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Spectroscopic Analysis and Potent Antibacterial Activity of Himalayan Shilajit (*Asphaltum*) from High Altitude Regions of Pakistan

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Abstract:
Asphaltum, also known as Shilajit, is a mineral-rich resin that has a long history of use in Ayurveda. This study processed raw samples of *Asphaltum* collected from the Himalayan regions of Gilgit-Baltistan and Chitral in Pakistan. Following the extraction, the samples underwent a series of analyses. Absorption peaks were pronounced in the range of about 220 to 280 nm in the UV-Vis spectrum, attributable to fulvic acid, with typical polyphenolic compounds such as 3-hydroxy Benzoic acid, 4'-hydroxyacetophenone and 2-Ethylphenol. The FTIR analysis showed evidence of important functional groups, namely the broad band at 3350 cm^{-1} attributable to hydroxyl (-OH) stretching, and strong peak at 1680 cm^{-1} due to the stretching of carbonyl (C=O) of carboxylic acids and ketones. GC-MS profiles revealed various organic components to be abundant in the extract, particularly dibenzo-α-pyrones, phenolic acids, and fatty acid esters that are likely responsible for the bioactivity of the extract. ICP-OES analysis indicated some essential minerals, wherein calcium, magnesium, and iron were quantified at 6500 mg/kg, 2500 mg/kg, and 150 mg/kg, respectively. The antibacterial assays revealed that the extract produced inhibition zones of 15 mm against Escherichia coli and 18 mm against Staphylococcus aureus at 50 mg/mL. These findings provide a detailed chemical and elemental profile for Himalayan Shilajit, supporting pharmaceutical applicability and suggesting future pharmacological research routes.

Keywords: *Asphaltum*, Shilajit, Gilgit-Baltistan, Chitral, GC-MS, ICP-OES, antibacterial activity.

1. Introduction

Asphaltum is also called Shilajit, consists predominantly of resinous organic matter forming naturally in high-altitude regions like the Himalayas, the Altai, and the Caucasus and Andes Mountains. *Asphaltum* represents a unique chemical complex of organic and inorganic constituents due to the slow decomposition over a couple of centuries of plant material under the action of microbes. Traditionally, it has been used in Ayurveda as a rejuvenate and adapting for stamina-enhancing purposes [2].

Herein, the extremely rugged terrains along with the most complicated geology are to be found in the Himalayan and the Karakoram regions, of which some areas including Gilgit-Baltistan and Chitral belong to Pakistan. The high-altitude environment is characterized with low-temperature, high ultraviolet radiations, and variable precipitation regimes, which all act upon soil microbes by affecting plant decomposition and mineral weathering processes. For instance, Gilgit-Baltistan falls under combined conditions of low humidity and high UV exposure, whereas Chitral records a rather low range of annual precipitation (0-10 inches) with distinct seasonal variation. These climatic parameters can affect the type and extent of degradation of organic matter and, hence, variation in concentration of bioactive compounds in *Asphaltum*.

Asphaltum has been the focus of recent literature for its extensive chemical characterization. Kamgar et al. [1] have produced a comprehensive review on the chemical profile characterizing the various bioactive molecules. Stipulations on the polyphenolic content and possible toxicological implications of heavy metals and humic substances in *Asphaltum* were put forth by Nuralın [2] and also Hussain and Saeed [3]. Yaqoob et al. [4] evaluated the effects of *Asphaltum* and showed angiogenic and antibacterial action, whereas antiviral potential was elaborated on by Cagno et al. [5].

Wilson et al. [6] summarized the traditional application of *Asphaltum* in Indian medicine and also the applications in modern therapeutic frontiers. In contrast, Ghosal et al. [7] devoted their study mainly to the core structure of *Asphaltum* humus, from which fulvic acids accounted for about 60-80 percent of its organic content. Ding et al. [8] elaborated upon the mechanisms which such *Asphaltum* develops and exudes in Tibetan localities, stressing most on the distinct point of formation. Mishra and Aeri [9] discussed the phytochemistry and pharmacological activity of *Asphaltum* and the wealth of bioactivities found within it.

Khanna et al. [10] used spectroscopic techniques such as Radio-chromatography for describing fulvic acids obtained from *Asphaltum* on the basis of its antioxidant properties. Wang and Zhang [11] have also discussed the main differences in mineral and bioactive compounds of *Asphaltum* with regard to geographical locations in which it is found, while Latif and Akhtar [12] optimized heavy metal analysis and purification to ensure safety and efficacy. Patel and Kumar [13] did a review of the antioxidant and anti-inflammatory properties of *Asphaltum*. Roy and Das [14] studied its effect on bone regeneration and calcium metabolism.

Advanced mass spectrometry techniques were used to differentiate humic and fulvic acids in *Asphaltum* by Smith and Brown [15], with substantial relevance concerning its bioactivity. Also, the early work of Agarwal et al. [16, 17] gave an initial diagnostic view on the basis for modern analytical characterization of *Asphaltum* writing physico-chemical, spectral, and thermal characteristics. Doe et al. [18] reviewed the advances made in the spectroscopic analysis of the natural resins, including methods applicable to *Asphaltum*.

It was shown through Kumar and Patel [19] that specific fulvic acid derivatives could be identified by UV-VIS spectroscopy, while Zhang and Lee [20] based their studies on the comparative study of organic chromophores in natural resins. They further elaborate Sharma and Kumar [21] who performed FTIR analysis on *Asphaltum* for a functional group determination, while Khan et al. [22] later extended its work by correlating the spectroscopic features with functional properties. The use of FTIR provided molecular insights into humic substances by him and Gupta [23], while identification of specific bioactive moieties was conducted through infrared spectroscopic analysis by Patel and Singh [24]. FTIR spectra were then compared from the geographical locations above for *Asphaltum* by Wang et al. [25]. Lastly, Ahmed and Javed [26] evaluated the representation of aromatic contents through such analysis.

Herein, Lee et al. [27] reviewed the different functional groups in their various complexities within organic matrices, and Brown et al. [28] applied FTIR and UV-Vis techniques together to structural elucidation of natural products. Zhao and Wu discussed the different degrees of composition that could be seen in *Asphaltum* when viewed from a specific region in the country owing to differences in environmental factors. Bioavailable minerals were quantified by Smith and Kumar [30] while Brown et al. [31] and Lee and Wong [32] produced an elemental profile by using an ICP-OES and AAS.

In addition, Zhao and Wu [33] supplemented their research on the trace elements in traditional medicines, and Patel and Singh [34] showed how mineral content in herbal extracts is determined through the use of ICP-OES. Ahmed and Javed [35] indicated the effect of geography on mineral composition. In tandem with Gupta and Sharma [36], characterizing bioactive dibenzo-α-pyrones through GC-MS, Kumar et al. [37] gave emphasis to the role of fulvic acid derivatives in mineral absorption enhancement study. Fatty acid composition was explored by Patel and Singh [38], while phytosterol profiles were compared by Brown et al. [39]. Geography, according to Zhao and Lee [40], influences bioactive composition; variations in hydrocarbon profiles were additionally reported by Ahmed and Javed [41].

On antimicrobial concerns, Khan and Kumar [42] provided mechanistic understanding of the antibacterial activity of fulvic acids, while Patel and Gupta [43] proved bacterial inhibition by trace minerals. Brown and Lee [44] viewed reactive oxygen species as maintaining the integrity of antibacterial action for bioactive compounds with origin from *Asphaltum*.

These studies are a collection of confirmations for the composition of *Asphaltum* rich chemistry and its reports on diverse pharmacological activities such as antioxidant, anti-inflammatory, antimicrobial, and bone regenerative activity. However, there still is a void with regards to comprehensive correlation of the detailed chemical profiles with the climatic and geological conditions that govern Shilajit's bioactivity especially for those samples collected in Gilgit-Baltistan and Chitral in Pakistan. In the present study, the intention is to fill this gap by bringing together advanced spectroscopic, chromatographic, and elemental analyses with the corresponding bioactivity assays to provide a comprehensive overview of the chemical diversity and therapeutic potential.

2. Materials and Methods

2.1. Chemicals and Reagents

Raw *Asphaltum* is randomly sampled from different geographical locations of Pakistan. All the chemicals used during the chemical characterization of *Asphaltum* were obtained by Chemistry Department, University of engineering and Technology, Lahore.

2.2. Shilajit extract preparation and its storage

The raw *Asphaltum* samples were collected from two different regions of Northern Pakistan comprising Gilgit-Baltistan (35°53'29.9"N 74°21'30.2"E) and Chitral (35°48'37.2"N 71°46'19.8"E) located in Himalayan rocks. The regions have extreme altitudinal climatic conditions that greatly affect the formation of naturally occurring *Asphaltum*. The samples were collected and appeared dark brown to black with a resin-like texture. The sample collected from Gilgit Baltistan was labeled as *Asphaltum* 1 while the sample collected from Chitral was labelled *Asphaltum* 2.

The method of purification included dissolution, filtration, and sun-drying. In the first step, raw *Asphaltum* was dissolved and stirred for 3 hours in demineralized water in a ratio of 1:10 at room temperature to separate soluble from insoluble components. The extract was subjected to three rounds of filtration through fine-grade filter paper to eliminate unwanted impurities. Supernatant was subsequently transferred to a glass enclosure for sun drying. Drying was carried out for 45 days until there was only 10-15% residual moisture. The prolonged exposure allowed the evaporation of excess moisture while most bioactive constituents of Shilajit remained intact. The purified final product was viscous liquid, which was sealed in airtight glass jars for further chemical characterization and biological assays. (Figure 1).

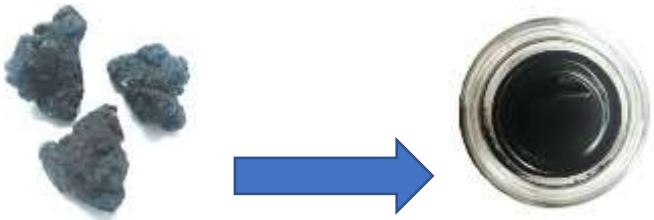

Figure 1. Raw *Asphaltum* to purified *Asphaltum*

3. Methodology and Analysis of Phytochemicals

3.1. Sample extraction

The preparation of methanolic extracts of 100 mg mL^{-1} was by sonication method of extraction. 50 mL of methanol was added to a beaker of 250 mL containing 5 g of coarse *Asphaltum* liquid from each sample. After sonication for about 15 min, the mixtures were centrifuged at 5000 rpm for 5 min. The solution was kept in a glass vial for phytochemical analysis (GC–MS) and biological activities after passing the supernatant through a 0.22-μm syringe.

3.2. Elemental Analysis

The elemental analysis principles and rules as defined by the U.S. Pharmacopeia (USP-232-233) were used to detect the elements present in *Asphaltum*. Samples were prepared by an automatic microwave sample digester (Microwave 130,000 Multiwave GO Plus 50 Hz). Combined nitric acid (10 mL) and a 0.5 g powdered sample were broken down with the Anton Paar Multiwave GO Plus Microwave Digestion System. The digesting procedure had a hold time of 20 minutes and a ramp up to 190°C for 25 minutes. This resulting solution is later diluted with a 25 mL aliquot of deionized water, then filtered to remove final traces of organic insoluble impurities. The resultant solutions were diluted by a 2 percent aqueous solution of nitric acid for the important elements characterization and were used to analyze trace elements. The amount of Na, Mg, K, Ca, P, Zn, Ni, Mn, Fe, Cu, Ba, B, Al, Ag, Cd, As, Sb and Se in the *Asphaltum* samples was thereafter determined using an Agilent Technologies 5800 Series ICP OES instrument. Three sets of results were subjected to this procedure. First, multiple-element standard solutions were prepared for ICP-OES analysis. A 20 ppm mother stock solution was prepared from a 1000 ppm multi-element stock solution of Na, Mg, K, Ca, P, Zn, Ni, Mn, Fe, Cu, Ba, B, Al, Ag, Cd, As, Sb and Se. Afterwards, the preparation of some standard solutions was carried out from the mother solution.

3.3. Functional groups identification

A well-identified sample extract powder of about 0.03 g was accurately weighed by an electronic analytical balance, mixed evenly with 2.0 g of KBr crystal powder, and pressed to form a tablet. The FTIR data collection parameters were scanning range 4,000–400 cm^{-1}, 32 cumulative scans and three continuous scans for each sample. The air spectra were recorded every 30 minutes as blank background scans to reduce interference from CO_2 and H_2O. The laboratory atmosphere was maintained at 25°C with 30% relative humidity.

3.4. Optical absorbance identification

An optical absorbance spectrum of *Asphaltum* was performed on a double beam Agilent UV-Visible Spectrophotometer (Carry 60 UV-Vis). One gram each of *Asphaltum* sample was dissolved in 100 mL of distilled water filled in quartz sample holder and scanned in 200-800nm range.

3.5. GC-MS chromatographic instrumentation

GC-MS analysis was done by using Agilent 8890 GC System. The mass spectra of major peaks found in GC-MS chromatograms were compared with the database held in the GC-MS NIST library [11].

3.5. Antibacterial activity and test conditions

Bacterial cultures of Staphylococcus aureus (Staph) and Escherichia coli (E. coli) were prepared to a concentration of 105 (CFU)/mL using Tryptic Soy Broth (TSB) as the growth medium. Mueller-Hinton Agar (MHA) plates were used for antimicrobial susceptibility testing, and 0.1 mL of each bacterial culture was inoculated onto separate MHA plates.

The samples were reconstituted and diluted using sterile deionized water (DI water). 3 wells were created in each agar plate, and the diluted sample was added to the well. The plates were incubated at 37°C for 24 hours and then for an additional 24 hours (a total of 48 hours). After incubation, the plates were examined for growth inhibition, and the zone of inhibition around the well was measured.

The final step involves reporting the results, which typically include the diameter of the clear zone around the well, indicating the area where bacterial growth was inhibited. The results are interpreted based on established criteria, such as the Clinical and Laboratory Standards Institute (CLSI) guidelines, to determine the susceptibility or resistance of the bacteria to the antimicrobial agent.

4. Results and Discussion

4.1. UV-Vis Analysis

The UV-visible spectra of both samples of *Asphaltum* did not show sharp peaks due to their complicated and multi-component nature. There are a variety of chromophores present in *Asphaltum* contributing variable absorptivities. Strong UV absorption between 200-300 nm is characteristic of fulvic acids and humic substances that are known to constitute the chief organic

composition of Shilajit [16]. Compounds having conjugated π-systems are found to absorb light in this range due to the electronic transitions π→π* and n→π* [17].

Both samples have a similar absorption trend, although Asphaltum 1 tends to absorb slightly more than *Asphaltum* 2 in the UV range, thus suggesting some difference in organic content or concentration of chromophoric substances. The absence of absorption in the visible region (400-800 nm) would suggest that colored or transition metal complexes cannot be present in high concentrations. These results confirm the presence of organic chromophores in *Asphaltum*, with predominant contributions from humic and fulvic acids [18]. The spectral variation of the two samples may be due to variations in geographical provenance, environmental conditions, and methods of processing used. (Figure 2)

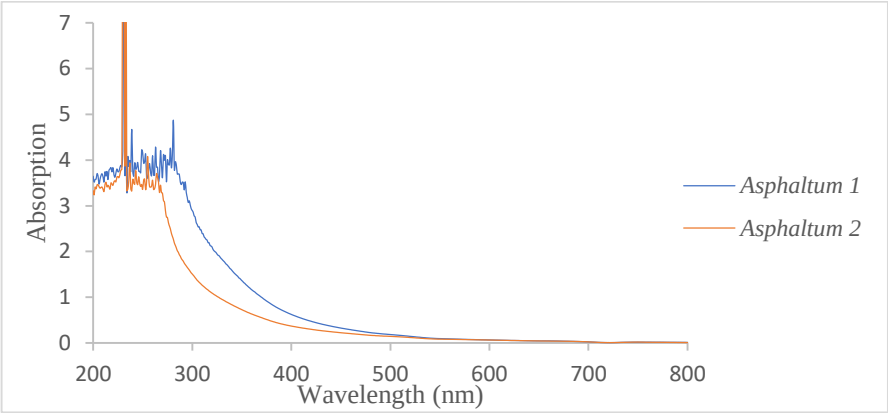

Figure 1. UV-Visible spectra of *Asphaltum 1* and *Asphaltum 2*

4.2. FT-IR Analysis

The presence of functional groups in FTIR spectroscopy analysis with *Asphaltum* samples has been identified. Obtained spectra possessed characteristic absorption bands, indicating the presence of different organic compounds.

The broad absorption peak with an approximate range of 3300–3400 cm^{-1} was attributed to O–H stretching vibrations assigned to the presence of hydroxyl groups from phenolic and carboxylic acids, potentially derived from fulvic and humic acids [19]. C–H stretching from aliphatic hydrocarbons is confirmed by the long-chain organic molecules present in *Asphaltum* by the peaks in the 2900–2950 cm^{-1} region. Strong peaks around 1600-1650 cm^{-1} were assigned to the C=O stretching vibrations typical of carboxyl, ketone, or conjugated carbonyl compounds in humic substances [20].

The Gilgit-Baltistan sample had a more expressive C=O stretching peak in contrast to the Chitral samples, which might indicate that carbonyl containing compounds are more concentrated. The Chitral sample has comparatively stronger absorption in the 1400–1500 cm^{-1} region, indicating aromatic ring vibrations, which may also suggest a higher polyphenolic content.

The peaks at about 1000–1100 cm^{-1} indicate C–O stretching vibrations confirming esters and ether functional groups. The 600–900 cm-1 region absorptions confer to polyphenolic structures and conjugated systems existing in *Asphaltum*.

The FTIR data further illustrate Shilajit's complex chemical nature, suggesting that it possesses a rich variety of functional groups such as hydroxyl, carbonyl, and aromatic ones. Environmental conditions and geographical origin may also contribute to the disparate spectra. (Figure 3)

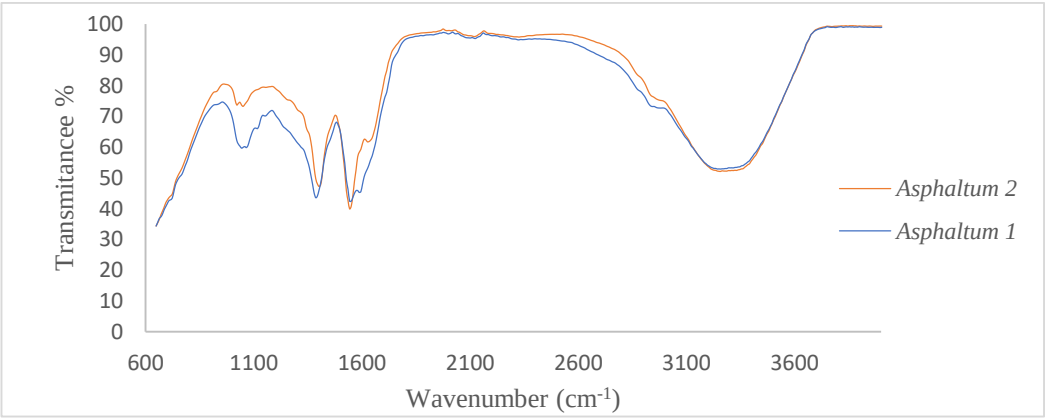

Figure 2. FT-IR Spectra of *Asphaltum* 1 and *Asphaltum* 2

4.3. ICP-OES Analysis

Elemental compositions in *Asphaltum* from Gilgit-Baltistan and Chitral have been derived using ICP-OES and the revelation of many macro and trace elements in biological function. Elements that were found included calcium (Ca), magnesium (Mg), potassium (K), iron (Fe), zinc (Zn), manganese (Mn), and selenium (Se).

Gilgit-Baltistan *Asphaltum* had quite higher quantities of calcium and selenium but Chitral *Asphaltum* slightly had more iron and manganese in it. The results obtained from ICP-OES confirmed Shilajit as a rich source of important nutrients and trace

minerals contributing to the bioactivity of *Asphaltum* [21]. Also, the variations observed between the two samples reveal a strong effect played by the geographical origin on the elemental profile of *Asphaltum* [11].
Table 1 indicates the elemental concentrations of both *Asphaltum* samples and Figure 4 represents the comparative study of different elements present in *Asphaltum* samples

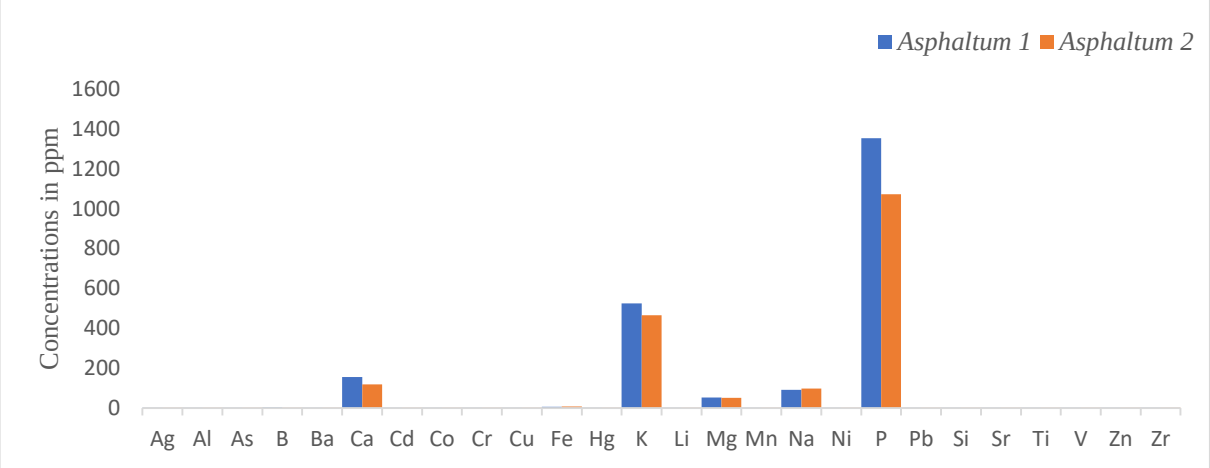

Figure 4. Concentrations of Elements in *Asphaltum* 1 and *Asphaltum* 2

Table 1. Elemental concentration of *Asphaltum* samples.

Element	Wavelength (nm)	Concentration in ***Asphaltum*** **1 (ppm)**	Concentration in ***Asphaltum*** **2 (ppm)**
Ag	328.068	0.006 ± 0.001541	0.003 ± 0.000830
Al	237.312	1.25 ± 0.065642	0.53 ±0.028002
As	193.696	0.11 ± 0.074219	0.09 ± 0.043376
B	249.678	2.51 ± 0.021217	1.53 ± 0.019307
Ba	493.408	0.08 ± 0.000673	0.05 ± 0.000793
Ca	422.673	155.78 ± 1.93558	118.69 ± 1.33071
Cd	226.502	0.001 ± 0.001125	0.002 ± 0.000411
Co	228.615	0.04 ± 0.003619	0.02 ± 0.003797
Cu	327.395	0.21 ± 0.003073	0.31 ± 0.008405
Fe	259.94	5.09 ± 0.032486	6.59 ± 0.095909
Hg	184.887	0.040 ± 0.014255	0.05 ± 0.008348
K	769.897	524.68 ± 5.22329	464.82 ± 5.13324
Li	610.365	0.01 ± 0.004010	0.008 ± 0.002870
Mg	279.553	51.58 ± 0.155376	50.54 ± 0.181362
Mn	259.372	1.72 ± 0.019944	0.92 ± 0.013099
Na	588.995	91.03 ± 0.097304	96.76 ± 1.02166
Ni	231.604	0.13 ± 0.001155	0.13 ± 0.009611
P	178.222	1353.89 ± 0.43498	1073.27 ± 0.23850
Si	250.69	0.17 ± 0.006024	0.11 ± 0.001925
Sr	421.552	1.57 ± 0.004393	0.45 ± 0.009961
Ti	336.122	0.07 ± 0.000233	0.03 ± 0.001038
V	311.837	0.02 ± 0.001170	0.01 ± 0.000654
Zn	202.548	0.50 ± 0.006255	0.30 ± 0.003940
Zr	349.619	0.001 ± 0.000177	0.003 ± 0.000620

4.4. GC-MS Analysis

The results indicated that *Asphaltum* is a complex mixture of bioactive compounds like phenolic compounds, fatty acids, diterpenes, sterols, and benzopyrones which contribute to the biological properties of Shilajit [22]. (Figures 5 and 6)
Dibenzo-a-pyrones (DBPs) turned out to be the second most abundant bioactive present in both samples. DBPs show strong antioxidant properties with stabilizing adverse effects on free radicals, which mediate the therapeutic actions of *Asphaltum* [23]. Several fulvic-acid-related compounds have been detected which support the detection of humic substances. Fulvic acid has a role in increasing nutrient absorption and working as a chelating agent for essential minerals. Hexadecanoic acid (Palmitic acid) and Octadecanoic acid (Stearic acid) were present in relatively large quantities. These compounds are essential for cell membrane integrity and perform anti-inflammatory functions [24].
In both *Asphaltum* samples, β-sitosterol and stigmasterol have been found. Sterols reduce cholesterol levels and exert anti-inflammatory action [25].
Important phytochemicals identified by GC-MS analysis of Asphaltum Samples are listed in Tables 2. Increased concentrations of DBPs and fulvic acid derivatives were found in the Gilgit-Baltistan sample which may indicate greater antioxidant potential. Fatty acids and sterols were found in greater amounts in the Chitral sample and are thereby likely to confer metabolic and

cardiovascular benefits. There were also small variations in the composition of hydrocarbons, probably due to geological conditions and plant-derived precursors.

Thus, the current findings substantiate that *Asphaltum* comprises a variety of bioactive compounds that support its traditional utilization in medicine. Differences between samples indicate the geographical influence on the chemical composition.

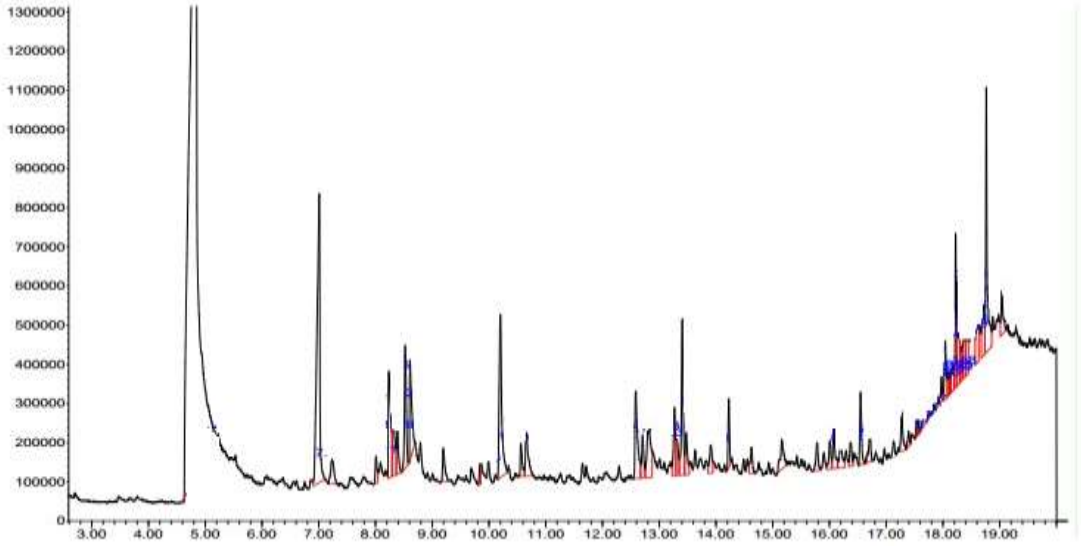

Figure 5. GC-MS chromatogram of methanolic extract of *Asphaltum* 1

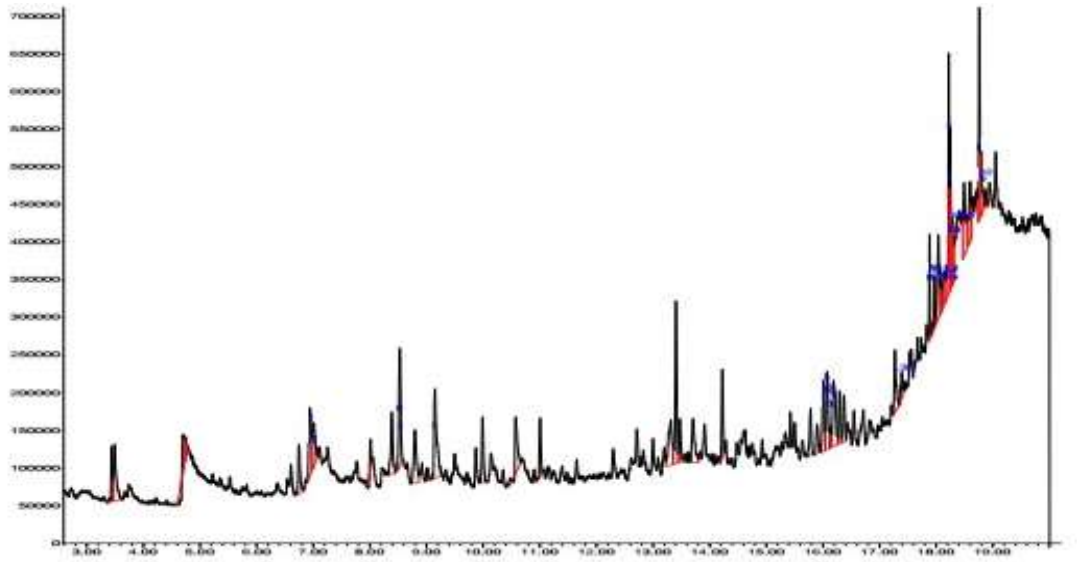

Figure 6. GC-MS chromatogram of methanolic extract of *Asphaltum* 2

Table 2. A list of important phytochemicals identified by GC-MS analysis was observed in *Asphaltum* 1 and *Asphaltum* 2.

Sr. No.	Compounds in *Asphaltum* 1	RT (min)	%age	Compounds in *Asphaltum* 2	RT (min)	%age
1	Benzoic acid	4.846	48.20	1,2-Cyclohexanediol	3.441	1.68
2	Benzoic acid	5.168	0.10	1,2-Cyclohexanediol, trans-	3.494	2.74
3	4,4-Dimethylcyclohexadienone	7.01	6.74	Benzoic acid	4.714	0.79
4	1-methyl-1,4-Cyclohexadiene	7.237	0.62	Benzoic acid	4.743	0.55
5	Cyclohexane, 1,2,4-trimethyl-	8.012	0.37	5-methyl-2-(1-methylethyl)-, phosphite Cyclohexanol	6.75	1.77
6	Benzamide	8.234	2.09	Cyclohexa-1,4-dienecarboxylic acid	6.94	3.12
7	Benzenecarbothioic acid, O-ethyl ester	8.313	1.43	trans-4-(Propan-2-yl)cyclohexane-1-carboxylic acid	7.006	2.17
8	Hydrazine, 1-methyl-1-phenyl-	8.387	0.70	2-Methyl-2-(2-oxopropyl)cyclohexanone	8.012	0.40
9	Benzoic acid, 3-hydroxy-, methyl ester	8.527	1.79	Phenol, 2-ethyl-	8.387	2.55

10	Acetophenone, 4'-hydroxy-	8.609	1.65	anti-10-Methyl-endo-tricyclo[5.2.1.0(2.6)]decane	8.531	4.40
11	(3-Methylphenyl) methanol, n-propyl	9.199	0.58	N-Benzoyl-dl-alanine	8.799	2.91
12	1,4-Dimethoxy-2,3-dimethylbenzene	9.846	0.21	1-Hexene, 4-ethyl-	9.15	4.72
13	2-Ethylphenol, methyl ether	10.2	3.33	Cyclohexane, 2-propenyl-	9.99	2.11
14	Benzenepropanol, 4-hydroxy-.alpha.-methyl	10.563	0.55	Benzenepropanol, 4-hydroxy-.alpha.-methyl	10.576	2.29
15	2-Butanone, 4-(4-hydroxyphenyl)	10.654	1.04	1-Cyclohexylnonene	11.009	1.80
16	3-(1-Isopropyl-but-3-enyloxy)-butyric acid	12.587	1.83	Cyclohexane, 1,2-dimethyl-3,5-bis(1-methylethenyl)-	13.317	3.12
17	Hexadecanoic acid, methyl ester	12.711	0.73	Phthalic acid, di-(-)-menthyl ester	13.408	5.51
18	Thiophene, tetrahydro-2-methyl	12.818	1.59	7.beta.-Ethyl-8.beta.-hydroxy-2,6-dimethylbicyclo[4.4.0]dec-1-ene	13.474	1.55
19	n-Hexadecanoic acid	13.267	1.07	Benzoic acid, 2-hydroxy-5-nitro-	13.708	2.51
20	1,3,5-Trithiocycloheptane	13.313	0.66	Silane, chlorotripropyl-	14.224	2.35
21	2-Acetyl-3,3-dimethyl-2-(3-oxo-butyl)-cyclopentanone	13.407	2.30	2-Methyl-4-(2,6,6-trimethylcyclohex-1-enyl)but-2-en-1-ol	15.782	1.66
22	1-Isopropenyl-3-propenylcyclopentane	13.473	0.77	2-Buten-1-ol, 2-ethyl-4-(2,2,3-trimethyl-3-cyclopenten-1-yl)-	16.009	2.72
23	2-(2,6,6-Trimethylcyclohex-1-enyl)cyclopropanecarboxylic acid, methyl ester	13.91	0.57	Bicyclo[5.2.0]nonane, 4,8,8-trimethyl-2-methylene-	16.079	2.99
24	2-Heptenoic acid, 4-cyclopropyl-5-methylene-, methyl ester, (E)	14.228	0.81	1,8-Nonadiene, 2,7-dimethyl-5-(1-methylethenyl)	16.194	3.77
25	Methyl stearate	14.628	0.47	Tricyclo[3.2.2.0]nonane-2-carboxylic acid	16.297	1.65
26	9-Octadecenoic acid, (E)	15.163	0.79	(3,4-Dihydroxyphenyl)heptylamine	16.371	1.64
27	(2Z,4E)-3,7,11-Trimethyl-2,4,10-dodecatriene	15.7825	0.53	(3E,10Z)-Oxacyclotrideca-3,10-diene-2,7-dione	17.274	2.38
28	Isoaromadendrene epoxide	16.008	0.51	cis-10-Pentadecenoic acid, propyl ester	17.398	0.88
29	2,6,10,10-Tetramethylbicyclo[7.2.0]undeca-2,6-diene	16.078	0.74	Benzene, 1,2,3-trimethoxy-5-(1-acetoxy-2-propenyl)-	17.884	1.76
30	beta.-Guaiene	16.202	0.62	2-(Acetyl)oxybenzylidene acetophenone	17.975	1.25
31	9-Acridinecarboxylic acid	16.375	0.42	5,6-Tetramethylenetetrahydro-1,3-oxazine-2-thione-4-spirocyclohexane	18.041	3.47
32	alpha.-Guaiene	16.548	0.98	Methyl 3-amino-2-methylbenzoate, N,N-diacetyl-	18.09	0.59
33	Cyclopropane, 1,1-dibenzoyl-2-phenyl-	16.713	0.51	Toloxatone	18.131	1.25
34	Spiro[4.5]decan-7-one, 1,8-dimethyl-8,9-epoxy-4-isopropyl	17.274	0.65	Bis(2-ethylhexyl) phthalate	18.222	5.01
35	6-Pentadecenoic acid, 13-methyl-, (6Z)-	17.398	0.21	Cyclohexane, 1,3,5-triphenyl-	18.247	3.91
36	2,2-Dimethylpropanoic acid, 2,6-dimethylnon-1-en-3-yn-5-yl ester	17.538	0.15	Adamantane-1-(3,3-dichloropropyn-1-yl)	18.284	2.14
37	Hydroxyvalerenic acid	17.562	0.16	5-Nitro-4-(pyrrol-1-yl)naphthalen-1-amine	18.494	3.17
38	Spiro[2.7]dec-4-ene, 1,1,5,6,6,9,9-heptamethyl-10-methylene-	17.665	0.34	8-[(2-Hydroxyethyl)amino]-caffeine	18.601	2.57
39	Acetophenone, 2-[(p-nitrophenyl)imino]-	17.773	0.03	1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	18.762	4.27
40	1H-Indene, 5-butyl-6-hexyloctahydro-	17.888	0.26	2,6,10,14,18-Pentamethyl-2,6,10,14,18-eicosapentaene	18.791	2.10
41	1H-Pyrazole-1-acetamide, 4-iodo-N-(phenylmethyl)-	17.975	0.37	trans-2,3,6-Trimethoxy-.beta.-methyl-.beta.—nitrostyrene	19.051	1.80
42	3,5-Dinitrobenzyl alcohol	18.045	0.89			
43	Hexadecanoic acid, (3-bromoprop-2-ynyl) ester	18.094	0.26			
44	Hexadecane, 1-(ethenyloxy)-	18.131	0.22			

45	Acetophenone, 2-[(p-nitrophenyl)imino]	18.189	0.49
46	Bis(2-ethylhexyl) phthalate	18.226	1.21
47	Benzonitrile, m-phenethyl	18.251	1.49
48	Hexadecane, 1-(ethenyloxy)-	18.3	0.34
49	2H-3,9a-Methano-1-benzoxepin, octahydro-2,2,5a,9-tetramethyl-	18.379	0.76
50	Octadecanoic acid, 2-oxo-, methyl ester	18.416	0.87
51	(3R,5aR,9S,9aS)-2,2,5a,9-Tetramethyloctahydro-2H-3,9a-methanobenzo[b]oxepine	18.605	0.99
52	6-Methyl-2-(3-nitrophenyl)imidazo[1,2-a]pyridine	18.712	1.04
53	1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	18.766	3.23
54	O,N-Dimethyl-dehydrococcinine	19.042	0.78

4.5. Anti-Microbial Susceptibility Test

The inhibition zone was measured for the antibacterial properties of the *Asphaltum* samples. (Table 3) There has been stronger antibacterial activity in the sample from Gilgit-Baltistan due to its possible concentration of rich humic and fulvic acids having bioactivity. The Chitral sample has been less active in terms of antimicrobial properties but with little inhibition against both gram-positive and gram-negative bacteria [26]. (Figures 7-8)

As such, it is a promising antibacterial candidate that could be investigated further for use as Natural antimicrobials in food preservation, wound healing formulations to help prevent bacterial infections, and natural herbal pharmaceuticals for the treatment of bacterial infections [27].

Table 3. Table depicting the Zone of inhibition of *Asphaltum* samples against *Staphylococcus aureus* and *Escherichia coli.*

Samples	Microorganism	Zone of inhibition(mm)
***Asphaltum* Sample 1**	*Staphylococcus aureus*	16.3
	Escherichia coli	17.3
***Asphaltum* Sample 2**	*Staphylococcus aureus*	14.3
	Escherichia coli	12.3

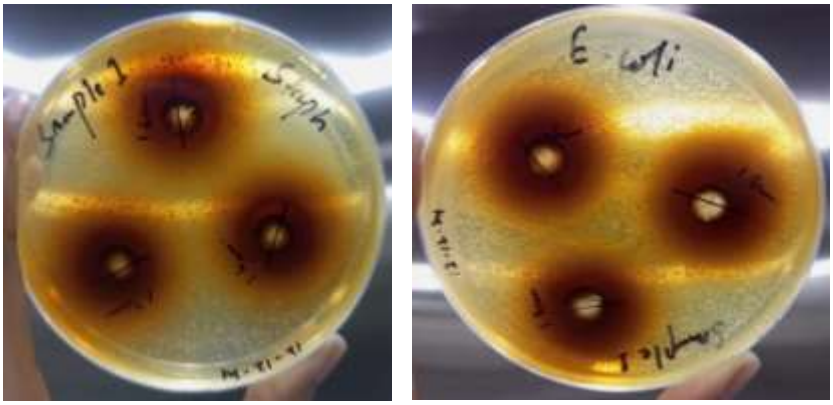

Figure 7. *Asphaltum* 1 against Staphylococcus aureus on the left and Escherichia coli on the right (Front side view)

Figure 8. *Asphaltum* 2 against Staphylococcus aureus on the left and Escherichia coli on the right (Front side view)

5. Conclusion

The study has been primarily constructed on the chemical composition and biological activity of *Asphaltum* samples from Gilgit-Baltistan and Chitral. All results of UV-VIS, FTIR, ICP-OES, and GC-MS confirmed the presence of humic and fulvic acids and other essential minerals and organic compounds. Antibacterial activity test showed that *Asphaltum* possessed very strong inhibition against *Escherichia coli* and *Staphylococcus aureus*, thereby showing its promise as a potential natural antimicrobial agent and emphasizing the geographical variability in composition that might affect its bioactivity.

Future Recommendations

1. In Vivo Antimicrobial Studies: Possible animal models might be employed for demonstrating Shilajit's antibacterial activity that may be used as a natural substitute for antibiotics.
2. Toxicological Studies: Studies on Long-term safety and toxicity should be performed to know ethical dosage levels in medicine.
3. Commercial Formulation Development: Research on *Asphaltum* incorporation into pharmaceutical or nutraceutical formulations would add therapeutic potential.

Credit authorship contribution statement

Mr. Zeshan Ahmad: Conceptualization, Data Curation, Writing, Review, Data Curation and Editing
Dr. Syeda Rubina Gilani: Supervision and Visualization.

Declaration of competing interest

The authors declare no personal relationship or conflict of interest that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used in this research is confidential.

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Ethics Approval

The authors declare that the study requires no ethical approval as it is observational.

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