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Short Communication

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Phytochemical Screening and Biological Studies of Shilajit (Asphaltum)

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Abstract

Shilajit (asphaltum) is produced by the long term humification of dead plant material and organic vegetable matter by different micro-organisms and has great potential for the treatment of a variety of human conditions. This treatise reviews its origin, sources, chemical composition, biological and commercial importance. Phytochemical analysis was done by standard methods to evaluate different Shilajit (asphaltum) classes of compounds in different samples of shilajit which are responsible for their biological activity. Shilajit`s anti-microbial activity has been evaluated against four different bacterial strains viz., *Escherichiacoli, Psuedomonas aeuroginosa*, *Klebisella pneumonia* and *Staphylococcus aureus.* Phytochemical analysis illustrated that shilajit contains terpenoids, cardiac glycosides, saponins and reducing sugars. Surprisingly, some classes of compounds are absent in shilajit viz., alkaloids, flavonoids, tannins and anthraquinones. Shilajit showed no response towards halophytic bacteria and negligible activity was shown towards other strains of bacteria. Since anti-microbial activity is based on environmental factors its activity varied between locations.

Keywords: Shilajit, phytochemical screening, anti-microbial activity.

Introduction

Shilajit is considered as a venerable ancient medicinal practice [1] currently being applied in the Himalayan hills from Nepal to Kashmir, Afghanistan, Russia, India, Bhutan, Mongolia, Tajikistan, Algeria, Japan, Tibet, and China [2,3]. There are essentially two major types of shilajit viz., karpura shilajit and gomuthira shilajit. The latter is further classified into four lesser types viz., savrana shilajit, tamra shilajit, rajat shilajit and lauha shilajit [4-6]. Shilajitis is considered to be a top rated and powerful adaptogen (Shukla *et al.*, 2009). It is used to treat chest problems, diabetes mellitus, nervous disorders, immune disorders, obesity, kidney disorders, asthma, gall stones, painful and bleeding piles, enlarged spleen and liver, fermentative dyspepsia, worms, renal and bladder calculi, nervous debility, sexual neurasthenia, hysteria, bone fracture, moorcha (fainting), female infertility, joint pains, wounds, chronic ulcers and skin diseases [7,8],

Shilajit has demonstrated good inhibition against viral enzymes and also demonstrated a degree of anti HIV activity [9-11].Shilajit is available in tablet form in medicines such as Abana, Cystone and Diabecon. It is also available in syrup form as Evecare and Geriforte [12]. Current knowledge on the phytochemical screening and antimicrobial activity of shilajit is sparse and thus there remains a wide gap in our knowledge of it and thus it needs to be explored further to include various other pathogenic microbes. Thus, the present study was carried out to obtain a more comprehensive knowledge of the phytochemical screening and antimicrobial activity of different samples of shilajit collected from Chitral in the Northern area of Pakistan against some pathogenic bacterial and fungal species. The selected pathogens are *Aspergillus niger*, *Pseudomonas aeruginosa*, halophytic bacteria, *Escherichia coli, Kleibesela pneumonie* and *Staphylococcus aureus*.

Material and Methods

Sample preparation

Two forms of shilajit (crude and commercially available) were collected from Chitral, in the Northern area of Pakistan, in the month of April 2013.The crude Shilajit was taken and immersed in methanol/water (4:1) for 15 days being stirred daily. After filtration, the filtrate was covered with aluminum foil. The pure shilajit was purchased from the local market in packets and had the consistency of a thick tar which did not dissolve in methanol. To overcome this, a small amount of the material (0.5 g) in the methanol:water (4:1) solvent in a large test tube was treated with acetic acid (0.5 mL) and vigorously shaken after which potassium carbonate was added to neutralize the solution. An ethyl acetate

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extraction was done to get the raffinate (organic layer) and the aqueous layer was retained. Removal of the solvent from the raffinate at a bath temperature of 40ºC gave a residue as did evaporation of the aqueous layer between 50-60 Cº on a rotary evaporator. All samples were subjected to biological activity analyses.

Chemicals and reagents

All chemicals used were of analytical grade. Methanol, ethyl acetate, water, ferric chloride hexahydrate ($FeCl_3.6H_2O$), Dragendorff's reagent, hydrochloric acid (HCl), sulphuric acid (H_2SO_4), ammonia, glacial acetic acid, mercuric chloride, potassium iodide, chloroform, acetic anhydride were used without further purification.

Phytochemical screening

Methanolic extracts of crude shilajit (Sample A), the organic portion of commercially available shilajit (Sample B) and the aqueous portion of commercially available shilajit (Sample C) were tested for the presence of different classes of phytochemicals. The qualitative results are expressed as (+) for the presence and (-) for absence of phytochemicals.

Test for Alkaloids

Each extract (15 mg) was separately stirred with 1%HCl (6mL) on a water bath at 60ºC for 5 minutes and filtered. The filtrates were divided in three equal parts.
(a) Dragondorff's test: To one portion of the filtrate, Dragendorff's reagent (Potassium bismuth iodide solution) (1 mL) was added; an orange red precipitate indicates the presence of alkaloids.
(b) Mayer's test: To one portion of filtrate, Mayer's reagent (Potassium mercuric iodide solution) (1mL) was added. Formation of a cream colored precipitate gives an indication of the presence of alkaloids.
(c) Wagner's test: Potassium iodide (2g) and iodine (1.27g) were dissolved in distilled water (5 mL) and the solution was diluted to 100mL with distilled water. A few drops of this solution were added to each filtrate; a brown colored precipitate indicates the presence of alkaloids [13,14]. Same procedure was adopted for each sample.

Tests for steroids and terpenoids

(a) Salkowski test: Each extract (100 mg) was separately shaken with chloroform (2mL) followed by the addition of concentrated H_2SO_4(2mL) down the side of the test tube. A reddish brown coloration at the interface indicates the presence of terpenoid [15].
(b) Liebermann-Burchard test: Each extract (100mg) was shaken with chloroform in a test tube. A few drops of acetic anhydride were added to the test tube and boiled in a water bath (2 min) and then rapidly cool in iced water. Concentrated H_2SO_4 (2mL) was added down the side of the test tube. Formation of a brown ring at the junction of two layers underneath an upper greenish layer shows the presence of steroids while the formation of a deep red color indicates the presence of triterpenoids [13].

Test for Tannins

Each of the extracts (0.5 g) were separately stirred with distilled water (10mL) and then filtered. A few drops of 5% ferric chloride were then added. A black or blue-green coloration or precipitate was taken as being positive for the presence of tannins.

Test for Saponins

Each shilajit extract (0.5g) was separately shaken with distilled water (10mL) in a test tube. The formation of frothing, which persists on warming in a water bath at 90 ºC for 5 minutes, shows the presence of saponins.

Tests for Glycosides

(a) Anthraquinone glycoside (Borntrager's test): To the extract solution (1 g), 5% H_2SO_4 (1 mL) was added. The mixture was boiled in a water bath for 5 min and then filtered. The filtrate was then shaken with an equal volume of chloroform and left to stand for 5 minutes. The chloroform layer was shaken with half of its volume with dilute ammonia. Formation of a rose pink to red color of the ammonical layer gives indication of anthraquinoneglycosides [13].
(b) Cardiac glycoside (Keller-killiani test): Each extract (0.5g) was shaken with distilled water (5mL). To this, glacial acetic acid (2mL) containing a few drops of ferric chloride was added, followed byH_2SO_4 (1 mL) down the side of the test tube. Formation of a brown ring at the interface gives a positive indication for cardiac glycosides and on occasion a violet ring may appear below the brown ring [15].

Test for Flavonoids

Three methods were used to determine the presence of flavonoids in the different samples of sholajit. Dilute ammonia solution (5 mL) was added to a portion of the aqueous filtrate of each extract followed by addition of a few drops of concentrated H_2S0_4. A yellow coloration observed would indicate the presence of flavonoids. The yellow coloration usually disappears on standing. Alternatively a few drops of 1% aluminium solution when added to a portion of each filtrate and generates a yellow coloration. Also indicates the presence of flavonoids. A portion (this has to be quantified ie 10 mg or 50 mg but not just a "portion") of each of the shilajit samples

was heated with ethyl acetate (10 mL) in a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow coloration indicates the presence of flavonoids.

Test for Reducing Sugar

The Fehling test proposed by (Khana *et al.*, 2008) is used to detect the presence of any reducing sugars in the sample. According to this method the appearance of red or violet coloration indicates the presence of a reducing sugar. Each sample (5 mg) of shilajit and 5mL of distilled water were taken in a beaker containing a few drops of Fehling solution and heated in a steam bath for 30 min to detect presence or absence of any reducing sugar.

Anti-bacterial screening of different samples of shilajit

Sample preparation

There were three samples for anti-microbial activity.
Sample A is methanolic extract of crude shilajit
Sample B is extracted organic layer (raffinate) of commercially available shilajit
Sample C is Aqueous layer of commercially available shilajit
Media preparation
Agar jell was prepared by dissolving28 gram nutrient ager in 1 liter distilled water. Solution was heated in a microwave oven for 1 minute and the light yellow solution was sterilized inan autoclave.
Cultures used
Anti-microbialactivity of shilajit samples was determined on the following pathogenic cultures:
Escherichiacoli
Psuedomonas aeuroginosa
Klebisella pneumonia
Staphylococcus aureus
These microorganisms were obtained from the culture collection of the Microbiology Laboratory, MUST University, Mirpur, Pakistan.

Anti-fungal activity of shilajit samples

Sample and media preparation

The method for sample preparation is similar to that for antibacterial activity. However, for anti-fungal activity 2 samples were taken viz., sample A and sample B. For antifungal activity PDA was used. Thus PDA (39g) was dissolved in 1litre of distilled water by heating. This solution was sterilized by autoclaving at 121^{o}C for 15 minute and mixed well before pouring. *Aspergillus* was used as a culture.
Disc diffusion assay The different samples of shilajit were screened for antimicrobial activity by using the disc diffusion method [16]. In the assay each inoculum suspension (108 CFU/mL) was spread evenly over the entire nutrient agar surface by sterile collection swab. Then, discs of diameter 6 mm were sterilized at 121^{o}C for 15 min and loaded with extract solutions of shilajit. The impregnated discs were dried for 3–5 min and dispensed onto the surface of the inoculated plates with flamed forceps. Each disc was pressed down firmly to ensure complete contact with nutrient agar surface. The discs were suitably placed apart and not moved once in contact with the agar surface. The plates were then labeled and incubated at 37 ^{o}C for 24 h for both bacteria and fungus (Espinel-Ingroff *et al.*, 2007; Zaidan *et al.*, 2005). The results were measured and expressed in terms of zone of inhibition (ZI) of bacterial and fungal growth around each disc in millimetres as low activity (1–6 mm), moderate activity (7–10 mm), high activity (11–15 mm), very high activity (16–20 mm), no activity (0).

Results and Discussion

Phytochemical screening

The result of phytochemical screening of shilajit sample A revealed the absence of some secondary metabolites such as alkaloids, steroids, tannins, flavonoids and anthraquinones (Table 1). However, terpenoids, saponins, reducing sugars and cardiac glycosides were present. The same result was obtained for shilajit sample B whereas all for sample C all the tested phytochemicals were absent except for cardic glycosides and saponines. The phytochemicals present are known to have medicinal importance. Absence of alkaloids in the current study may be the consequence of different geographical locations in which soil minerals and environmental factors influence the plant's abilities to produce a greater diversity of phytochemicals in the shilajit [17]. The cardiac glycosides have been used to treat congestive heart failure and cardiac arrhythmia [18]. There is thus no doubt that the phytochemicals identified in the shilajit extracts are indeed responsible for the biological activities demonstrated by shilajit and is furthermore the reason for their use as a traditional medicine by the natives of Pakistan.

Table 1: Phytochemical constituents of different samples of shilajit

Phytochemicals	Shilajit		
	Sample A	Sample B	Sample C
Alkaloids	(-)	(-)	(-)
Terpenoids	(+)	(+)	(-)
Cardiac glycosides	(+)	(+)	(+)
Tannins	(-)	(-)	(-)
Flavonoids	(-)	(-)	(-)
Steroids	(-)	(-)	(-)
Saponins	(+)	(+)	(+)
Anthraquinone	(-)	(-)	(-)
Reducing sugar	(+)	(+)	(-)

Antimicrobial assays

The antimicrobial activity of shilajit extracts is shown in Table 2. This behavior is attributed to the presence of certain active compounds and the absence of others viz., alkaloids in shilajit extracts of the current study [19]. Also, low concentrations or selectivity of active principles towards a particular fungus could also be one of the reasons of the poor activity. The selective activity of compounds in the extracts towards certain bacteria might be due to the presence of lipopolysaccharides in the outer membrane of gram negative bacteria, which acts as a permeability barrier and restricts diffusion of these active compounds through its lipopolysaccharide covering [20]. Unlike gram negative bacteria, gram positive bacteria allow direct contact of the constituents in the extract with the phospholipid bilayer of the cell membrane, causing either enhanced ion permeability, leakage of vital intracellular constituents, or impairment of the bacterial enzyme systems [21]. All the samples were resistant towards *E. Coli* cultural plate except sample D (pure shilajit+silica gell). Sample D showed 11 mm inhibition zone.

In *Pseudomonas aeuroginosa* cultural plate all samples were resistant except sample B (organic layer of pure shilajit) that showed 7 mm inhibition zone.

In *Klebisella pneumonia* cultural plate every sample displayed an inhibition zone with that of sample C having a maximum of 12 mm.

In *Staphylococcus aureus* cultural plate only sample B displayed an inhibition zone of 12 mm while samples A and C were inactive.

When viewed as a whole, sample B is the most active of all three followed by sample C and then sample A. Anti-microbial activity varies from region to region and is due due to the organic constituents within the plant (Galimov *et al.*, 1986). For instance, rayma shilajit shows strong anti microbial activity, while al-jouf, Indian shilajit samples displayed no anti microbial activity while the Russian shilajit again showed strong anti –microbial activity. Some researchers in this area have reported that this difference is aligned to climatic conditions (Kotb *et al.*, 2012).

Conclusion

Shilajit (asphaltum) is an exudation of variable consistency found at high altitudes. From the present phytochemical analysis of shilajit the following groups of organic compounds were identified: terpenoids, cardiac glycosides, saponins and reducing sugars. Some classes of compounds are absent in shilajit viz., alkaloids, flavonoids, tannins and anthraquinones . Anti-microbial activity of shilajit varies from place to place. Shilajit is resistant towards halophytic bacteria but a very mild activity was shown towards other strains of bacteria.

Research is warranted to determine the efficacy of these extracts against various other pathogenic bacterial and fungal species. There is thus an urgency for the active principles of the shilajit extracts to be isolated and identified which are responsible for antimicrobial activity in order to develop future pharmaceuticals. It is concluded from the above study that the medicinal properties of shilajit might be due to the presence of some compounds and other phytochemicals in this plant and this ongoing study is necessary to comprehensively investigate its potential biological activities.

Table 2: Antimicrobial activity of different samples of shilajit

Shilajit Samples	Bacterial culture	Inhibition zone (mm)
Sample A	*Escherichia coli*	---
Sample B	*Escherichia coli*	11
Sample C	*Escherichia coli*	---
Sample A	*Pseudomonas aeuroginosa*	---
Sample B	*Pseudomonas aeuroginosa*	7
Sample C	*Pseudomonas aeuroginosa*	---
Sample A	*Klebisella pneumonia*	8
Sample B	*Klebisella pneumonia*	12
Sample C	*Klebisella pneumonia*	11
Sample A	*Staphylococcus aureus*	---
Sample B	*Staphylococcus aureus*	12
Sample C	*Staphylococcus aureus*	---

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