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Inorganic Analysis and Antioxidant Activity of Shilajit

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***Abstract* —**Shilajit is an ancient Hindu medication of Ayurveda which has many applications worldwide. This study aims to investigate the inorganic content of two common samples from Pakistan and Afghanistan used in the treatment of many diseases in Kingdom of Bahrain. The inorganic phosphate content of Afghani and Pakistani shilajit samples was determined to be 0.133 ±0.004 mg/g and 0.174 ±0.002 mg/g, respectively. Elemental analysis of solid Afghani and Pakistani shilajit samples was determined by energy dispersive x-ray fluorescence (ED-XRF). Afghani shilajit sample mainly comprised of calcium (50.298%), sulfur (21.299%), potassium (17.194%) and chlorine (8.405%) while the Pakistani shilajit mainly comprised of potassium (24.309%), calcium (20.933%), chlorine (18.614%), and silicon (15.197%) with relatively close percentages. Determination of the concentration of minerals and trace metals; As, Pb, Cd, Ag, Al, B, Ba, Cr, Cu, Fe, Mn, Ni, Sb, Se, Zn, P, Ca, K, Mg and Na, in each sample was carried out by inductively coupled plasma-optical emission spectrometry (ICP-OES). According to the ICP-OES analysis, the most significant outcome was the concentration of Ca and K in Afghani shilajit, which were determined to be 30292 and 21587 ppm (equivalent to 30.292 and 21.587 g/L), respectively. The antioxidant activity of both shiljait samples were evaluated by determining 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. The antioxidant activity of Afghani and Pakistani shilajit samples were determined to be 96.797 ± 0.561% and 95.297 ± 2.884%, respectively which may indicate strong antioxidant actions.

***Keywords* —** shilajit composition, minerals, trace metals, phosphate content, antioxidant activity

I. INTRODUCTION

Shilajit is a herbomineral found in the Himalayans, central Asia and India and is widely used by Indian Ayurvedic and Siddha systems as folk medicine [1], [2]. Shilajit, which is also known as mumijo, is a blackish-brown powder or an exudate found in mountain rocks, it is hypothesized that shilajit is formed as a product of decomposition of some plant species such as *Euphorbia royleana* and *Trifolium repens* [1].

In terms of composition and chemical constituents of shilajit, it has been noticed that shilajit samples of different regions vary in terms on organic composition, but commonly would comprise of 80-85% humic substances (including humins, humic acids and fulvic acids) and 15-20% of non-humic substances [1], [3]. Overall, shilajit samples would contain 14-20% humidity, 18-20% of different types of minerals, 13-17% proteins (with a wide range of amino acids), 18-20% nitrogen-free compounds, 4-4.5% lipids, 3.3-6.5% steroids, 1.5-2% carbohydrates, and 0.05-0.08% alkaloids [3]. Trace elements are responsible for approximately 5% of the composition of shilajt [4]. The organic compounds present in shilajit have been investigated, it has been reported that shilajit contains fulvic acid, a bioactive compound, that has been shown significant results in neurological treatments, precisely in the treatment of Alzheimer [1]. Yet, the inorganic composition and content of shilajit has not been explored.

Shilajit was found to have considerable radioprotective and antioxidant improving potential in a research experimenting the exposure of X-ray radiation on Zebrafish, *Danio rerio*, by studying the morphological, behavioural, clinical symptoms, antioxidant levels and DNA damage of the fish [5]. Shilajit was also found to be effective in improving the antioxidant activity, immunity and disease resistance in fresh water prawn, *Macrobrachium rosenbergii* (de Man), against a heterotrophic, gram-negative bacterium, *Aeromonas hydrophila*, by evaluating the total hemocyte count, respiratory burst, phagocytic activity, glutathione peroxidase activity, glutathione reductase activity, phenoloxidase activity, superoxide dismutase activity and cumulative mortality [6]. The antiviral potential of shilajit has also been investigated. Shilajit was found to have antiviral activity against herpes simplex type 1 and 2, human cytomegalovirus, human respiratory syncytial virus, human rotavirus, and vesicular stomatitis virus [2].

The current information available on inorganic content and mineral profile of shilajit are basic and limited. In this research, the inorganic content of two types of shilajit from two different locations, Afghanistan (Figure 1) and Pakistan (Figure 2) has been determined in terms of inorganic phosphate content by a spectrophotometric method, elemental composition by energy dispersive x-ray fluorescence (ED-XRF), and mineral profile (metal content) by inductively coupled plasma-optical emission spectrometry (ICP-OES). The pH and the ultraviolet/visible radiation absorbance of shiljait samples were also determined. In addition, the antioxidant potential of shilajit was also evaluated by determining the radical scavenging activity towards 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical.

Figure 1: Afghani shilajit

Figure 2: Pakistani shilajit

The rest of this paper is organized as follows, Section II describes the methodology followed in this research (measurement of pH, ultraviolet/visible spectra of shilajit, estimation of inorganic phosphate content, composition determination by elemental analysis using ED-XRF, determination of elemental content using ICP-OES, and determination of antioxidant activity), in Section III the results obtained are illustrated in the form of tables and figures with appropriate discussion of the findings, Section VI summarizes the findings and suggests the possible areas to explore in the future based on the findings of this research.

II. METHODOLOGY

Measurement of pH

One gram of each type of shilajit was dissolved in a voume 100 mL of distilled water, then the pH of each solution was measured using ORION STAR A111 pH meter.

Ultraviolet/visible spectra of shilajit

One gram of each type of shilajit was dissolved in 100 mL distilled water and heated for few minutes to ensure that most of the sample has dissolved in water. Then, the solutions were filtered through filter paper to remove undissolved solids. The UV/VIS absorbance spectra (200-800 nm) of each sample was measured using ORION AQUAMATE 8000 UV/VIS spectrophotometer.

Estimation of inorganic phosphate content

One gram of each type of shilajit was dissolved in a volume of 50 mL distilled water and heated for few minutes. Then, the solutions were filtered through filter paper and centrifuged at 5000 rpm for 5 min. A volume of 1 mL of the supernatant was mixed with 3 mL of copper acetate buffer (pH = 4) (prepared by dissolving 2.5 g of copper sulphate and 46 g of sodium acetate in 1 L of 2 M acetic acid, pH adjusted with sodium hydroxide), 0.5 mL of 50 g/L ammonium molybdate, 0.5 mL of metol reducing agent solution (prepared by dissolving 20 g of *p*-methyl aminophenol sulphate in 100 g/L sodium sulphite and diluted to 1 L) and 1.0 mL trichloroacetic acid (100 g/L) and kept to stand for 10 minutes at room temperature. Then, the absorbance of each solution was measured at 880 nm using ORION AQUAMATE 8000 UV/VIS spectrophotometer. Three experimental replicates were done for each sample (samples required dilution by a dilution factor of 10). For the blank reading, 1 mL of trichloroacetic acid (instead of test sample) was mixed with the same reagents mentioned above. The concentration of inorganic phosphate was determined using a standard curve of phosphate (phosphate standard solutions were prepared by diluting 10 mg/L phosphate solution to 0.1, 0.3, 0.6, 0.8, 0.9 and 1.0 mg/L using trichloroacetic acid (100 g/L) and treated as samples above). The concentration was determined in mg/L and then converted to mg/g.

Composition determination by elemental analysis using ED-XRF

The composition of crude solid shilajit samples was measured using Philips diffractometer equipped with Cu Kα radiation (λ = 1.542 Å) with an acceleration voltage of 40 kV. The data were recorded with a counting rate of 1°/min. This procedure was repeated 3 times for triplicate set of results.

Determination of elemental content using ICP-OES

An amount of 0.500 g of each shilajit sample was dissolved in 25 mL distilled water by heating the solution for 20 minutes at 95 °C, this was followed by gravity filtration. To each sample, 1.0 mL of nitric acid was added. The samples were diluted and completed to 100 mL by distilled water. Further dilution was done by diluting the sample with a dilution factor of 100. The samples were then analysed by Agilent Technologies 700 Series, inductively coupled plasma-optical emission spectrometry (ICP-OES) instrument to determine the amount of As, Pb, Cd, Ag, Al, B, Ba, Cr, Cu, Fe, Mn, Ni, Sb, Se, Zn, P, Ca, K, Mg and Na in shilajit samples. This procedure was repeated 3 times for triplicate set of results. Prior to that, multi-element standard solutions were prepared for ICP-OES analysis. From a multi element stock solution of 1000 ppm containing As, Pb, Cd, Ag, Al, B, Ba, Cr, Cu, Fe, Mn, Ni, Sb, Se, Zn, P, Ca, K, Mg and Na, a mother stock solution with concentration of 20 ppm was prepared. Then, a series of standard solutions (50, 100, 200 and 400 ppb) were prepared from the mother solution.

Determination of antioxidant activity

One gram of each type of shilajit was dissolved in 100 mL distilled water and heated for few minutes. Then, the solutions were filtered through filter paper. A volume of 50 µL of filtrate was added to 2.95 mL of DPPH solution (prepared by dissolving 4.5 mg of DPPH in 100 mL ethanol). The samples were kept standing at room temperature for 30 min, then their absorbance was measured at 517 using ORION AQUAMATE 8000 UV/VIS spectrophotometer [7]. Three replicates were done for each sample. The antioxidant activity of each sample was calculated as the percentage scavenging activity towards DPPH radical according to the following formula:

$$\text{Percentage Scavenging Activity towards DPPH radical} = \left(\frac{\text{Absorbance of fresh DPPH solution} - \text{Absorbance of test sample}}{\text{Absorbance of fresh DPPH solution}}\right)$$

III. RESULTS AND DISCUSSION

pH of shilajit solutions

The pH of Afghani and Pakistani shilajit samples was determined to be 5.13 and 4.74, respectively. The Pakistani shilajit was slightly more acidic compared to the Afghani shilajit, generally both were weak acids.

Ultraviolet/visible spectra of shilajit

The UV/VIS spectra of both shilajit samples was obtained as shown in Figure 3 and 4. The maximum wavelength at which absorption took place, i.e. lambda max., of Afghani shilajit was at 229 nm, while that of Pakistani shilajit was at 228 nm. As seen in the spectra of each sample (Figure 3 and 4), shilajit primary absorbs radiation in the range of 200-400 nm, which is the referred to the ultra-violet region. Within the ultraviolet range, the highest amount of absorption of UV radiation by shilajit takes place within the ultraviolet C (UVC) region (200-290 nm) [8]. UVC is the most energetic type of UV radiation having the strongest biological damage effect, fortunately it is filtered by the ozone layer [8]. Nevertheless, with ozone layer depletion, it could be predicted that minor fractions of UVC could reach the surface of the earth and thus sunblock and sunscreen products may require using UVC absorbents. Shilajit, as a natural source and a common medical herbomineral could be evaluated in future studies as a UVC absorbent in health products.

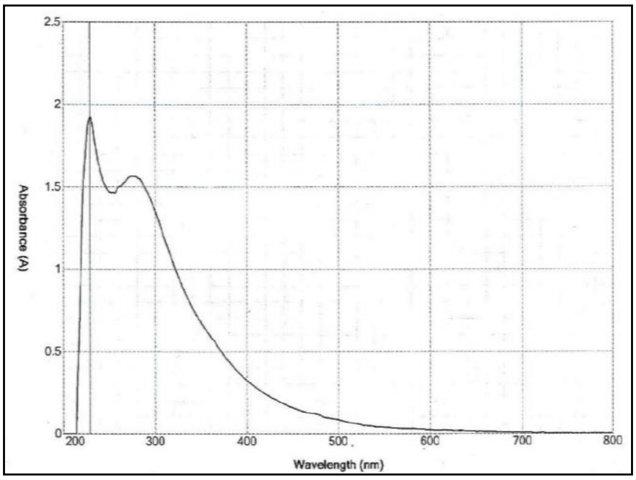

Figure 3: UV/VIS spectra of Afghani shilajit

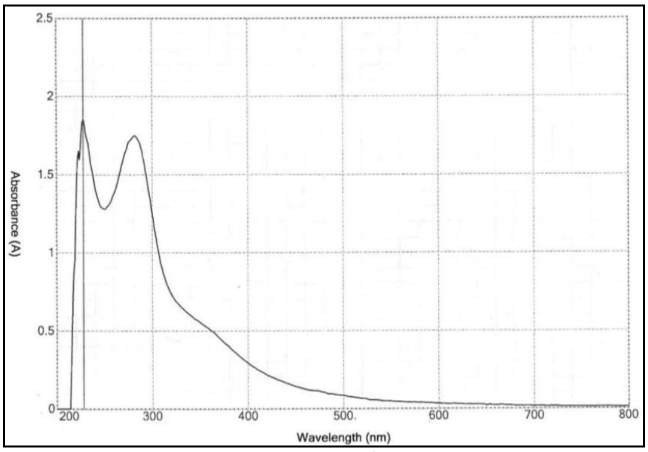

Figure 4: UV/VIS spectra of Pakistani shilajit

Inorganic phosphate content

The inorganic phosphate content of shilajit samples was determined by a colorimetric method. The phosphate content of Afghani shilajit was determined to be 0.133 ±0.004 mg/g, and the phosphate content of Pakistani shilajit was determined to be 0.174 ±0.002 mg/g. The difference in the inorganic phosphate content of both samples is significant at $p < 0.05$ according to statistical analysis.

Solid composition of shilajit determined by ED-XRF

The elemental composition of both types of shilajit was determined using ED-XRF. The solid composition of the two types of shilajit are outlined in Table 2 and illustrated in Figures 1 and 2. The elements rubidium, strontium and zirconium were only detected in Afghani shilajit sample. The Afghani shilajit sample mainly comprised of calcium (which is accounts for half of its composition), sulphur, potassium, and chlorine. On the other hand, the Pakistani shilajit contained potassium, calcium, chlorine, and silicon with relatively close percentages.

Table 3: Elemental composition of shilajit samples

Sample	Elemental Composition	Percentage (%)
Afghani Shilajit	Silicon (Si)	0.781 ±0.004
	Sulphur (S)	21.299 ±0.010
	Chlorine (Cl)	8.405 ±0.008
	Potassium (K)	17.194 ±0.002
	Calcium (Ca)	50.289 ±0.021
	Iron (Fe)	0.574 ±0.002
	Rubidium (Rb)	0.015 ±0.001
	Strontium (Sr)	1.433 ±0.006
	Zirconium (Zr)	0.008 ±0.000
Pakistani Shilajit	Aluminium (Al)	4.727 ±0.004
	Silicon (Si)	15.197 ±0.011
	Sulphur (S)	9.120 ±0.010
	Chlorine (Cl)	18.614 ±0.017
	Potassium (K)	24.309 ±0.013
	Calcium (Ca)	20.933 ±0.010
	Iron (Fe)	7.100 ±0.006

Mineral profile of shilajit samples

The amount of As, Pb, Cd, Ag, Al, B, Ba, Cr, Cu, Fe, Mn, Ni, Sb, Se, Zn, P, Ca, K, Mg and Na in shilajit samples was determined by ICP-OES as outlined in Table 3 and Figures 7 and 8. The detected and quantified elements can be divided into two categories: (a) elements present in concentration of approximately 0-200 pm and (b) elements present in concentration of above 500 ppm. The elements As, Pb, Cd, Ag, Al, B, Ba, Cr, Cu, Fe, Mn, Ni, Sb, Se, Zn and P are categorized in group (a), while Ca, K, Mg and Na are categorized in group (b). The overall trend on the mineral profile and illustrations (Figures 7 and 8) suggest that the mineral and elemental content of Afghani shilajit is higher than the Pakistani shilajit. The concentrations of Pb, B, Fe, Mn, Zn, P, Ca, K, Mg and Na in Afghani shilajit were higher than that in Pakistani shilajit. On the other hand, the concentrations of As, Al, Ba and Cu in Pakistani shilajit were higher than that in Afghani shilajit. The elements Ag and P were only present in Afghani shilajt. On the other hand, Ni and Se were only present in the Pakistani shilajit. The elements Cd, Cr and Sb were absent in both types of shilajit. A research has concluded that the amount of C, Ca, P, Al, Mn and Fe present in shilajit samples is affected by the altitude from which shilajit was collected [9]. The amount of P diminishes with increasing altitude, while the amount of C, Ca, Al, Mn and Fe increase with increasing the altitude from which shilajit was collected [9].

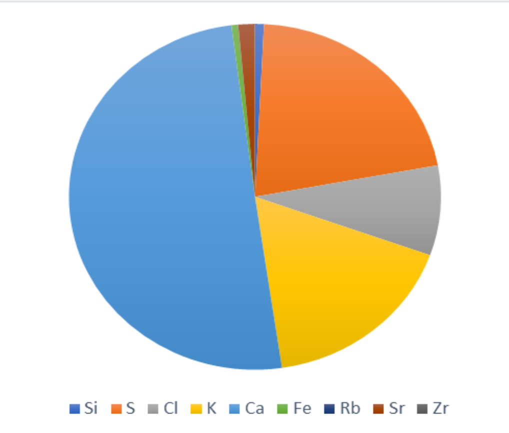

Figure 5: Elemental composition of Afghani shilajit

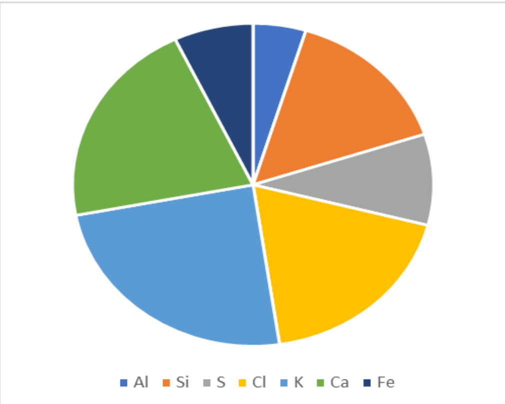

Figure 6: Elemental composition of Pakistani shilajit

The most significant outcome of this analysis is the concentration of Ca and K in Afghani shilajit, which were determined to be 30292 and 21587 ppm (equivalent to 30.292 and 21.587 g/L), respectively. The significant Ca content of Afghani shilajit was also proved by the ED-XRF analysis in Section 3.4. Other than the well-known importance of Ca for bone strength, based on a recent study Ca has shown anti-obesity potential by preventing adipocyte dysfunction and resulting in improved blood glucose, insulin and glucose tolerance by the activation of some pathways, such as activation of hepatic and muscle insulin signaling pathways [10]. This can suggest the possible potential of shilajit's (specifically Afghani shilajit) anti-obesity activity, which can be a new medicinal area to be explored. In fact, the possibility of using shilajit in fertilizers due to its rich elemental content can also be investigated.

Table 4: Elemental content of shilajit samples

Element	Concentration of Element (ppm)	
	Afghani Shilajit	Pakistani Shilajit
As	68.078 ±0.024	73.654 ±0.053
Pb	66.509 ±0.063	60.292 ±0.046
Cd	0.000 ±0.000	0.000 ±0.000
Ag	2.864 ±0.012	0.000 ±0.000
Al	1.848 ±0.002	5.854 ±0.025
B	114.191 ±0.042	66.491 ±0.032
Ba	4.566 ±0.022	35.802 ±0.044
Cr	0.000 ±0.000	0.000 ±0.000
Cu	9.098 ±0.030	13.476 ±0.018
Fe	145.514 ±0.010	15.028 ±0.029
Mn	16.343 ±0.058	1.617 ±0.016
Ni	0.000 ±0.000	12.068 ±0.021
Sb	0.000 ±0.000	0.000 ±0.000
Se	0.000 ±0.000	93.595 ±0.050
Zn	31.481 ±0.022	18.324 ±0.020
P	204.27 ±0.47	0.000 ±0.000
Ca	30292 ±21	877.09 ±0.63
K	21587 ±13	1433.8 ±9.2
Mg	2079.8 ±8.4	147.96 ±0.51
Na	1580.3 ±7.7	513.08 ±7.8

Antioxidant activity

The antioxidant potential of shilajit has previously been explored and evaluated by different methods and biological assays. In this research, the activity of shilajit samples was determined by the DPPH method in which the antioxidant activity is calculated as the percentage scavenging activity towards DPPH radical. The antioxidant activity of Afghani and Pakistani shilajit samples were determined to be 96.797 ± 0.561% and 95.297 ± 2.884%, respectively. Both samples have shown a very high antioxidant activity, which suggests their significant potential in reducing free radicals. The difference in the antioxidant of both samples is not significant at $p < 0.05$ according to statistical analysis.

According to a previous research, it was found that the aqueous extract of shilajit exhibited a DPPH radical scavenging activity with value of 11.9 µg/mL [11].

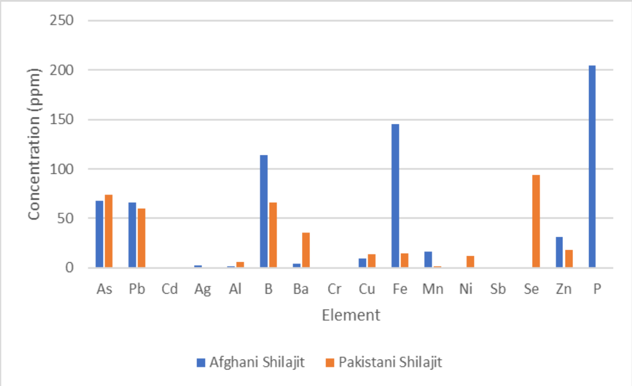

Figure 7: Mineral content present in shilajit samples of concentrations between 0 to 200 ppm

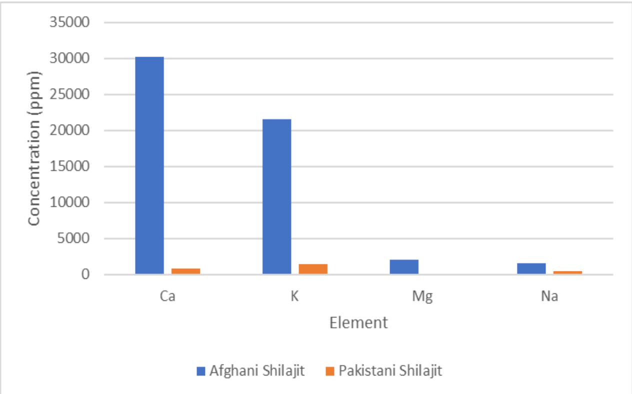

Figure 8: Mineral content present in shilajit samples of concentrations above 500 ppm

IV. CONCLUSION AND FUTURE SCOPE

To conclude, through this research the inorganic content of Afghani and Pakistani shilajit samples were determined. In addition, the antioxidant activity of both samples was determined. The most significant outcome of the inorganic analysis was determination of high amounts of calcium and potassium in Afghani shilajit. This important finding can trigger several biochemical analyses and led to medicinal investigations with respect to shilajit focusing on these properties.

V. ACKNOWLEDGMENT

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