Acetophenone were present in all 7 samples **Table 8**. Thus, we have summarised the findings of SS01, for validation with the other 6 samples of shilajit, and we found that SS01 (Upakarma Ayurveda) has the highest value of mean AUC of total metabolites, reported in the HRAMS datasheet, mean AUC of major 14 novel metabolites and exclusive presence of Urolithin A.

TABLE 8: ANALYSIS OF COMMON METABOLITES AMONG 5 RESIN-SHILAJIT AND ALL 7 SAMPLES INCLUDING 2 CAPSULES

Sample	Metabolites of Shilajit as reported in HRAMS data sheet	
SS01 (Upakarma Ayurveda)	Common in 5 resinShilajit	Common in 7 Samples
2149 Unique	260	129
30 Major	14	4
10 Acidic	6	3
20 Non-Acidic	8	1
10 BBB	7	3
Acidic metabolites crossing BBB	4	3
Non-acidic metabolites crossing BBB	3	0

Urolithin A	Not Found	Not Found
Urolithin B	Found in all samples	Found in all samples
Acetophenone	Found in all samples	Found in all samples

Drug Likeness of 14 Major Common Metabolites of Shilajit: We further determined the drug-likeness by using Swiss ADME and all of them showed a good degree of drug-likeness with passive transport across the GIT membrane **Table 9**, but only 7 metabolites could cross the blood-brain barrier, as shown in the egg model **Fig. 4.** Among them, 4 were acidic and 3 were non- acidic.

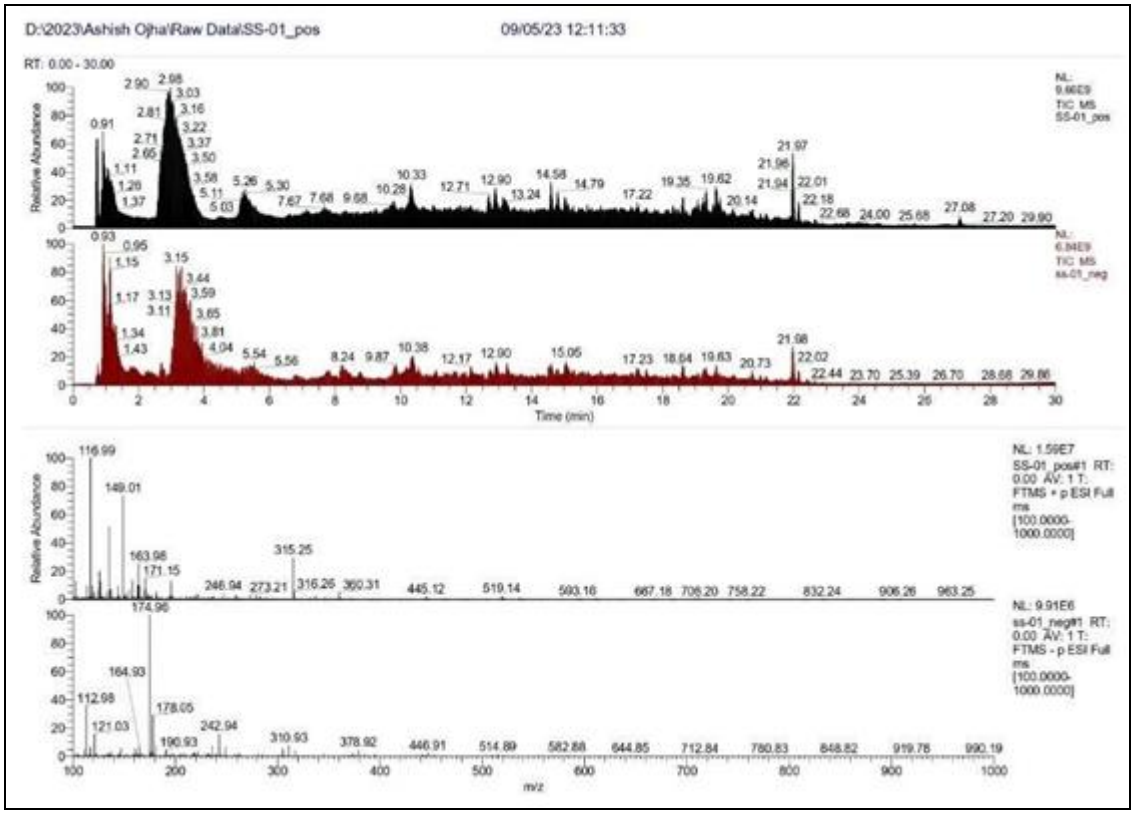


FIG. 3: ORIGINAL CHROMATOGRAM OF HRAMS SHOWING RELATIVE ABSORBANCE VS RT OF SS01 (UPAKARMA AYURVEDA)

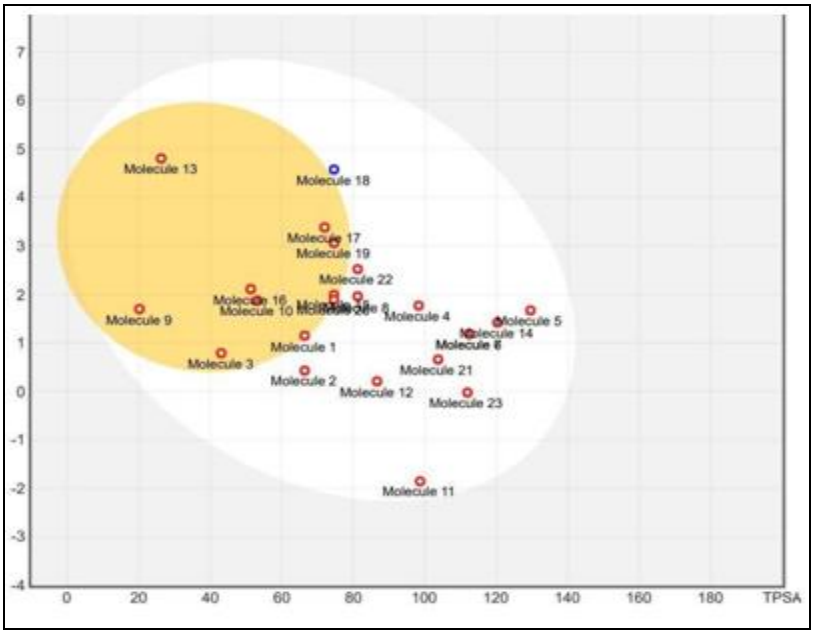


FIG. 4: POSITION OF METABOLITES IN BOILED EGG PICTURE, SHOWING THE LOCATION OF THOSE MOLECULES WHICH CROSSED BBB

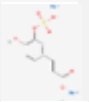
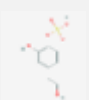
TABLE 9: DRUG LIKENESS OF 13 MAJOR METABOLITES OF HRAMS DATA OF SS01 (SHILAJIT OF UPAKARMA AYURVEDA)

Sr. no.	Name	BBB permeant	PGP substrate	Calc.MW	RT (min)	x^9
1	4-Acetamidobenzoic acid	Yes	No	179.0574	3.326	85.75
2	Phenylacetylglycine	No	No	193.074	6.874	32.91
3	Benzamide	Yes	No	121.0529	1.873	21.41
4	3-(Sulfooxy)benzenepropanoic acid	No	No	246.0196	2.157	8.89
5	4-(2-Hydroxyethyl)phenyl hydrogen sulfate	No	No	218.0243	8.232	7.39
6	N-Benzylformamide	No	No	135.0685	6.09	6.86
7	p-Cresol	Yes	No	108.0578	1.828	4.75
8	2-Hydroxyhippuric acid	No	No	195.0524	1.209	3.47
9	3-tert-Butyladipic acid	Yes	No	202.1198	15.05	2.93
10	thymol sulfate	Yes	No	230.0608	14.533	2.73
11	Prostaglandin B1	No	Yes	336.23	21.997	2.67
12	Dodecanedioic acid	Yes	No	230.1514	18.626	2.54
13	Azelaic acid	Yes	No	188.104	13.26	2.23

Note: one metabolite NP-020214 was not considered because it had no CID number in PubChem.

Pharmacological Properties of unique Metabolites: Since we have identified 14 major metabolites of SS01 as "novel/unique," we explored their pharmacological claims by using PubChem. Most of them showed anticancer, antioxidant and anti-inflammatory properties **Table 10**. Since NP-020214 had no record in the PubChem Database, that was excluded. The molecular weights of all these metabolites were in the range of 179 -336 Da, and most of them had a lower range of retention time (RT), indicating their high polarity.

TABLE 10: BIOLOGICAL PROPERTIES OF 14 COMMON METABOLITES SELECTED AS NOVEL MARKER MOLECULES FOR STANDARDIZATION AND QUALITY CONTROL

Sr. no.	Name	Biological Claims	Structure
		Acids	
1	4-Acetamidobenzoic acid	Agonists of the farnesoid-X-receptor (FXR), Anti- Cancer,Inhibition of the ß-class carbonicanhydrases	
2	3-(Sulfooxy)benzenepropanoic acid	Nil	
3	2-Hydroxyhippuric acid	Colorectal cancer	
4	3-tert-Butyladipic acid	Anti-Leukemia	
5	Dodecanedioic acid	Colorectal cancer, Diabetes mellitus type 2, Metabolic syndrome,Ulcerative colitis	
6	Azelaic acid	Antineoplastic Agents, Dermatologic Agents	
		Non-Acids	
7	Phenyl acetyl glycine	Arthritis, Heart failure, Eosinophilicesophagitis	
8	Benzamide	Acute Kidney Injury, Acute Lung Injury, Amnesia, Anterograde, Brain Injuries, Drug-Related Side Effects and Adverse Reactions	
9	4-(2-Hydroxyethyl) phenyl hydrogen sulfate	Nil	

10	N-Benzyl formamide	alcohol dehydrogenase	
11	p-Cresol	Cardiovascular Diseases, Dermatitis, Exfoliative, Hyperplasia, Kidney Diseases	
12	NP-020214	Nil	
13	Thymol Sulfate	Colorectal cancer	
14	Prostaglandin B1	Inhibitors of Chronic Active B-Cell Receptor Signaling& G Protein Regulator, Modulation ofHypoxia Response Element Signaling Pathway	

DISCUSSION: In Ayurveda, an Indian system of traditional medicine, shilajit is being used solo or in combination with other drugs for various therapeutic claims, like antiaging, aphrodisiac and as an effective drug vehicle [19, 20].

Shilajit is a good source of essential minerals and metals, which help to maintain good health/wellness. Some clinical studies have reported its beneficial effects for physical strengthening, improving urinary tract functioning, stabilizing blood sugar, immune modulation, enhancement of brain potency, anti-arthritis, anti-ageing and anti-hypertension activity [21]. These therapeutic claims are attributed to 'Fulvic Acid', which is a mixture of organic compounds, associated with several therapeutic claims. While analyzing the list of these metabolites, many of them showed strong antioxidant and anti-inflammatory properties, which may be attributed to their antiaging potential, Alzheimer's disease, neural health, skincare and the management of other lifestyle-linked degenerative diseases. Its reported mood elevator activity may be attributed to the modulation of gamma-aminobutyric acid (GABA) and serotonergic systems [26]. Some reports indicate the capability of shilajit to modulate the activity of testosterone in males [22, 23]. One study has indicated its role in wound healing, attributed to Ferulic Acid (FA) [24] and the normalization of heart functions [25]. Regarding the genesis of shilajit in nature, the earlier reports define it as a product of the continuous microbial decay of bryophytes.

Though we have not done any ligand-receptor docking studies against those targets, which are responsible for these therapeutic claims, there could be a very high possibility of their modulation, because of the presence of a high number of metabolites (5467). These molecules, primarily belong to (a) long-chain aliphatic compounds (hydrocarbons and their derivatives at different levels of oxidation), alkyl-cycloalkanes, aromatics, polyphenolics, carboxylic acids and N- and S-containing heterocyclic compounds, 4′-methoxy- 6-carbomethoxybiphenyl (MCB), 3,8-dihydroxy-dibenzo-α-pyrone (DDP) etc. attributing to various therapeutic claims of shilajit [27]. The skin care, anti-allergic and management of respiratory/immunological abnormalities may be linked to its potential to stabilize the mast cells [28]. It is also reported to enhance spermatogenesis in patients with oligospermia [29].

However, due to a lack of suitable markers for its standardization, consumers are hesitant to use this product. For the first time, we have used the HRAMS data to identify some novel metabolites, which can be used for the quantitation of shilajit in different preparations. This finding has been validated by testing these novel metabolites in seven brands of shilajit, available in the market. Since there were a huge number of metabolites, so we filtered 30 major metabolites having AUC>$2x10^9$. Among them, 14 metabolites were found in all the tested samples, so they were chosen as novel metabolites **Table 8**. These are in addition to the existing 3 metabolites, i.e. urolithin-A, urolithin B and acetophenone.

Limitations of this Study: For this study, we have selected only 30 major metabolites, however further study is required for all metabolites reported in the HRAMS data, to find out more common metabolites, though less in abundance but unique.

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