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Modernization of Shilajit Processing: Comparative Phytochemical Screening, Antioxidant Potential, and Phenolic Content in Standardized vs. Non-Standardized Extracts

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Abstract: Shilajit, an Ayurvedic medicinal substance, is recognized for its rich humic content and various health benefits, including anti-inflammatory and neuroprotective effects. This study involves a comparative evaluation of phytochemical composition, antioxidant potential, and total phenolic content in standardized and non-standardized Shilajit extracts. The standardized Shilajit was processed through a controlled workflow including extraction, filtration, distillation, and drying, aimed at reducing heavy metal contamination while preserving bioactive compounds. Methodologies employed included qualitative biochemical screening, high-resolution mass spectrometry (HRMS), UV-visible spectrophotometric analysis to determine the E4/E6 ratio, Fourier-transform infrared spectroscopy (FTIR) for functional group identification, and scanning electron microscopy coupled with energy-dispersive X-ray spectroscopy (SEM-EDX) to assess elemental composition and confirm the elimination of heavy metals. The E4/E6 ratio of standardized Shilajit ranged from 2.84 to 2.93, significantly lower than that of non-standardized Shilajit samples, indicating a higher concentration of aromatic components. Additionally, its IR analysis revealed distinct functional groups, whereas non-standardized samples lacked the humic acid band, while SEM-EDX confirmed the absence of heavy metals in all samples. Phytochemical screening indicates the abundance of different compounds, such as flavonoids, phytosterols, terpenoids, phenols, and coumarins, which were further validated by the HRMS study. The phytochemical richness is responsible for significantly higher antioxidant activity (via DPPH and phosphomolybdate assays) and phenolic content in Standardized Shilajit than non-standardized Shilajit samples. These results demonstrate that the standardized production process enhances the safety and therapeutic efficacy of shilajit, supporting its application in health and wellness.

Keywords: *VCA-964, Standardized Shilajit, Heavy Metals, Antioxidant, Physiochemical Properties.*

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I. INTRODUCTION

In Ayurvedic medicine, shilajit has been used down the ages to boost vitality and treat a range of ailments. It is also called mumijo, which means ‘saving body’ or ‘protecting organism in Greek. In Sanskrit, shilajit signifies ‘destroyer of weakness’[1]. Due to its Rasayana (rejuvenating) nature, Shilajit enhances the function and vitality of the body’s seven fundamental tissues (Sapta Dhatus), which include plasma (Rasa), blood (Rakta), Muscle (Mamsa), fat (Meda), bone marrow (Majja), and reproductive tissue (Shukra). It is also traditionally recognized by the terms Dathuras or Dathusara, indicating its nourishing influence on these essential bodily components [2]. Shilajit is formed by the slow decomposition of plant materials by microbes and molds such as Barbula, Fissidens, Minium, and Thuidium along with Asterella,

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Dumortiera, Marchantia, and Pellia. Shilajit made up of humified organic material, encompasses fulvic acid, along with minerals. [3]. Shilajit has been reported to possess therapeutic potential in the management of peptic ulcers and demonstrates an anti-inflammatory, antioxidant, antibacterial, anti-arthritic, anticancer, memory improvement, and neurocurative activity [4],[5],[6],[7]. In different pharmaceutical studies, Shilajit has shown nootropic, antianxiety, neuroprotective, antioxidant, hypnosedative, glycine, and GABA-mimetic effects on the central nervous system (CNS) [8]. Also, Shilajit is known to raise blood testosterone levels and sperm count in both rats and humans [9]. Other effects of Shilajit include improving blood flow to the reproductive organs, reducing mental and physical stress, and improving muscle tissue turnover, elasticity, and the extracellular matrix that facilitates healing and regeneration, and skeletal muscle adaptation through the upregulation of ECM-related genes [10],[11],[12] It is likely that therapeutic attributes of Shilajit are ascribed to the notable concentrations of fulvic acids, in light of the fact that fulvic acids are recognized for their potent antioxidant properties and is likely to exert systemic effects as a complement activator. Various studies highlighted the presence of additional substances found in shilajit, including benzoic acid, ellagic acid, benzo coumarins, hippuric acid, fatty acids, ichthyol, resin, triterpenes, sterols, amino acids, and phenolics. [13]. Nonetheless, concerns have been raised regarding heavy metals and toxins in various asphalt products. Ingestion of raw Shilajit poses significant health risks. One major concern is its inherently high content of stable free radicals, which may exert pro-oxidant effects rather than the intended antioxidant benefits. Moreover, unpurified Shilajit may serve as a medium for mycotoxin-producing fungi, introducing the potential for fungal contamination and associated toxicities. [14] These issues are particularly critical when Shilajit is consumed without adequate purification or quality control measures, as is often the case with some commercial or traditional preparations. Conventional purification practices, such as hot water extraction, sedimentation, filtration, and sun drying, often integrated with herbal or milk-based treatments, are still widely employed in indigenous systems. The lack of standardized purification protocols further contributes to batch-to-batch variability, undermining both therapeutic efficacy and product safety. These limitations underscore an urgent need for the adoption of modern purification strategies that incorporate low-temperature techniques, advanced analytical validation (e.g., HPLC, FTIR), and compliance with Good Manufacturing Practices (GMP) and pharmacopoeial guidelines [15]. Standardization not only ensures consistency and safety but also facilitates regulatory acceptance and enhances the global credibility of Shilajit-based formulations within evidence-based healthcare systems. This study compares standardized and non-standardized Shilajit extracts. The aims include physicochemical characterization (UV spectra, HRMS, FTIR), heavy metal analysis, qualitative phytochemical screening, antioxidant activity, and phenol content of both types of Shilajit powder.

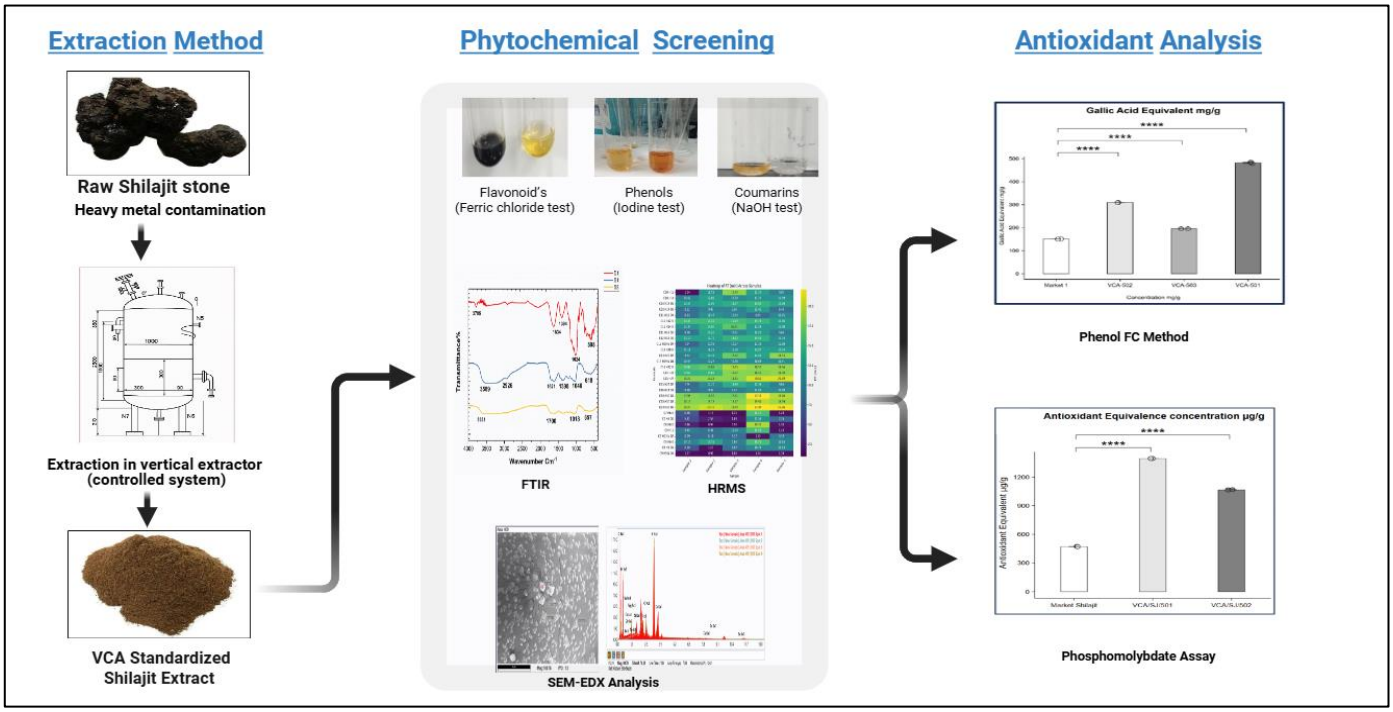

Fig 1 Graphical Abstract

II. MATERIALS AND METHODS

➢ *Chemicals*

DPPH reagent (TOKYO CHEMICAL INDUSTRY CO., LTD; LOT: 32JHO-DL)*[Shamirpet Mandal, Building 189, MN Park, Synergy Square, 1, Genome Valley Rd, Turkapally, Hyderabad, Telangana 500078], Phenol reagent (Qualigens; Batch No: 7758030623), Molybdate reagent (Qualigens; Batch No: 8091351223), and Gallic acid (Loba CHEMMIE PVT. LTD; Batch No: L438152211) were purchased from Science House (Varanasi, Uttar Pradesh).

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➢ *Test Materials*

Three batches of standardized Shilajit (Shilajit-964; Batch No.: 2024501, 240502, and 240503) were obtained from VCA Healthcare Pvt Limited (G-91 Agro Food Park, MIA, Alwar, Rajasthan, India), with percentages of total fulvic acid and dibenzopyrones of 40% and 5%, respectively (according to the manufacturer's certificate of analysis). Additionally, two other Shilajit samples purchased from a local store, along with the VCA standardized Shilajit, were used for further analysis.

➢ *Sample Analysis*

- *UV-VIS Spectroscopy:*

The E4/E6 ratio, or the ratio of the solution's absorbance at 465 and 665 nm, was calculated for the different samples since humic substances tend to generate atypical spectra in the UV and visible range.

- *Fourier Transform Infrared Spectroscopy:*

IR spectra of different Shilajit batches were done using (FT/IR-4700 JASCO) FTIR analyser and Origin software. Shilajit dried extracts were used for FTIR analysis using the standard pellet technique. Then, scanning of all samples was done in the range of 4000 to 450 cm-1, and the spectrum of these pellets was recorded.

- *Scanning Electron Microscopy with Energy Dispersive X-Ray Spectrometry (SEM–EDX)*

Surface morphology examination and element analysis of standardized and non-standardized shilajit powder were accomplished via SEM (ZEISS EVO, Carl Zeiss Microscopy). The method detailed in [16] was employed for all batches. Imaging of all Shilajit batches was conducted utilizing a beam setting of 124 keV for the acceleration voltage and 0. 1 nA for the probe current. A specific beam setting of 124 keV for the acceleration voltage and 0. 8 nA for the probe current was utilized for EDX analysis to guarantee a high signal-to-noise ratio and to acquire X-ray signals from heavier elements.

- *High-Resolution Mass Spectrometry (HR-MS):*

Owing to their superior activity in terms of phytochemical and antioxidant activity, VCA-964 standardized Shilajit extract samples from batches 501, 502, and 503, 601, along with the non-standardized shilajit, were selected for high-resolution mass spectrometry-based metabolomic profiling. The HRMS research on Standardized shilajit samples involved carefully selecting compounds to ensure accurate data. The process started with identifying 205 molecular formulas. The number of molecules was then narrowed down using multiple steps to find the most suitable & important compounds for analysis. In the first step, compounds with an Area (Max) less than 10,000,000 were removed, leaving 194 molecules. Then the compounds were sorted manually to mark the compounds that were most responsible for the properties of Shilajit; a total of 46 compounds were selected in this step. Ultimately, 30 compounds, which were common in all the datasets, were analyzed, leading to a total of 150 compounds examined. Compounds with the formula C7H7NO were excluded because their Area (Max) was significantly larger than others, resulting in 145 compounds (29 distinct metabolites common in each dataset) remaining for in-depth evaluation.

➢ *Biochemical Profiling*

- *Qualitative Phytochemical Screening:*

To evaluate the presence of major chemical constituents in shilajit samples (Phenols, Flavonoids, coumarin, Phytosterol, triterpenes, etc.), qualitative phytochemical screening was performed using the method reported in [17],[18],[19].

- *Antioxidant Analysis*

- *DPPH Assay*

The antioxidant molecules can turn DPPH free radicals into a colourless or bleached result by quenching them (reducing absorption). [13]. Based on the method mentioned in [20], we used DPPH method to measure the radical scavenging property of several shilajit samples. Standard solution prepared by mixing 2400 µg of DPPH in 10000 µL of methanol. Later, 100 µL of shilajit samples were mixed with 3 ml of DPPH working solutions. All samples were kept in dark place for half an hour. Consequently, the absorbance at 517 nm was calculated.

Proportion of antioxidants, or RSA percentage = $[Abs_{(control)} - Abs_{(sample)} \div Abs_{(control)}] \times 10$

- *FRAP*

This approach relies on the shilajit sample capacity to reduce Fe^{3+} ions to Fe^{2+} ions. Under acidic conditions and in the presence of TPTZ, the ferric-tripyridine-trazine (Fe^{3+}-TPTZ) complex is converted to the ferrous (Fe^{2+} - TPTZ) form, producing an intense blue color with maximum absorption at 593 nm. The method outlined in [21] was utilized to estimate the antioxidant power of the Standardized shilajit. We standardized a protocol for FRAP assay at LAPCOM (Psychopharmacology and Behaviour laboratory, UFRGS). This method measures the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) by antioxidant molecules, forming a blue Fe^{2+} - TPTZ dissolved in hydrochloric acid (usable for 2 days), ferric chloride hexahydrate, and ferrous sulfate heptahydrate, prepared freshly. Working solutions, with or without TPTZ depending on sample characteristics, are prepared in a definite ratio. Samples and controls are incubated in microtubes at a controlled temperature for 20 minutes, followed by 5 minutes rest at room temperature. Absorbance readings are taken in a 96-well microplate reader, and data analysis includes generating a standard curve (Fe^{2+} concentration vs absorbance) with an R^2 value > 0.9. Sample absorbance values are corrected for blanks, and Fe^{2+} concentrations are calculated using the standard curve. Analytical outcomes were represented as micromoles of Fe^{2+}/milliliter of all Shilajit batches, providing a reliable measure of antioxidant capacity [22].

- *Phosphomolybdenum Assay*

Antioxidant capacity of all shilajit batches was assessed using the Jan & Bokhari [23] phosphor-molybdenum technique. In this process, 1000 µl of molybdate reagent (0.6M

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H_2SO_4), 0.028 M sodium phosphate (NaH_2PO_4), and 0.004 M ammonium molybdate ($(NH_4)_6Mo_7O_{24}$). $4H_2O$ was taken in each test tube and 100 µl of the sample & standard solution (Vit. C)was added. Test tubes were covered and incubated for 90 min at 95 °C. Lastly, the absorbance at 765 nm of the samples was noted and the antioxidant contents in standardized and non-standardized shilajit samples were ascertained using an ascorbic acid standard curve.

- *Determination of Total Phenolic Content*

The overall phenolic compounds in Shilajit were measured utilizing the FC reagent, employing gallic acid as a standard [24]. Phenolic compounds constitute components of specialized metabolites primarily prevalent in plant species exhibiting significant structural diversity. Particular emphasis has been directed towards phenolic compounds due to their antimicrobial, antioxidant, anti-inflammatory, anticancer, and cardiovascular protection activities [25]. To summarize, 100 µl of the extract was mixed with 1 ml of diluted FC reagent and left at room temperature for 5 minutes. 1 ml of sodium carbonate was added to the reaction mixture, which was kept at room temperature for 90 minutes. Then, O.D was noted at 750 nm using a UV-visible spectrophotometer (JASCO V 730). Estimated phenol concentration was expressed as gallic acid equivalent in µg/g of Shilajit powder.

➢ *Statistical Significance:*

Data were analysed using one-way ANOVA with a subsequent t-test to determine statistical significance, performed by Graph Robot [26], with a p-value of less than 0.05 considered significant, confirming validity of the observed differences.

III. RESULTS AND DISCUSSION

➢ *UV-VIS Spectra:*

UV-VIS spectra analysis of standardized shilajit powder (VCA 501, VCA 502 & VCA 503) showed that the E4/E6 ratio ranged between 2.84 and 2.93, while the ratio of the non-standardized shilajit sample (market 1) ranged between 6.70 and 6.78. Humic substances with a more condensed structure and more aromatic components are associated with a lower E4:E6 ratio value (see Fig. 2). [27] Thus, it can be asserted that Standardized shilajit possesses a greater abundance of aromatic compounds than non-standardized shilajit.

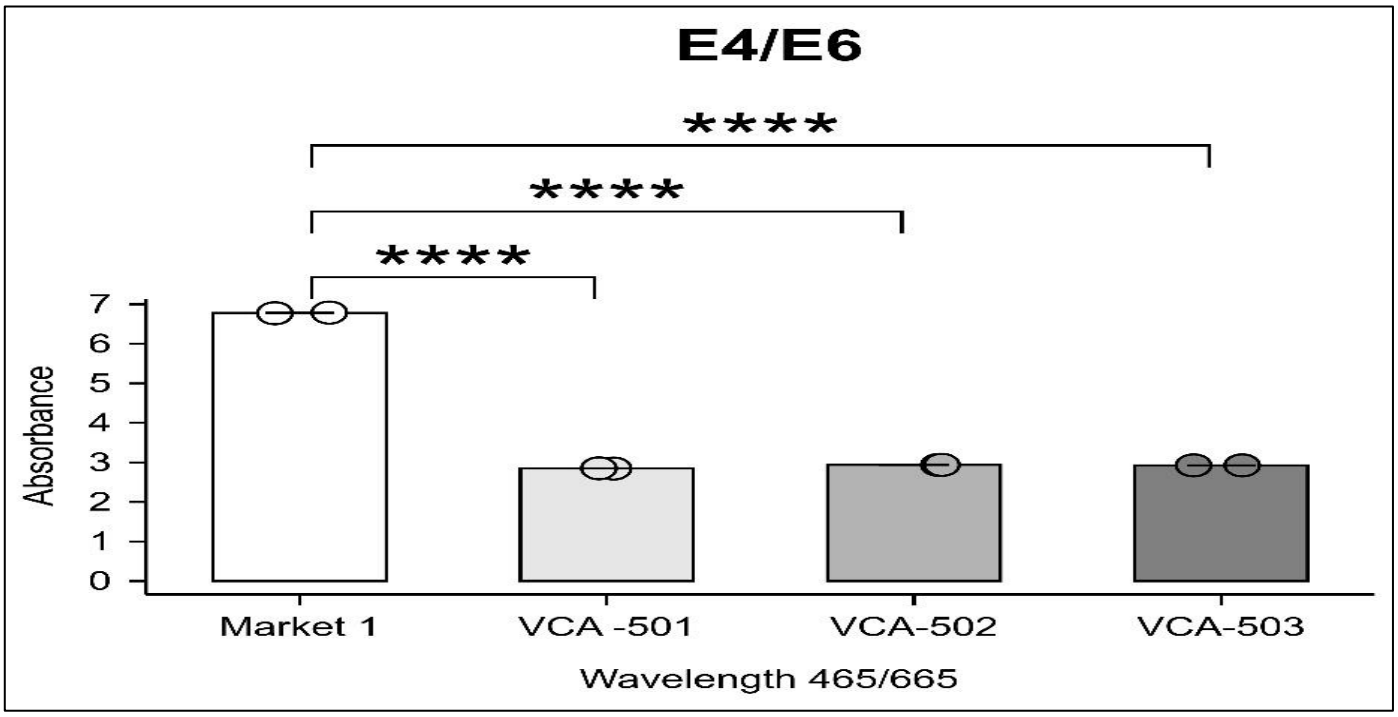

Fig 2 E4/E6 Ratio of Standardized Shilajit Powder
* P < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001

➢ *FTIR Analysis*

Shilajit displayed broadband at a certain wavelength, as demonstrated by several investigations of shilajit IR spectra analysis (as shown in Table 1). Humic acid was found to have a distinctive band at around 3,400 cm−1 (hydrogen-bonded OH group), as well as bands in the 1,640 cm−1 (conjugated C=C double bond), 1,400 cm−1 (OH bending of carboxylic acid), and 1,140 cm−1 (C–O stretching) regions [28],[29]. In this study, we have examined various functional groups found in VCA-standardized & non-standardized shilajit. FT-IR absorption spectra of heavy metal-free Standardized shilajit powder (Fig. 3B & 3C) revealed specific absorption bands at 1,625 cm−1 & 1,615 cm−1 (Oxygen-containing functional groups in HA). The presence of polysaccharides was confirmed by 1389 & 1385 (C-O stretching), while a band at about 3,400 cm−1 (hydrogen-bonded OH group) resembles the Humic acid broadband. The 1050 & 1035 cm-1 band corresponds to C–O stretches of alcohol, phenol-like compounds. non-standardized shilajit (fig 3 A) peaked at 1020,1398, 1634 & 2930 cm-1, indicating the occurrence of (C-O, C-H stretching) alcohol, and phenol-like compounds, but lacks a humic acid band.

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Table 1 Functional Groups at a Specified FTIR Frequency Range are Reported in Different Shilajit-Based Studies.

SI NO	Wavenumber	Functional group/Bonds	References
1	3300 - 3600 cm−1	OH, N-H bond (HA, phenolic, carboxylic acid)	[30]
2	2800 -3000 cm−1	C–H bond elongation	[31]
3	900 to 1400	C–O bond elongation	[32]
4	2902–2976 cm−1	C–H bond elongation	[31]
5	1049–1234 cm−1	C–O stretches of alcohol, phenol	[30]
6	700 to 900 cm−1	Aromatic structures	
7	1900 to 1000 cm−1	Oxygen-containing functional groups in HA	

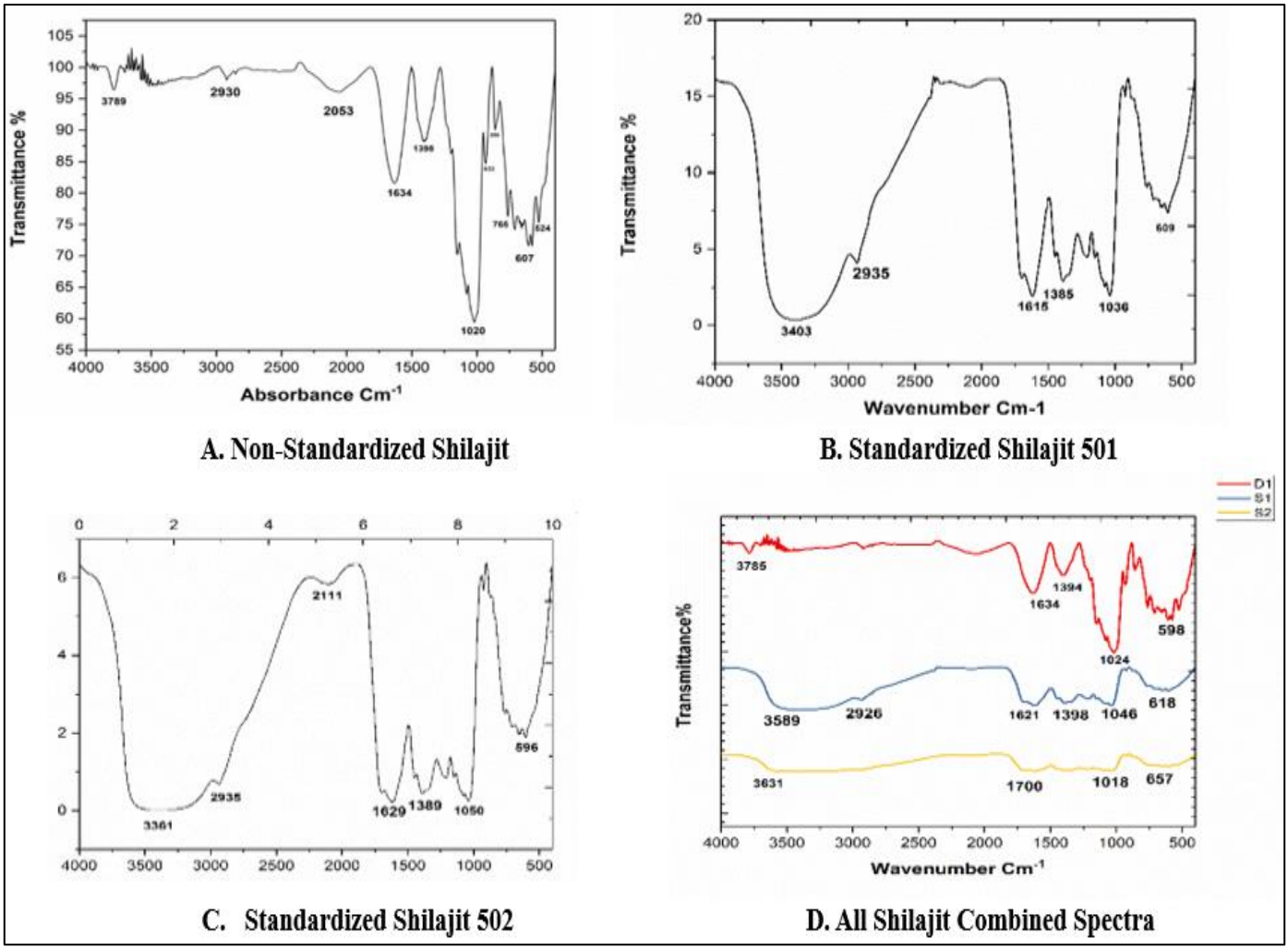

Fig 3 FTIR Absorption Spectra of A. Non-Standardized Shilajit, B. Standardized Shilajit 501, C. Standardized Shilajit 502, D. All Sample Combined Spectra

➢ *Scanning Electron*

- *Microscopy with Energy Dispersive X-Ray Spectrometry (SEM–EDX):*

FESEM images of the Standardized shilajit samples are presented in figure A, B, & C. It displayed Shilajit particle ranges of 5-7 µm, exhibiting a spongy HS structure with internal space. Figure (4B) represents rough, rounded surface made up of condensed particles. It also exhibited a round and rough surface of condensed particles demonstrated in figure. Due to similar processing and the nature of shilajit, there is less variation in morphologies among VCA samples. Additionally, EDS shows that all batches contained Mg, Si, S, k, Ca, Cl, Cu, and Se (Table 2), but no heavy metals such as As, Cd, or Pb, were detected in any shilajit samples. All these elements, detected in shilajit samples, possess notable medicinal significance; for instance, as Cu and Si exhibit antibacterial properties, while Ca and Mg are essential for maintaining bone health [33],[34].

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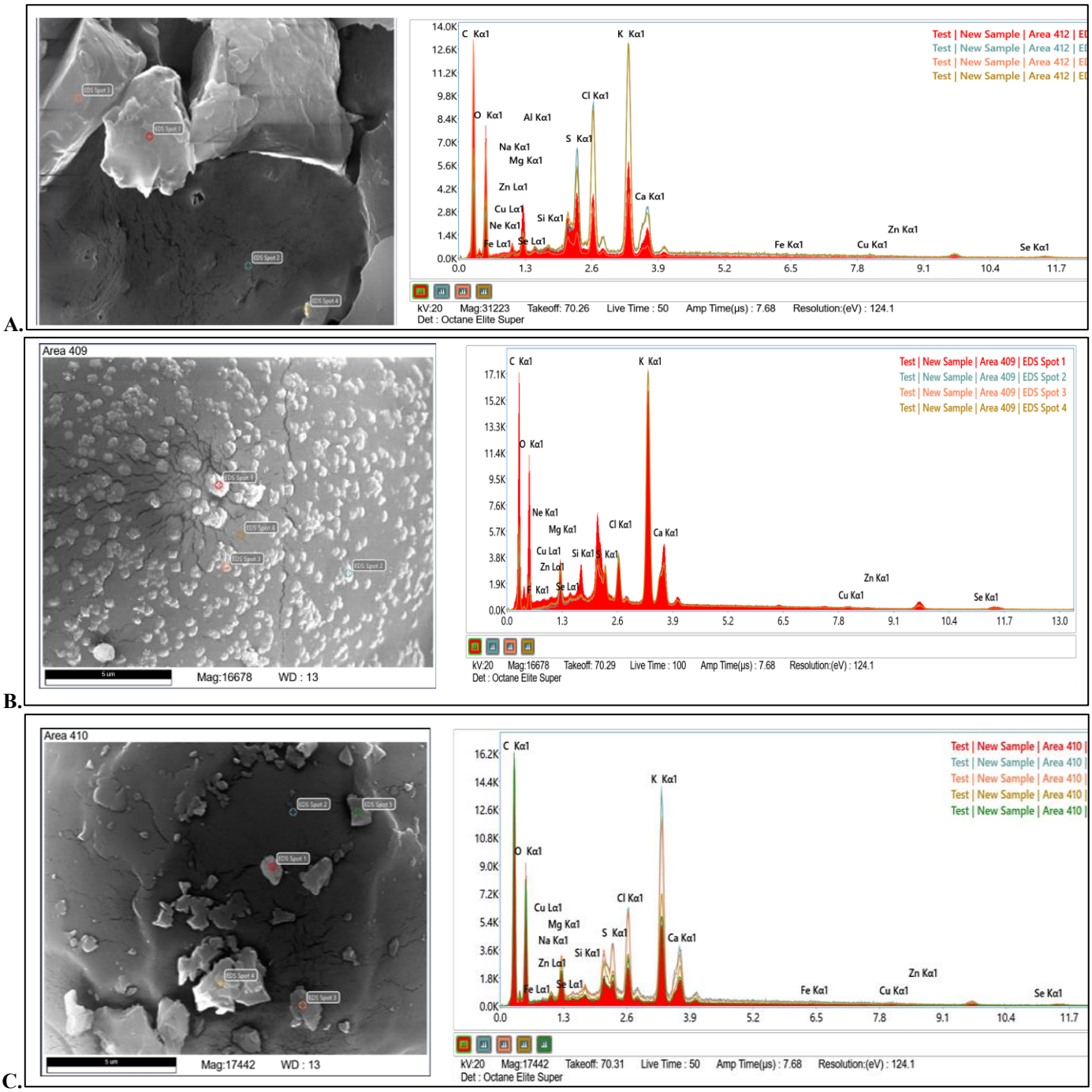

Fig 4 EDX Elements Analysis in A. VCA 501 B. VCA 502 & C. VCA 503

Table 2 Elements Present in Different Shilajit Samples.

S.I NO.	Elements	Atomic % VCA/SJ/501	Atomic % VCA/SJ/502	Atomic % VCA/SJ/503
1	C	71.8	66.7	71.1
2	O	21.7	12.3	24.4
3	Mg	0.8	1.3	0.5
4	Si	0.1	0.2	0.1
5	S	1.4	2.7	0.7
6	Cl	1.0	1.8	0.6
7	K	8.2	11.3	4.3
8	Ca	2.3	2.7	1.3
9	Cu	0.1	0.1	0.1
10	Se	0.6	1.1	0.3

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➢ *HRMS*

Graph plotted by comparing the Area (Max) of all compounds (29) across all datasets using a line chart (Fig. 5) and concentration values [RT (min)] across all datasets using a heatmap (Fig. 6). These metabolites included different primary as well as secondary metabolites such as benzene derivatives (Benzaldehyde, 2,4-Dimethyl-1-vinylbenzene, 4-Hydroxybenzaldehyde, 1_2_4-Trimethylbenzene), lactone (beta-Ionone, Acetophenone, N-(3-oxo-octanoyl)-homoserine lactone), fatty acids derivatives(Mycinonic acid III, Pinolenic acid, Docosahexaenoic acid ethyl ester), organic acids (2-Hydroxyhippuric acid), phenolics (2,6-Di-tert-butylphenol, Valerophenone, 2-tert-Butyl-4-methoxyphenol), terpenoids(Trans-Cinnamaldehyde, p-cymene, (-)-alpha-Cedrene), etc (See Table 3).

Table 3 Metabolites in Different Shilajit Samples

S I No.	Formula	Compounds	Sources ((HMDB)	RT [min] VCA501	VCA 502	VCA503	VCA 60 1	Non-Standardized Shilajit
1	C 10 H12	2,4-Dimethyl-1-vinylbenzene	Coffea & Poaceae (HMDB0040359)	1.24	11.51	18.40	12.72	9.86
2	C 10 H14	p-cymene	Herb and spice Common sage (FOOD00165)	10.42	12.88	10.89	11.71	11.69
3	C 10 H14 O2	6-Pentyl-2H-pyran-2-one	Plants (HMDB0031085)	12.43	12.99	14.60	41.07	13.20
4	C 10 H14 O3	2-Oxo-delta3-4_5_5-trimethyl cyclopentenyl acetate	Herb and spice (HMDB0301821)	8.22	9.97	9.96	12.45	8.42
5	C 11 H10 O4	Scoparone	Herbs, spices & citrus fruit (HMDB0030818)	8.33	11.47	10.43	8.0	10.65
6	C 11 H12 O	5,6-undecided-8,10-diyn-1-ol	-	15.35	15.5	15.34	14.78	12.76
7	C 11 H 14 O	Valerophenone	green vegetables, and wild celeries (Apium graveolens (HMDB0031208)	11.34	14.00	16.61	12.39	12.40
8	C 11 H16 O2	2-tert-Butyl-4-methoxy phenol	Biofluid (HMDB0059925)	8.58	13.20	10.01	12.25	9.68
9	C 12 H18 O2	Sedanolide	Food, Herbs, and spices (HMDB0302242)	13.15	12.75	14.63	14.42	11.34
10	C12 H19 N O4	N-(3-oxo-octanoyl)-homoserine lactone	Biofluid (HMDB0255213)	7.24	10.59	10.57	11.16	11.20
11	C13 H20 O	beta-Ionone	Plants & herbs HMDB0036565	11.18	11.20	11.58	13.07	12.64
12	C13 H20 O4	Mycinonic acid III	FA& Fatty acid derivatives	9.72	12.48	17.22	12.85	18.53
13	C15 H22	alpha-Curcumene	Biological fluid (HMDB0061838)	14.68	14.89	18.22	18.11	18.50
14	C 15 H24	(-)-alpha-Cedrene	Herb and spice Spice: Ginger (FOOD00206)	18.22	18.29	18.15	20.80	20.79
15	C 18 H 30 O2	Pinolenic acid	PUFA n-6(HMDB0340922)	17.98	17.52	17.51	22.11	19.70
16	C 24 H 36 O2	Docosahexaenoic acid ethyl ester	DHA, Biofluid (HMDB0251557)	19.23	20.46	19.40	21.94	21.76
17	C7 H 6 O	Benzaldehyde	Both plants & animals (HMDB0006115)	6.30	1.13	1.15	15.71	1.28
18	C7 H 6 O2	4-Hydroxybenzaldehyde	Bacteria, plants & animals (HMDB0011718)	5.41	2.54	1.83	11.56	7.75
19	C9H13 N O3	Ecgonine	Coca leaves (HMDB0006548)	8.09	8.18	8.17	1.30	9.33

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20	C8H8O	Acetophenone	Chicory plants (HMDB0033910)	2.46	0.94	0.95	19.32	1.70
21	C9 H 12	1_2_4-Trimethylbenzene	All eukaryotes, ranging from yeast to plants to humans (HMDB0013733)	9.45	8.58	10.0	15.53	1.25
22	C9H8O	Trans-Cinnamaldehyde	cinnamon trees (HMDB0003441)	10.18	13.31	9.69	15.71	10.74
23	C9 H9 N O4	2-Hydroxyhippuric acid	Biofluid or Excreta (HMDB0000840)	1.07	0.91	1.65	1.24	1.34

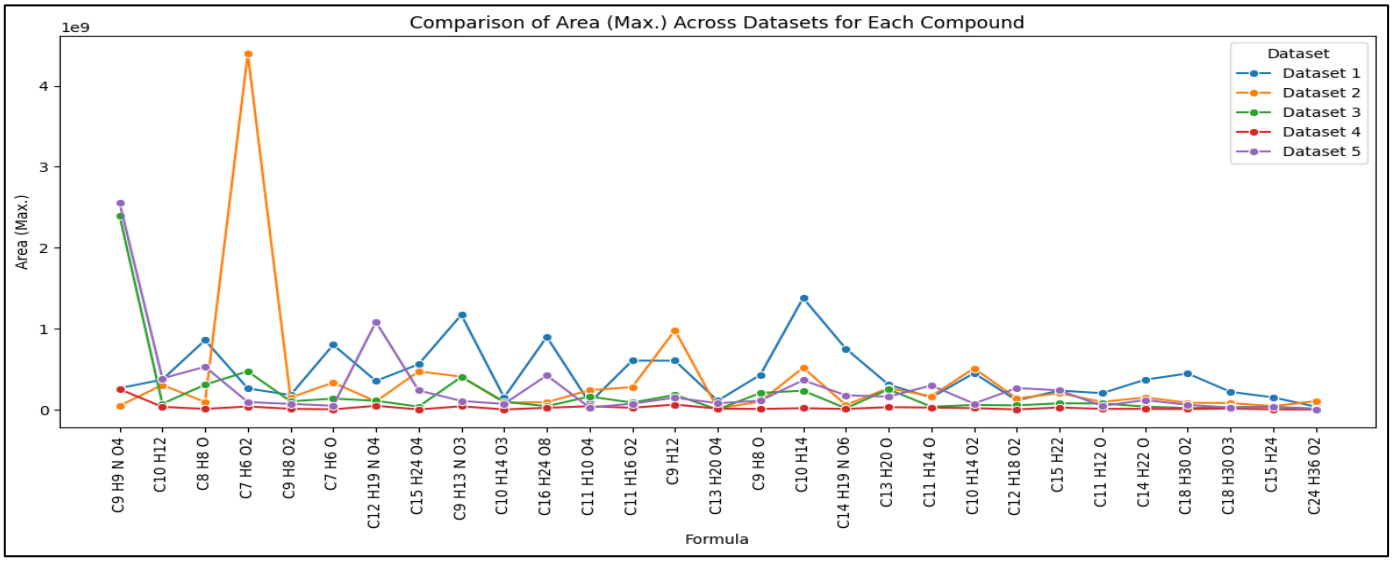

Fig 5 Comparison of Area [Max] of All Compounds [29] Across All Datasets Using Line Chart

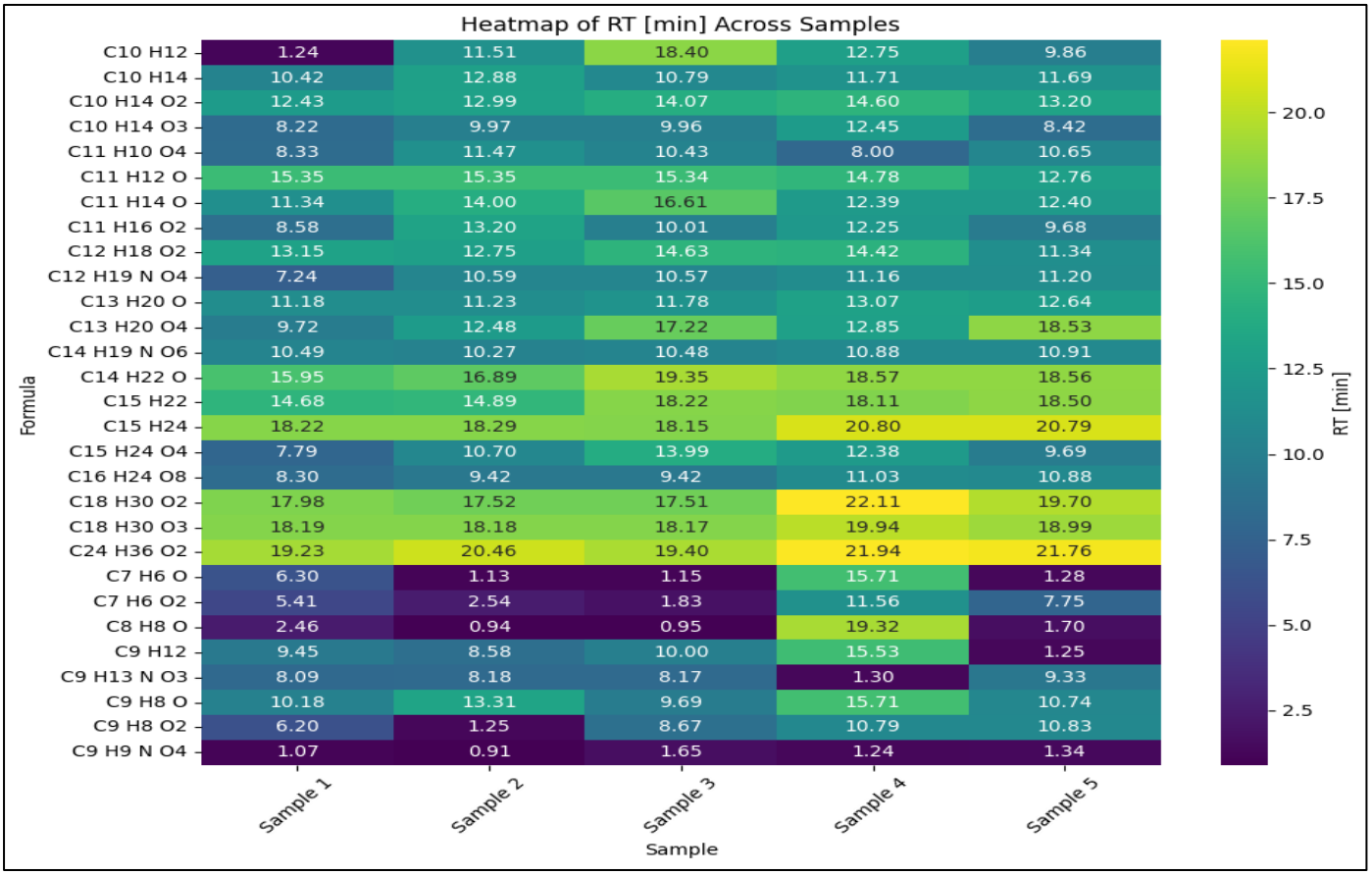

Fig 6 Comparison of Concentration Values [RT (min)] Across All Datasets Using Heatmap

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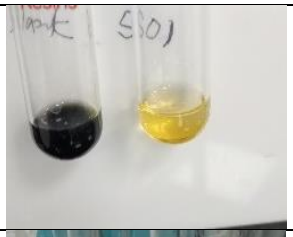
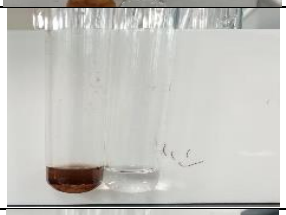
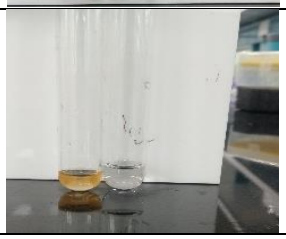
➢ *Qualitative Phytochemical Screening:*

Phytochemical test result of the non-standardized Shilajit showed that several secondary metabolites, including terpenoids, polysterol, and coumarins, were absent (See Table 5). But there were phenols and flavonoids. However, the VCA 501 and 502 standardized Shilajit samples contained every phytochemical examined. The presence of phytochemicals is recognized to have therapeutic value. Therefore, there is ample evidence that the biological activities are caused by the phytochemicals found in the standardized Shilajit samples.

Table 5 Phytochemical Screening of Different Shilajit Samples.

S. No.	Name of The Test	Observation	240501	240502	B1	Result	References
1.	Flavonoid's (Ferric chloride test)	Dark green color	+	+	+		[18].
2.	Phenols (Iodine test)	Transient red color appears	+	+	+		[35].
3.	Test for Phytosterol	Red color appears at lower layer	+	+	+		[35]
4.	Test for Terpenoids	Reddish brown color appears	+	-	-		[18]
5.	Coumarins (NaOH Test)	Yellow color appears	+	+	-		[36]

➢ *Antioxidant Analysis*

- *DPPH Method*

Radical quenching capacity of all shilajit batches were quantified via DPPH method. It was noted (from Fig. 7) that Standardized shilajit 501 & 502 exhibited the percentage of antioxidants being 81.173 % & 80.86 %, respectively, while non-standardized Shilajit (Market shilajit) showed 77.41 % of antioxidants (see supplementary Table S2). EC50 is determined using the "Quest Graph™ EC50 Calculator [37]. It has been found that VCA/SJ/501 & VCA/SJ/502 EC50 is 0.823 & 1.64, whereas the EC50 of market shilajit is 1.67 (Fig.8). However, the difference in RSC% and EC50 between standardized shilajit and non-standardized market samples was not statistically significant; nonetheless, standardized shilajit shows slightly better antioxidant properties than market shilajit.

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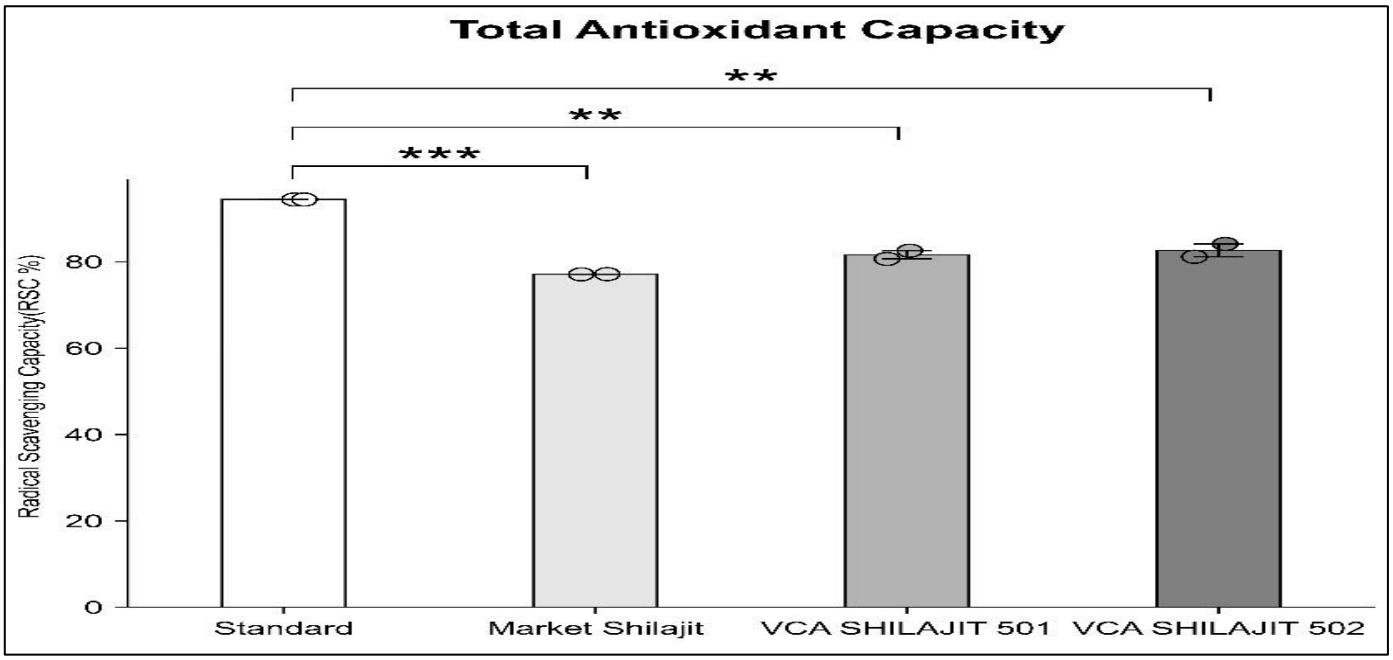

Fig 7 RSC% of Standard vs Standardized and Non-Standardized Market Shilajit.
* P < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001

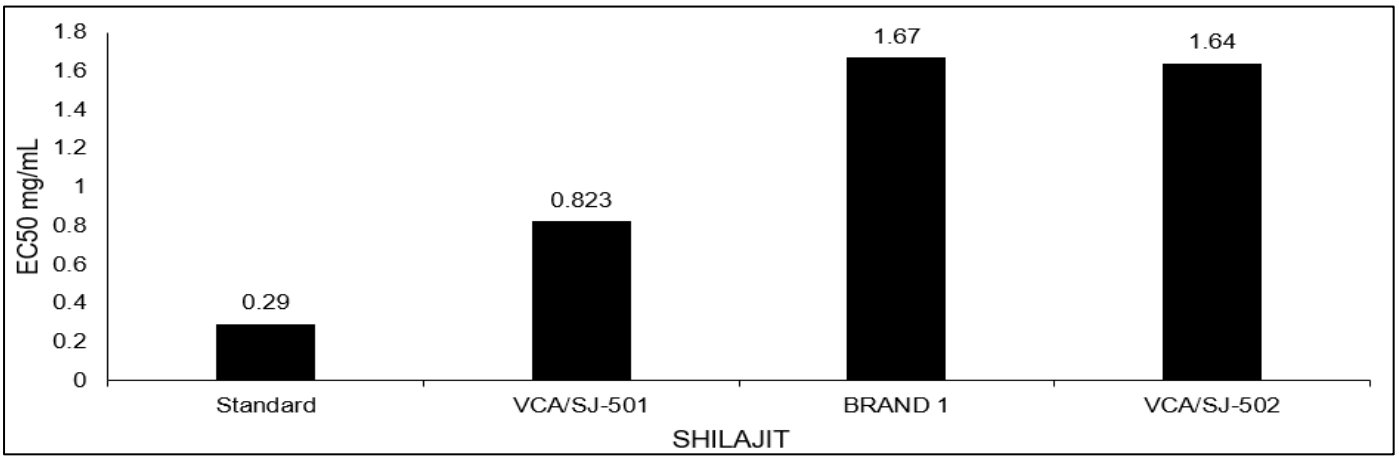

Fig 8 EC50 of Different Shilajit Samples.

- *FRAP:*

The antioxidant equivalent concentration (µg/g) of various Shilajit samples was quantified using an ascorbic acid standard curve. The antioxidant levels observed were 0.496 µg/g, 0.500 µg/g, and 0.503 µg/g for non-standardized Shilajit (Brand1), 501, and 502, respectively, as presented (See figure 10). No significant difference in Fe2+ micromoles/ml, among all shilajit samples as well as standard (ascorbic acid) was detected (as illustrated in Figure 10)

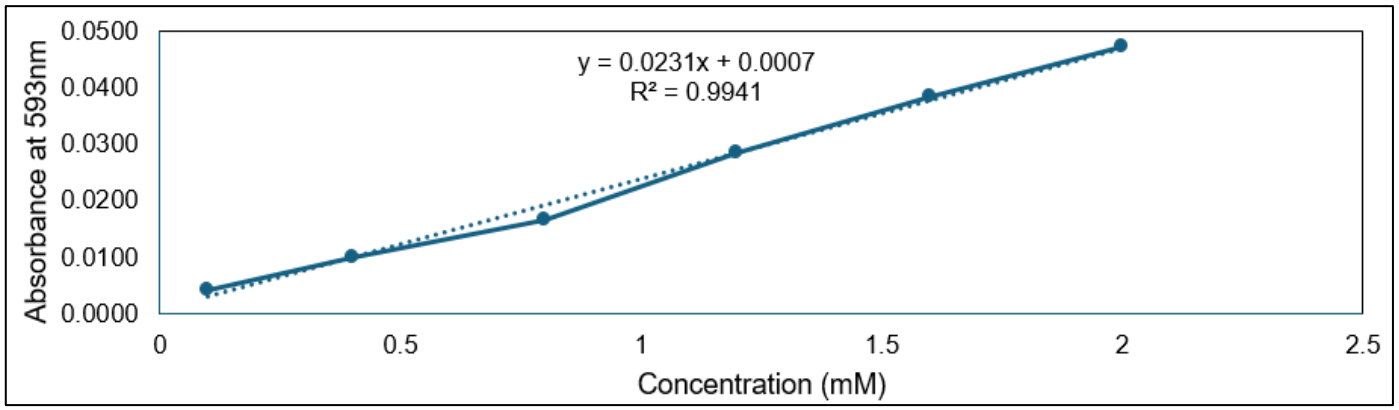

Fig 9 Standard Curve of Ascorbic Acid

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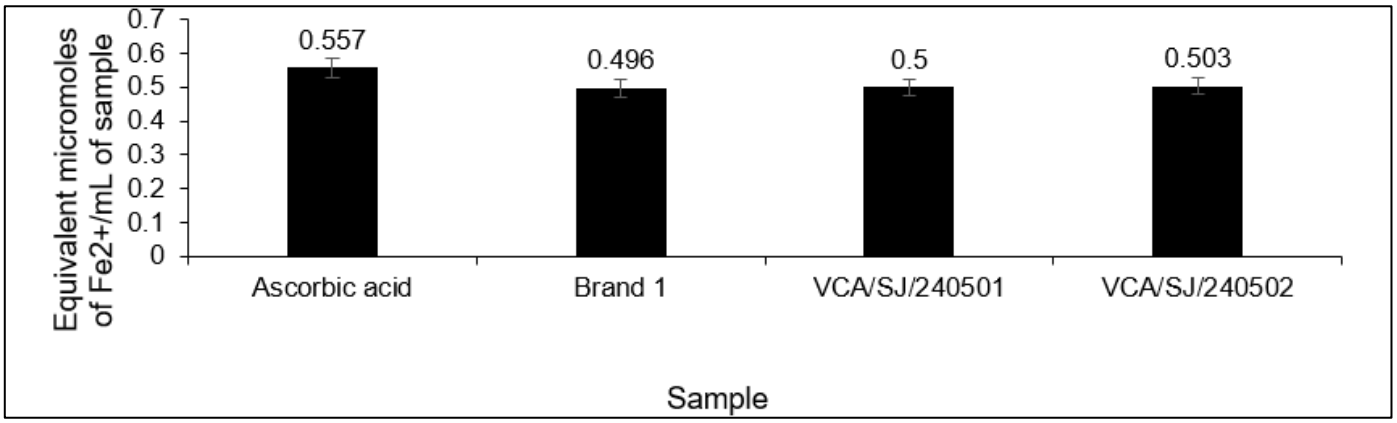

Fig 10 Equivalent Micromoles of Fe2+/mL of Sample

- *Phosphomolybdenum Assay*

An ascorbic acid standard curve was used to determine the antioxidant equivalent concentration in various shilajit samples, measured in µg/g (Standardized Shilajit 501, 502, and non-standardized). The antioxidant levels were found to be 1393.89±0.15, 1066.28±1.10, and 472.19±1.47 µg/g in VCA501, VCA502, and Non-Standardized Shilajit Brand 1, respectively (see supplementary table). It was observed that standardized shilajit contains significantly higher antioxidants compared to the non-standardized sample (see Fig. 12).

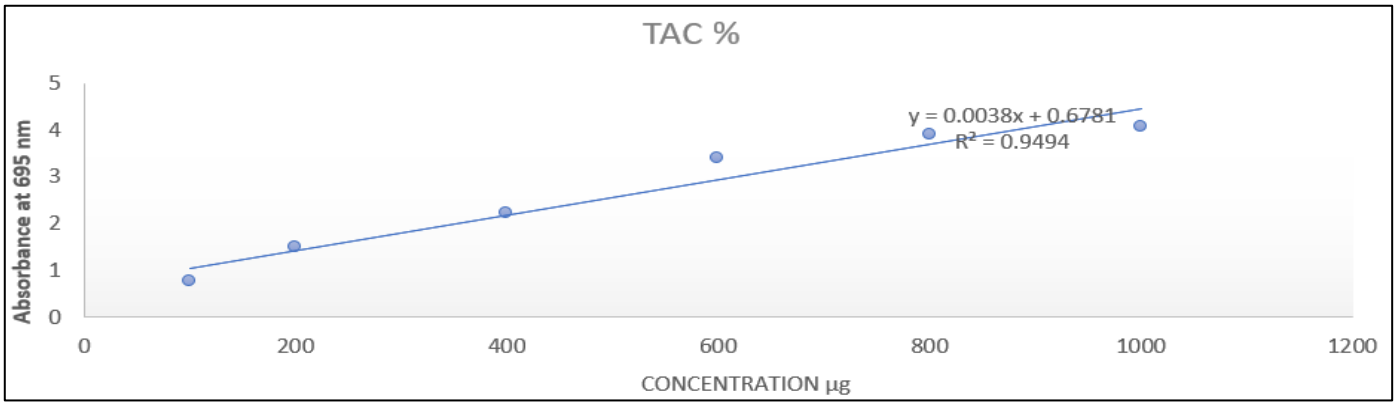

Fig 11 Standard Curve of Ascorbic Acid

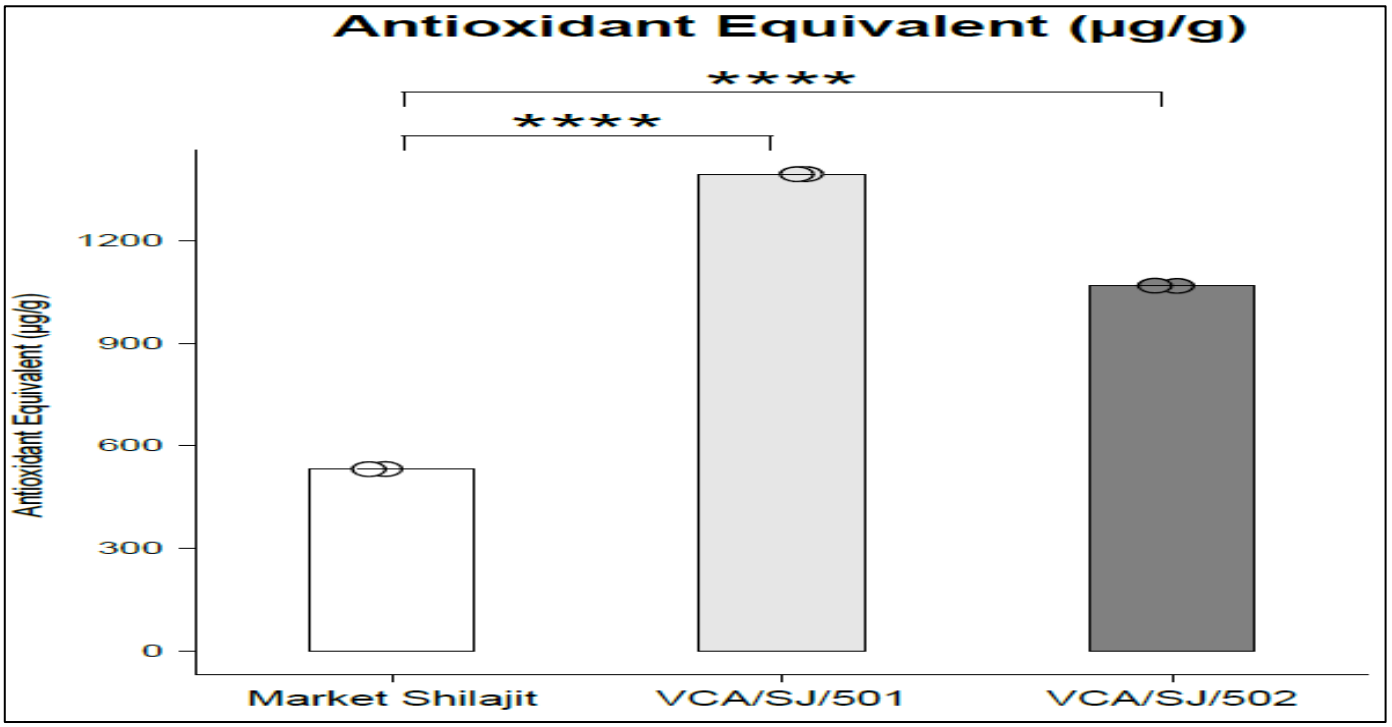

Fig 12 Antioxidant µg/g of Different Shilajit Extracts

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➢ *Phenol Content by FC Reagent*

The FC method assessed the phenol concentration of many shilajit samples using a standard curve of ascorbic acid (Fig. 13) (VCA/SJ/501, VCA /SJ/502, and market 1). The amounts of GAE/g were 150.97 ±0.26, 309.55±0.089, and 483.83±1.51, respectively, in market 1, VCA/SJ/502, and VCA/SJ/501(shown in Fig. 14). The Standardized shilajit shows statistically significantly higher phenol content in GAE/g than non-standardized market shilajit samples. (Fig. 13)

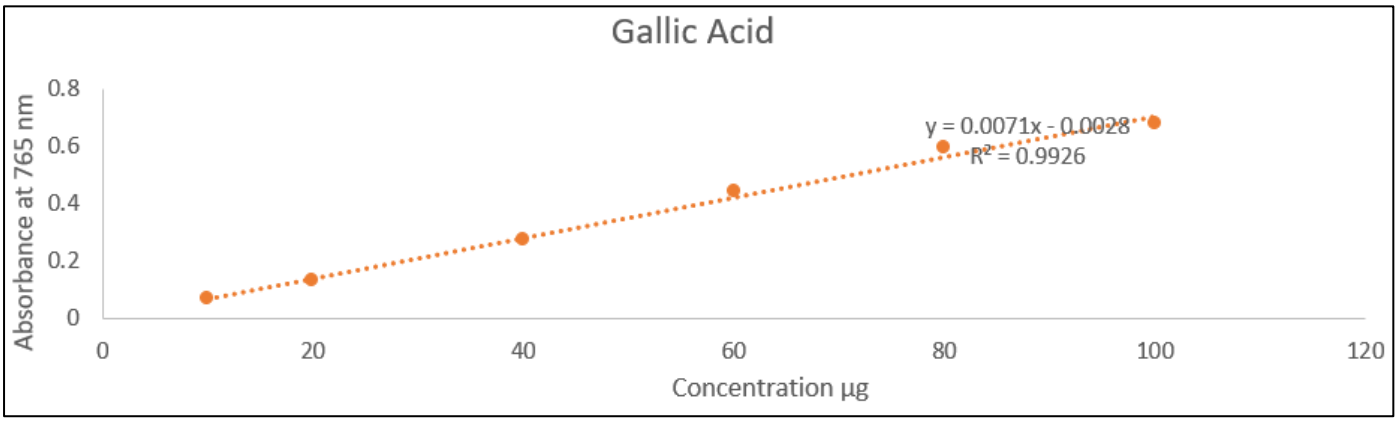

Fig 13 Gallic Acid Standard Curve

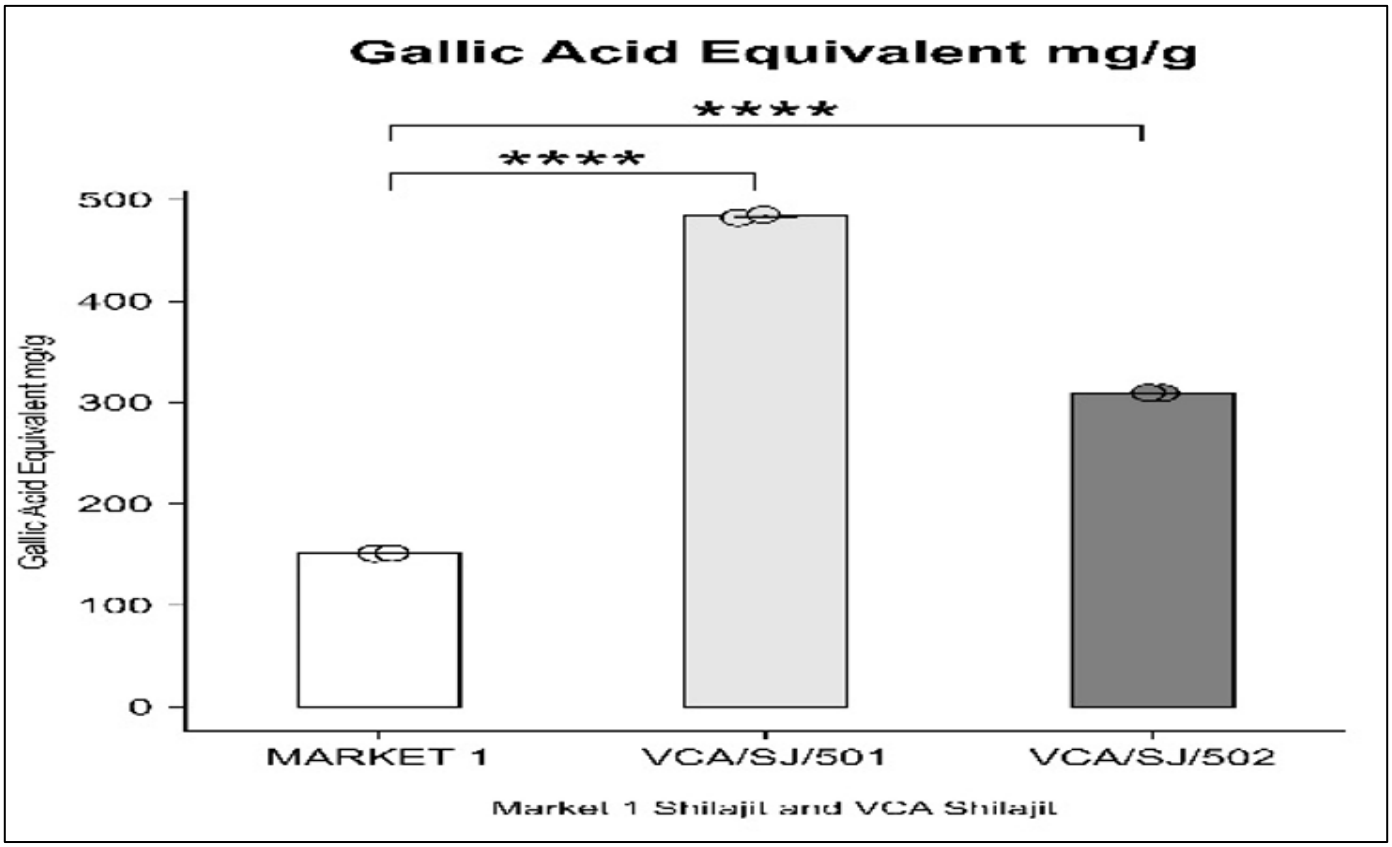

Fig 14 GAE mg/g Present in Different Shilajit Samples

IV. CONCLUSION

Two different types of Shilajit samples (Standardized Shilajit from VCA Healthcare and other market-available shilajit powders) were characterized, and their physicochemical properties, nutritional content, and elemental content were compared. The lower E4/E6 ratio of VCA-964 standardized shilajit indicates a higher concentration of aromatic components. IR spectrum analysis revealed a distinct band of humic acid in standardized shilajit, which was lacking in non-standardized samples. Additionally, SEMEDX analysis confirms the absence of heavy metals in VCA-964 standardized samples. Higher antioxidant percentages were detected in VCA-964 standardized shilajit than in market variants. further, various qualitative & Fc method-based tests validated their phytochemical richness and higher phenol content. So, we can conclude that standardization of shilajit powder enhances both safety and therapeutic potential, supporting its use in health and wellness applications.

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➢ *Credit Authorship Contribution Statement*

V. K. Tripathi: Conceptualization, Supervision. Komal Kumari: Conceptualization, Data curation, Writing – original draft, Formal analysis. Md. Zeyaullah: Data curation, Writing – review & editing. Harsh Jain: Data curation. Preeti Suman Saxena: Supervision, Writing – review & editing.

➢ *Conflict of Interest*

The author declares no conflict of interest.

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