ISSN 2228-9860 eISSN 1906-9642 CODEN: ITJEA8

International Transaction Journal of Engineering, Management, & Applied Sciences & Technologies

http://TuEngr.com

LC/MS Profiling of Shilajit Extract for Antimicrobial & Antifungal and Cytotoxic Activities

Fawzia AlShubaily[1*] and Ebtihaj Jambi[1,2]

[1] *Department of Biochemistry, Faculty of Science, King Abdulaziz University, P.O. Box 9515, Jeddah, 21589, SAUDI ARABIA.*

[2] *Biomedical Physics Center, King Fahd Medical Research Center, Box 80216, King Abdulaziz University, Jeddah, 21589, SAUDI ARABIA.*

**Corresponding Author (Tel: +966559198333, Email: falshibli@kau.edu.sa).*

Paper ID: 13A5N

Volume 13 Issue 5

Received 14 December 2021
Received in revised form 09 April 2022
Accepted 23 April 2022
Available online 28 April 2022

Keywords:
LC-HRESIMS; Shilajit bioactive compounds; Antimicrobial; Cytotoxicity; LC/MS; Bioactive compounds; Hep G2; Candida albicans; Staphylococcus aureus; DMSO; Microbial strain; Herbal traditional medicine.

Abstract

Enormous amounts of bioactive compounds incorporated in the shilajit extracts are accountable for many therapeutic properties. However, little is acknowledged concerning the chemical content and its correlation to the antimicrobial and cytotoxic properties of shilajit extract. Therefore, the current experiment aimed at the profiling of shilajit bioactive compounds with the aid of LC-HRESIMS technology, and assessing the antimicrobial and cytotoxic properties of in vitro and in vivo models. This method allowed the identification of a variety of bioactive compounds, which include fulvic acid, gallic acid, ferulic acid, naphsilajitone, fraxin, 3,8-dihydroxydibenzo-α-pyrone, and pregnane. The results confirmed significant antifungal activity against Staphylococcus aureus at a concentration of 100 µg disc^{-1}, and Candida albicans at concentrations down to 25 µg disc^{-1} and gave inhibition zones of 13±0.3 and 12±0.3 mm diameter, respectively. There was low inhibition detected at a concentration beneath 25µg disc^{-1}, and null activity of shilajit crude extract in opposition to all the different microbes at the distinct concentrations used in the current study. Cytotoxic percentage inhibition of applied cell lines was elevated via increasing extract concentration and significant percent inhibition (IC50: 19 µg mL^{-1}) of the investigated test extract was revealed by the applied cell line Hep G2. These statistics supply a molecular foundation to explain at least a section of the advisable therapeutic properties of shilajit extract.

Disciplinary: Biochemistry and Biochemical Engineering.

Cite This Article:

AlShubaily, F. and Jambi, E. (2022). LC/MS Profiling of Shilajit Extract for Antimicrobial & Antifungal and Cytotoxic Activities. *International Transaction Journal of Engineering, Management, & Applied Sciences & Technologies, 13*(5), 13A5N, 1-13. http://TUENGR.COM/V13/13A5N.pdf DOI: 10.14456/ITJEMAST.2022.98

Nomenclature and Symbols

LC-HRESIMS: liquid chromatography-high resolution electrospray ionization mass spectrometry,
CFU: colony-forming units,
DMSO: dimethyl sulfoxide,
MIC: minimal inhibitory concentration,
MFC: minimal fungicidal concentration,
LTQ: linear trap quadrupole,
HPLC: high-performance liquid chromatography,
PTFE: polytetrafluoroethylene,
LC/MS: liquid chromatography-mass spectrum,
PDA: photodiode array,
MeOH: methanol,
DMEM: Dulbecco's Modified Eagle Medium,
FBS: fetal bovine serum,
MCF-7: *Homo sapiens* adenocarcinoma epithelial breast mammary gland cell line,
T-47D: *Homo sapiens* ductal carcinoma epithelial mammary gland cell line,
Hep G2: *Homo sapiens* hepatocellular carcinoma liver cell line,
SKOV-3: ovarian carcinoma,
ATCC: American type culture collection,
MTT: tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide),
SDS: sodium dodecyl sulfate,
ANOVA: analysis of variance,
SEM: standard error of the mean,
$[M+H]^+$: the ions observed by mass spectrometry may be quasi-molecular ions created by the addition of a hydrogen cation.

1. Introduction

Shilajit, *Asphaltum punjabianum* is a natural black greasy substance formed by the degradation of plant materials from species such as *Euphorbia royleana* and *Trifolium repens* in the high-altitude mountains [1, 2]. This vegetation has undergone compression and high temperature for many years underneath several mountains' rocky layers to be transformed into its greasy perfusion from the rock of the mountains [1]. Shilajit is used as a traditional medicine for the remediation to cure many diseases.

Traditional medicine had been an essential part of health care in many middle eastern areas such as Yemen and Saudi Arabia, and in many Asian countries such as China and India for thousands of years [1]. Therefore, significant conformational testimony was intended to document the mode of action for herbo-medicinal extracts [1]. Shilajit has potential uses such as anti-

inflammatory, anti-fungal, anti-ulcerogenic, anxiolytic activity, anti-allergic, analgesic, anti-diabetic, memory enhancer, and antioxidant [3-6].

Various studies have evaluated the properties of shilajit extracts obtained from different countries [7, 8]. The ecological nature of the mountain's rocks, variation in local humidity and temperature, and speciation of plants impact the chemical composition of shilajit [9] for which the organic compounds, fulvic acid, 3,4-benzocoumarins, hippuric acid, resin, benzoic acid, fatty acids, ellagic acid, amino acids, and certain alkaloids are the main bioactive constituents. Abundant organic compounds of the shilajit extract exist in varied ratios corresponding to different countries, which affect the physical properties and chemical composition [10]. Studies had found that in India, Pakistan, and Russia, the ratios of shilajit's fulvic acid were 21.4%, 15.5%, and 19%, respectively [1]. Therefore, the local source of the shilajit extract is significant and directly correlated to its therapeutic impact [1, 2].

There are increasing awareness and interest in using traditional herbo-medicine like shilajit because of its efficient therapeutic impact on the treatment of common diseases such as anemia, chronic pain, diabetes, osteoporosis, and digestive disorders. The effects of several environmental and economic factors such as the plant source, temperature, transport, and storage have influenced the chemical composition and efficacy of the commercial shilajit. Therefore, the present study was performed to investigate the chemical composition of commercial shilajit extract using liquid chromatography-high resolution electrospray ionization mass spectrometry (LC-HRESIMS) analysis and determine its antimicrobial and cytotoxic potential using *in vitro* and *in vivo* models. There were limited studies and published information regarding the investigation of commercial shilajit specifically in providing the concentrations of shilajit that need to be utilized. Therefore, different concentrations of local commercial shilajit were tested to identify the highest non-toxic concentration that could be used in the *in vivo* studies on human carcinogen cell lines. These statistics supply a molecular foundation to explain at least a section of the advisable therapeutic properties of shilajit extract.

2. Methods

2.1 Microbial Strains and Growth Media

Bacterial species, *Staphylococcus aureus* (NCIMB 6571), *Pseudomonas aeruginosa* (NCIMB 6750), *Escherichia coli* (NCIMB 10214), and fungal species, *Aspergillus niger* (IMI 51433), and *Candida albicans* (NCPF 3255) were obtained from purchased from the American Type Culture Collection (ATCC). The specified fungal and bacterial cultures were kept at 4°C on Sabouraud dextrose and Mueller-Hinton agar media. The antifungal and antibacterial properties were analyzed by Sabouraud dextrose and Mueller Hinton agar and broth, respectively. All media were obtained from Oxoid Ltd, Basingstoke, Hampshire, UK. The fungi and bacteria cultures were grown for 24 hours and the standard MacFarland turbidity of 10^6 colony forming units (CFU) mL^{-1} was achieved by mixing with sterile physiological saline. A quantity of 100 µL of each microbial suspension was

added into 25 mL of (40 ºC) Sabouraud dextrose and Muller Hinton sterile broth media for fungi and bacteria respectively.

2.2 Disk Diffusion Method

Shilajit was purchased from Amazon India. The antimicrobial property of shilajit extract was assessed by the disc diffusion method [11, 12]. The crude methanolic extract of shilajit was dissolved in a sterile 10% dimethyl sulfoxide (DMSO) to make a 50 mg mL^{-1} stock solution before being tested for antimicrobial activity. Using a sterile cotton swab, 100 µL of (24 - 72 hrs) isolate inoculums were spread evenly on the surface of solid media plates. Blank 6mm-diameter disks were saturated with dilutions of the stock shilajit crude extract to make concentrations of 200, 100, 50, 25, and 12.5 µg $disc^{-1}$ and were placed on the surface of the agar plates and then incubated 24-48 hrs at 37°C and 28°C for bacteria and fungi, respectively. The inhibition zone's diameters were measured in milliliters from the disc margin to the inner edge of the encircled pathogens. A disc impregnated with 10% DMSO was used as a negative control and positive controls were assessed with standards nystatin and amoxicillin drugs (Sigma Chemical Co., St. Louis, MO, USA) which were dissolved in DMSO 10% (10 µg $disc^{-1}$) and served as positive antifungal and antibacterial controls; respectively. Duplicate repeats were conducted for each experiment.

2.3 Broth Micro-Dilution Method

Antimicrobial analysis and assessment of the minimal inhibitory concentration (MIC) of the extract against different bacterial and fungal strains were achieved using the broth micro-dilution method. As only *S. aureus* and *C. Albicans* have shown inhibitory effects down to 100 mg mL^{-1} and 12.5 mg mL^{-1}, respectively, MIC was calculated for these species only. To determine the MIC of the shilajit crude extract, 5 dilutions for each concentration of 100 mg mL^{-1} and 12.5 mg mL^{-1} were prepared in 5% DMSO. A quantity of 100 µL of the microbial suspensions was inoculated into the prepared dilution tubes and incubated at 28 and 37°C for 48 hrs for the fungi and bacteria strains respectively. The tubes comprising only the substrate with either diluted extract or microbial suspension were designated as negative and positive controls, respectively. The least concentration of each extract representing a clear inhibition was reported as the MIC concentration. To verify the presence or absence of growth turbidity, samples were also measured spectrophotometrically after 48 hrs at 540 nm. The determination of minimal fungicidal concentration (MFC) values for the fungi were evaluated as the highest dilutions (lowest concentrations) at which no growth occurred on the plates. All serial dilutions of the shilajit were performed in duplicates.

2.4 LC-HRESIMS Analysis

The liquid chromatography-high resolution electrospray ionization mass spectrometry (LC-HRESIMS) was assessed to determine the bioactive compounds in the examined extract. A quantity of 100 mg of shilajit crude extract was dissolved in 100 mL of 10% methanol. This mixture was filtered through a 0.2 µm PTFE filter, and a quantity of 1ml was placed into LC/MS vials. High-resolution mass spectrometric data were obtained using a Thermo LTQ Orbitrap coupled to an

HPLC system (PDA detector, PDA autosampler, and pump). The following conditions were used: capillary voltage of 45 V, capillary temperature of 260 °C, auxiliary gas flow rate of 10–20 arbitrary units, sheath gas flow rate of 40–50 arbitrary units, spray voltage of 4.5 kV, and mass range of 100–2000 amu (maximal resolution of 30000). For LC/MS, a Sunfire C18 analytical HPLC column (5 µm, 4.6 mm × 150 mm) was used with a mobile phase of 0 to 100% MeOH over 30 min at a flow rate of 1 mL/ min^{-1}. Analysis of data was performed using Xcalibur 3.0 and dereplication using a dictionary of natural products database V. 23.1 on DVD.

2.5 *In vitro* Cytotoxicity Assay

Cell lines of MCF-7, T-47D, and Hep-G2 were purchased from the American Type Culture Collection (ATCC, USA). The cells were nurtured in DMEM culture media containing antibiotics (100 U mL^{-1} of penicillin and 100 µg mL^{-1} of streptomycin) and 10% FBS (Gibco) at 37°C in a CO_2 incubator (5% CO_2 and 95% relative humidity). The inhibitors were tested for their cytotoxicity properties against MCF-7, T-47D, and Hep-G2 cells [13]. The analysis comprised distributing the cell aliquots for MCF-7, T-47D cells (3.6×10^5 cells mL^{-1}, 3.5×10^5 cells mL^{-1}, and 3.8×10^5 cells mL^{-1}, 95µL) in 96 well plates. The 96 well plates were aliquoted with the prepared inhibitor stock solutions in DMSO (150µL, 5µL) and incubated for 24 hrs. (37ºC, 5% CO_2). The control samples were the wells that contain the cells and only DMSO (5µL). The tested plate was incubated with the MTT solution (final concentration 0.5mg mL^{-1}, 10 µL) at 37 ºC in 5% CO_2 for 180 min. A solution of SDS (10% in 0.01M HCl, 100 µL) was added to each well of the 96 well plates and further incubated overnight (37ºC, 5%CO_2). The percent toxicity was assessed by measuring the optical density at 570 nm using a Biotek Lx800 microplate reader.

3. Statistical Analysis

The statistical analysis had been performed using a one-way ANOVA and t-test. The results were calculated as means ± S.D. to indicate the experimental variations. The difference is considered significant when $^*P$-value ⩽ 0.05.

4. Results

The antimicrobial activity of shilajit extract against the microorganisms considered in the present study was assessed by evaluating the presence of inhibition zones, as shown in Table 1. The crude extract showed significant antibacterial activity against *Staphylococcus aureus* NCIMB 6571 at a concentration of 200 µg $disc^{-1}$ and gave inhibition zones of 13±0.3 mm diameter, and antifungal activity against *Candida albicans* NCPF 3255 at concentrations of 200, 100, 50, and 25 µg $disc^{-1}$ and gave inhibition zones of 29±0.3, 18±0.3, 15±0.3 and 12±0.3 mm diameter, respectively. There was negative antimicrobial inhibition detection at a concentration below 25 µg $disc^{-1}$, and null activity of shilajit extract against all the other microbes at the specified concentrations used in the current study. The results obtained from amoxicillin and nystatin exhibited resistance to all selected microbial strains representing inhibition zones ranging from 10 to 22 µg $disc^{-1}$. As the extract was diluted, its antifungal activity level decreased markedly. It is feasible; therefore that elevation of

the antifungal property may be detected if the separated pure bioactive constituents of the extract were assessed [14]. The antifungal property of the herbo-medicinal extracts is mostly associated with their chemical compound structure and functional groups. Synergistic interactions between the molecular compositions of the herbo-medicinal extract could plausibly affect the antimicrobial activity [15].

The assessment of the minimal inhibitory concentration (MIC) task relies on the incidence that the lower the MIC value, the more efficient the herbo-medicinal extract as a potential disease remedy. Many studies speculated the prospected that diminished toxicity and side effects would be the results output of the low doses of herbo-medicinal extract needed to acquire a therapeutic response [16]. Table 2 summarizes the results of the antimicrobial activity of shilajit as MIC and MFC using the broth micro-dilution method. Results showed inhibitory antibacterial effects >50 mg mL^{-1} and a fungicidal effect was the plant extract (MIC: 12.5 mg mL^{-1} and MFC:50 mg mL^{-1}) was found on *C. Albicans* and null activity against all the other microbes. The inhibitory action of the extract on *C. sssssss* growth could be attributed to the presence of active compounds such as fulvic, gallic, and ferulic acids.

Table 1 Antimicrobial activities of shilajit crude extract, nystatin, and amoxicillin standards against microbial isolates mean ± SD of measured inhibition zone (mm) in experimental groups (n = 2 in each group).

Microbial Isolate	Mean Diameter of Inhibition Zone of inhibition (mm) ± SEM*					Nystatin	Amoxicillin
	200 µg $disc^{-1}$	100 µg $disc^{-1}$	50 µg $disc^{-1}$	25 µg $disc^{-1}$	12.5 µg $disc^{-1}$	10 µg $disc^{-1}$	10 µg $disc^{-1}$
Staphylococcus aureus NCIMB 6571	13± 0.3	-	-	-	-	-	22 ± 0.2
Pseudomonas aeruginosa NCIMB 6750	-	-	-	-	-	-	10 ± 0.3
Escherichia coli NCIMB 10214	-	-	-	-	-	-	12 ± 0.3
Aspergillus niger IMI 51433	-	-	-	-	-	15 ± 0.3	-
Candida albicans NCPF 3255	29 ± 0.3	18 ± 0.3	15 ± 0.3	12 ± 0.3	-	22 ± 0.2	-

SEM, standard error of the mean. Mean ± SEM in each column with no common superscript differ significantly ($P \leq 0.05$). (-): not detected activity at this amount of shilajit or standards.

Table 2 Minimal inhibitory concentration (MIC) of the shilajit extract, nystatin, and amoxicillin against microbial isolates.

Microbial Isolate	Shilajit (mg mL^{-1})	
	MIC	MFC
Staphylococcus aureus NCIMB 6571	50.0	-
Pseudomonas aeruginosa NCIMB 6750	-	-
Escherichia coli NCIMB 10214	-	-
Aspergillus niger IMI 51433	-	-
Candida albicans NCPF 3255	12.5	25

Mean ± standard error of the mean with no common superscript differ significantly (*$P \leq 0.05$) (-): not detected activity at this amount of shilajit or standards.

The liquid chromatography-high resolution electrospray ionization mass spectrometry (LC-HRESIMS) analysis of shilajit crude extract revealed the presence of 17 bioactive compounds explicated with their calculated mass spectrum m/z, retention time, and frequencies as shown in

Table 3. This technology allocated the recognition of distinct classes comprising phenolic acids, organic acids, alkaloids, tri-terpenoids, and flavonoids. Figure 1 showed the chromatogram of the shilajit with major peaks at the retention times (min.) of 0.99, 1.36, 4.97, 5.21, 11.6, 16.59, and 31.25 of fulvic acid, gallic acid, ferulic acid, naphsilajitone, fraxin, 3,8-dihydroxydibenzo-α-pyrone, and pregnane, respectively (Table 3). The utilization of the shilajit extract in traditional medicine was consistent with the results of the antifungal properties of several crude extracts. A wide-range spectrum of antibiotics can be obtained from the roots and shoot parts of plants. Bauer and Tittel [17] and Springfield, Eagles [18] indicated in their studies that chemical characterization is best achieved throughout HPLC spectrum analysis technology. Therefore, the plant extract's bioactive constituents that can act as antibacterial, antifungal, anti-inflammatory, and antioxidant in the current experiment were identified using LC/MS fingerprint. Phenolic acids exhibited various pharmacological properties such as ferulic acid with an antibacterial [19], antifungal [20] and anti-inflammatory properties [21]; gallic acid has anti-inflammatory [22], caffeic acid showed antibacterial, antifungal [19] and anti-inflammatory [21].

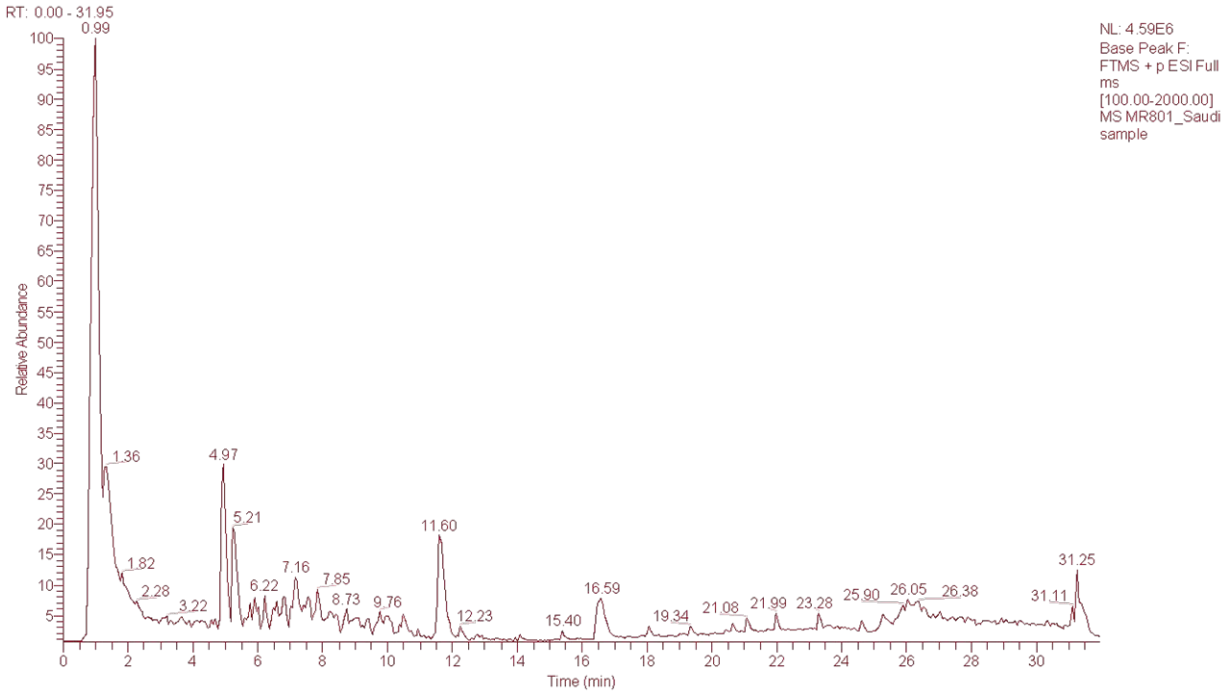

Figure 1: The liquid chromatography-high resolution electrospray ionization mass spectrometry (LC-HRESIMS) chromatogram of bioactive compounds in shilajit.

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was conducted to evaluate cell proliferation of shilajit crude extract on cell lines of Hep G2, MCF-7 and T-47D, A549. As shown in Figure 2, significant (*$P<0.05$) percent inhibition (IC_{50}: 19 µg mL^{-1}) of the investigated test extract is revealed by the applied cell line Hep G2. The shilajit extract showed an increased though non-significant ($P>0.05$) proliferation inhibition percentages for MCF-7 and T-47D cell lines for all the administered concentrations in the present study.

Table 3 The liquid chromatography-high resolution electrospray ionization mass spectrometry (LC-HRESIMS) analysis of shilajit extract.

Ret. (min.)	Accurate Mass	Molecular Formula *	Tentative Identification **
0.99	639.1190	$C_{27}H_{26}O_{18}$	Fulvic acid
1.36	171.0290	$C_7H_6O_5$	Gallic acid
1.82	181.0497	$C_9H_8O_4$	Caffeic acid
4.97	195.0650	$C_{10}H_{10}O_4$	Ferulic acid
5.21	201.0544	$C_{12}H_8O_3$	Naphsilajitone
7.16	249.1696	$C_{12}H_{24}O_5$	Shilajityl acetate
7.85	193.1224	$C_{12}H_{16}O_2$	Shilacatechol
11.60	371.0996	$C_{16}H_{18}O_{10}$	Fraxin coumarin
16.58	229.0497	$C_{13}H_8O_4$	3,8-dihydroxydibenzo-α-pyrone
16.61	213.0545	$C_{13}H_8O_3$	3-hydroxydibenzo-α-pyrone
18.04	311.0552	$C_{17}H_{10}O_6$	Shilaxanthone
19.34	311.1268	$C_{19}H_{18}O_4$	6-methoxy-2-[2-(3-methoxyphenyl)ethyl]chromone
21.08	309.0759	$C_{18}H_{12}O_5$	Shilanthralin
21.99	179.1430	$C_{12}H_{18}O$	Shilajitol
23.28	231.0769	$C_{17}H_{10}O$	No natural hits
31.09	237.1858	$C_{15}H_{24}O_2$	Geranyl tiglate
31.25	289.2892	$C_{21}H_{36}$	Pregnane

* Based on the quasimolecular ion $[M+H]^+$

** Based on Dictionary of Natural Products Database ver. 23.1 and Reaxys online database.

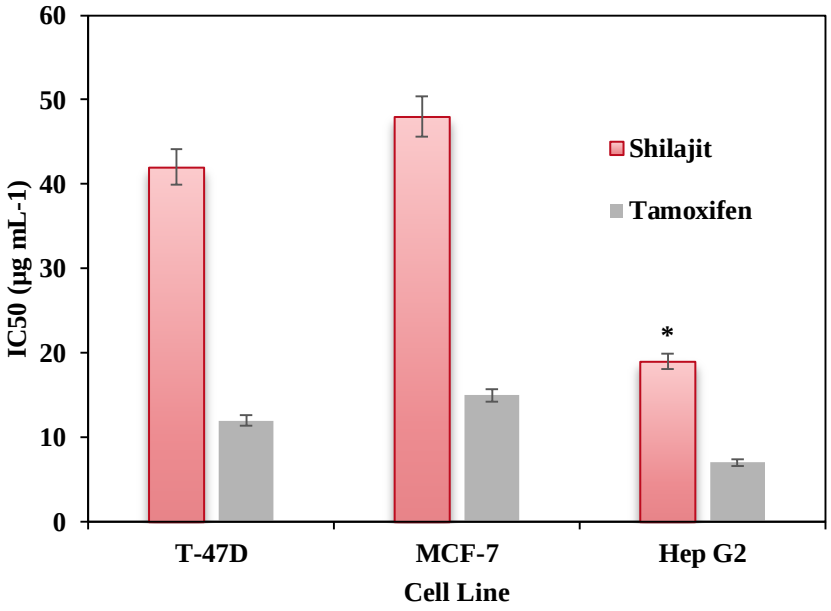

Figure 2: The effects of shilajit extract on the proliferation of MCF-7, T-47D, and Hep-G2 cell lines. Cells were incubated with increasing concentrations of tested extract in a culture medium, and the proliferative response was then assessed by MTT assay. Data presented are the mean of three independent experiments (*$P>0.05$*).

5. Discussion

The LC-HRESIMS analysis of shilajit extract revealed the presence of 17 bioactive compounds, including fulvic acid, gallic acid, ferulic acid, naphsilajitone, fraxin, 3,8-dihydroxydibenzo-α-pyrone, and pregnane. Previous researches have demonstrated that the shilajit extracts therapeutic effect against diseases was correlated to their bioactive phytochemical constituents [23-26]. Herbal extracts constitute various bioactive compounds, and it is crucial to perceive their mechanism of action on diverse targets for cure and disease remedy. Researchers have studied the chemical structure and

biological properties of many of these bioactive compounds. Shilajit is considered a distinct phytocomplex because it contains various significant bioactive compounds such as aromatic carboxylic acids, phenolic lipid, latex, sterols, gums, albumins, ellagic acid, 3,4-benzocumarins, triterpenes, amino acids, fulvic acid, some fatty acids, resins and polyphenols [27]. The technology of HPLC has attributed to the immense pharmacological activities of phenolic acids such as ferulic acid with anti-inflammatory [28] and antifungal [29]; gallic acid having anti-inflammatory [22], antibacterial [28]; tannic acid with astringent and antioxidant property [30, 31] and caffeic acid with anti-inflammatory properties [28]. Many biological and medicinal properties were attributed to fulvic acid, the main organic constituent of shilajit extract [32, 33]. Fulvic acid is efficient in the cure of diseases such as stomach ulcers, gastritis, diabetes mellitus, diarrhea, colitis and dysentery [1, 33] and induction of immunity role in lymphocytes and neutrophils [34, 35].

Efficient *In vitro* antimicrobial activities against phytopathogenic fungi have been observed with various medicinal plant extracts [36]. The results of the current study showed that the crude extract of shilajit had an antimicrobial effect on the tested *Staphylococcus aureus* NCIMB 6571 (>50 mg mL^{-1}) and *Candida albicans* NCPF 3255 (MIC: >12.5 mg mL^{-1} and MFC: >50 mg mL^{-1}). A previous study indicated that the MIC of Russian shilajit for *Staphylococcus Aureus* is 125 µg mL^{-1}, *Bacillus Subtilis* is 1000 µg mL^{-1} and for both *Escherichia Coli* and *Candida Albicans* 62.5 µg mL^{-1}, while no MIC for both Pseudomonas Aeruginosa within diluted concentrations [37]. Spore inhibitory activity of 95% against *Alternaria cajani* had been observed with the methanolic extract of shilajit at the concentration of 5000 µg mL^{-1} [38]. The varying concentration and antimicrobial impact of fulvic acidic attributed to the antimicrobial effects of the studied shilajit sample. A significant inhibition zone of 50 mm was observed in a strain of *Candida albicans,* among other examined microbes [39]. Most of the literature has confirmed the particularly antifungal activity of shilajit extracts. Rachana [38] has verified the inhibitory properties of shilajit against phytopathogenic fungi. Shakirov [40] has documented the antimicrobial activities of various prevalent pyogenic microbial strains such as *coliform bacteria*, *staphylococci*, *Proteus, enterococci*, and *streptococci*, as well as Muratova and Shakirov [41] who appointed the utilization of shilajit extract for the cure of suppurative wounds. Van Rensburg, Van Straten [42] reported the correlation of the antimicrobial activities with the shilajit extract constituents, fulvic, and benzoic acids. Variation in herbs and plants regional species and climate change lead to a difference in the discrepancy of its antimicrobial and antiulcer properties. Therefore, markedly diverse physiological activities of shilajit samples could be attributed to their source regions in the world based on their major organic constituents, as reported by many authors [9, 43]. Broad antimicrobial spectrum had been confirmed with major shilajit component, fulvic acid on various microbial strains of *S. mutant P. gingivalis*, *S. mitis*, *E. faecalis*, *A. actinomycetemcomitans*, *F. nucleatum*, and also possesses an anticancer cytotoxic effect *in vivo* [44].

In the current study, shilajit extract's cytotoxic activity revealed a significant increase in inhibition percentage for the concentrations of 19 µg mL^{-1} on cell lines of Hep G2 (hepatocellular carcinoma). Similarly, another study confirmed inhibitory anticancer properties of mineral pitch on

four cancerous cell lines with concentrations ranging from 25-250 µg mL^{-1} [45]. The 50% effective concentration (ED_{50}) are 89, 96, 225, and 286 µg mL^{-1} for A_{549} (lung cancer), HepG2 (liver cancer), MDA-MB-231 (human breast cancer), and SKOV-3 (ovarian carcinoma), respectively [45]. As indicated previously that the chemical constituents of shilajit extract exist in diverse ratios among different sources, which influenced their physical and chemical properties [10, 43]. Previous research studies reported that commercial shilajit purchased from different sources had varying cytotoxic impacts. Therefore, all traditional shilajit samples substantially demanded cytotoxicity assessment. A previous study revealed that the concentration of 1.6 mg mL^{-1} was the highest non-toxic level of shilajit conferring to the WST-1 analysis, whereas, a lower concentration of 19 µg mL^{-1} was obtained in the present experiment. This concentration was the 50% effective inhibitory concentration percentage of cell proliferation/viability compared to the control sample.

6. Conclusion

This study provides new scientific information about the commercial extract of shilajit based on its chemical profiling, antimicrobial, and cytotoxic potentials, which were attributed to the various phytochemical constituents present in the crude extract. The further purification process of the shilajit chemical components may have even more potency concerning inhibition of microbes and cytotoxic efficacy and encourage the development of a novel broad-spectrum antimicrobial and cytotoxic herbal formulation in the future.

7. Availability of Data and Materials

Data sharing does not apply as all information has been included in this study.

8. Acknowledgment

This project was funded by the Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah, under grant no. (G-617-247-1439). The authors, therefore, acknowledge with thanks to DSR for technical and financial support.

9. Authors' Contributions

Fawzia Alshubaily: study design, acquisition of data, analysis and interpretation of data, drafting of the manuscript.

Ebtihaj Jambi: study design, analysis and interpretation of data, writing and critical revision of the manuscript. All authors read and approved the final manuscript.

10. Ethics Approval and Consent to Participate

Not applicable.

11. Competing Interests

The authors declare that they have no competing interests.

12. References

[1] Agarwal SP, et al. Shilajit: a review. Phytotherapy research: PTR. 2007;21(5):401-405.

[2] Carrasco-Gallardo C, Guzmán L, and Maccioni RB. Shilajit: a natural phytocomplex with potential procognitive activity. International Journal of Alzheimer's disease. 2012.

[3] Bhattacharya SK. Shilajit attenuates streptozotocin-induced diabetes mellitus and decrease in pancreatic islet superoxide dismutase activity in rats. PTR Phytotherapy Research. 1995;9(1):41-44.

[4] Ghosal S. Chemistry of shilajit, an immunomodulatory Ayurvedic rasayan. Pure and Applied Chemistry. 1990;62(7):1285-1288.

[5] Acharya, SB, et al. Pharmacological actions of Shilajit. Indian journal of experimental biology, 1988. 26(10):775-7.

[6] Jaiswal, AK, and Bhattacharya, S. Effects of Shilajit on memory, anxiety and brain monoamines in rats. Indian J Pharmacol. 1992;24(1):12-7.

[7] Ghosal, S. Shilajit in Perspective, Alpha Science International Limited. 2006, Oxford, United Kingdom.

[8] Wilson, E, et al. Review on shilajit used in traditional Indian medicine. Journal of ethnopharmacology. 2011;136(1):1-9.

[9] Ghosal S, Lal J, and Singh SK. The core structure of Shilajit humus. Soil Biology and Biochemistry, 1991;23(7):673-80.

[10] Schepetkin IA, et al. Complement-fixing activity of fulvic acid from Shilajit and other natural sources. Phytotherapy research: PTR. 2009;23(3):373-84.

[11] Ferraro MJ. Performance standards for antimicrobial disk susceptibility tests. 2000: NCCLS.

[12] Tepe B, et al. Screening of the antioxidative and antimicrobial properties of the essential oils of Pimpinella anisetum and Pimpinella flabellifolia from Turkey. Food Chemistry. 2006;97(4):719-24.

[13] Denizot F, and Lang R. Rapid colorimetric assay for cell growth and survival: modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. Journal of immunological methods. 1986;89(2):271-7.

[14] Dellavalle PD, et al. Antifungal activity of medicinal plant extracts against phytopathogenic fungus Alternaria spp. Chilean J. Agric. Res. Chilean Journal of Agricultural Research. 2011;71(2):231-9.

[15] Naeini A, et al. Anti- Candida albicans activity of some Iranian plants used in traditional medicine. Journal de Mycologie Médicale Journal de Mycologie Médicale. 2009;19(3):168-72.

[16] Srivastava G, et al. Antimicrobial activity of the methanolic extract, fractions and isolated compounds from citrullus colocynthis (l.) schrad. Intl. J. Pharma Bio Sci. International Journal of Pharma and Bio Sciences. 2013;4(3):B825-33.

[17] Bauer R, and Tittel G. Quality assessment of herbal preparations as a precondition of pharmacological and clinical studies. Phytomedicine. International journal of phytotherapy and phytopharmacology, 1996;2(3):193-8.

[18] Springfield EP, Eagles PKF, and Scott G. Quality assessment of South African herbal medicines by means of HPLC fingerprinting. Journal of Ethnopharmacology Journal of Ethnopharmacology. 2005;101(1-3):75-83.

[19] Ravn H, et al., Caffeic acid esters as in vitro inhibitors of plant pathogenic bacteria and fungi. Biochemical Systematics and Ecology Biochemical Systematics and Ecology. 1989;17(3):175-84.

[20] Tawata S, et al. Synthesis and Antifungal Activity of Cinnamic Acid Esters. Bioscience, Biotechnology, and Biochemistry Bioscience, Biotechnology, and Biochemistry. 1996;60(5):909-10.

[21] Fernandez MA, Saenz MT, and Garcia MD. Anti-inflammatory activity in rats and mice of phenolic acids isolated from Scrophularia frutescens. Journal of pharmacy and pharmacology. 1998;50(10):1183.

[22] Kroes BH, et al. Anti-inflammatory activity of gallic acid. Planta medica. 1992.58(6):499-504.

[23] Mutalik S, et al. Effect of Dianex, a herbal formulation on experimentally induced diabetes mellitus. Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives. 2005;19(5):409-15.

[24] Pillai GKG, et al. Antidiabetic potential of polyherbal formulation DB14201: Preclinical development, safety and efficacy studies. Journal of ethnopharmacology. 2017;197:218-30.

[25] Yadav D, et al. In vitro toxicity and antidiabetic activity of a newly developed polyherbal formulation (MAC-ST/001) in streptozotocin-induced diabetic Wistar rats. Protoplasma. 2013;250(3):741-49.

[26] Yuan H, et al. How can synergism of traditional medicines benefit from network pharmacology? Molecules. 2017;22(7):1135.

[27] Chopra R, et al. Indigenous drugs of India, UN Dhur and sons Pvt. Ltd., Calcutta, 1958;289:665.

[28] Binutu OA, Cordell GA. Gallic acid derivatives from Mezoneuron benthamianum leaves. Pharmaceutical Biology. 2000;38(4):284-6.

[29] Mehrotra R. Defense mechanism in plants. Plant pathology. Tata-McGrew Hil, New Delhi, 1997:544p.

[30] Khan NS, Ahmad A, and Hadi S. Anti-oxidant, pro-oxidant properties of tannic acid and its binding to DNA. Chemico-Biological Interactions. 2000. 125(3):177-89.

[31] Windholz, M., et al., The Merck Index. Rahway, NJ: Merck & Co. Inc, 1983. 82:1417-9.

[32] Ghosal, S., et al., Anti-ulcerogenic activity of fulvic acids and 4′-methoxy-6-carbomethoxybiphenyl isolated from shilajit. Phytotherapy Research, 1988. 2(4):187-91.

[33] Schepetkin, I., A. Khlebnikov, and B.S. Kwon, Medical drugs from humus matter: Focus on mumie. DDR Drug Development Research. 2002. 57(3):140-59.

[34] Joone, G.K., J. Dekker, and C.E.J. van Rensburg, Investigation of the Immunostimulatory Properties of Oxihumate. ZEITSCHRIFT FUR NATURFORSCHUNG C. 2003. 58:263-7.

[35] Schepetkin, I.A., et al., Characterization and Biological Activities of Humic Substances from Mumie. J. Agric. Food Chem. Journal of Agricultural and Food Chemistry. 2003. 51(18):5245-54.

[36] Basha, S.A., et al., Effect of Berberine and (+/-)-Bicuculline Isolated from Corydalis chaerophylla on Spore Germination of Some Fungi. FOLIA MICROBIOLOGICA -PRAGUE-. 2002. 47:161-6.

[37] Kotb-El-Sayed, M.k., H. Amin, and A. Al-kaf, Anti-Microbial, anti-oxidant and anti-ulcerogenic effects of Shilajit on gastric ulcer in rats. Am. J of Biochem Biotech. 2011. 8(1):26-39.

[38] Rachana, S., Antifungal activity screening and HPLC analysis of crude extract from Tectona grandis, Shilajit, Valeriana wallachi. Electronic Journal of Environmental, Agricultural and Food Chemistry.

2009;8(4):218-29.

[39] Hayat, A., Antimicrobial Activity of Shilajit. RADS Journal of Biological Research & Applied Sciences. 2013;4(2):10-2.

[40] Shakirov, D., Experimental treatment of infected wounds with mumie asil'. Eksperimental'naia khirurgiia i anesteziologiia, 1969;14(6):36-9.

[41] Muratova, K. and D. Shakirov, Clinical treatment of suppurative wounds with Mumie. Khirurgiia, 1968;44(9):122-4.

[42] Van Rensburg C, Van Straten A, and Dekker J. An in vitro investigation of the antimicrobial activity of oxifulvic acid. Journal of Antimicrobial Chemotherapy. 2000; 46(5):853-4.

[43] Galimov E, et al., Geochemistry of mummiyo. Geochemistry. 1986;1494-505.

[44] Leighann S, et al. Investigating the biological properties of carbohydrate derived fulvic acid (CHD-FA) as a potential novel therapy for the management of oral biofilm infections. BMC Oral Health. 2013;13(1):47.

[45] Phaechamud T, et al. Some Biological Activities and Safety of Mineral Pitch. Science, Engineering and Health Studies. Silpakorn University Science and Technology Journal. 2008;2(2):7-17.

Dr.Fawzia A. Alshubaily is an Associate Professor of Protein Structural Bioinformatics, Biochemistry Department, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia.

Dr.Ebtihaj J. Jambi is an Associate Professor of Pharmacy and Biotechnology (Fermentation Technology), Biochemistry Department, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia. She is interested in the Bioprocessing Engineering