



**International Journal of Biology, Pharmacy  
and Allied Sciences (IJBPAS)**

*'A Bridge Between Laboratory and Reader'*

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## SHILAJIT LOADED NANOPARTICLES: A QBD BASED APPROACH FOR IMPROVED THERAPEUTIC POTENTIAL

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Received 14<sup>th</sup> June 2025; Revised 20<sup>th</sup> July 2025; Accepted 18<sup>th</sup> Aug. 2025; Available online 1<sup>st</sup> July 2026

<https://doi.org/10.31032/IJBPAS/2026/15.7.10241>

### ABSTRACT

Shilajit, a sticky black exudate of herbomineral origin, is commonly found in mountainous regions and generally consists of a complex mixture of organic humic substances and traces of plant and microbial metabolites. Humic acid and fulvic acid which are important constituents of Shilajit are responsible for its various pharmacological actions. Shilajit has been used popularly and safely as a rejuvenator and adaptogen for millennia as an integral part of traditional systems of medicine globally.

The current research aims to improve the therapeutic efficacy of Shilajit for cardiovascular health by formulating the drug as zinc nanoparticles while simultaneously working to conserve natural mineral resources by significantly decreasing the dose required using this approach.

The formulation was optimised using QbD and characterized using parameters such as entrapment efficiency, particle size, and zeta potential. *In vitro* diffusion studies, chorioallantoic membrane assay and trypan blue test for cell viability indicated better biological activity of the nanoparticles as compared to the extract thus indicating a significantly improved activity. When traditional medicinal agents such as Shilajit are combined with modern formulation techniques, a significant improvement is seen in its activity. Hence there is great potential for the development of novel drug delivery systems for traditional medicinal systems

**Keywords: Shilajit, fulvic acid, zinc nanoparticles, cardioprotective, anti-oxidant, QbD**

## INTRODUCTION

Shilajit, an important Ayurvedic rasayana is a sticky brown to blackish-brown substance, of herbomineral origin obtained as an exudate in many mountainous areas of the world, especially the Himalayas and Hindukush ranges. It is known by various local names and can be found in many regions of the world including central, south-east, and eastern Asia, Australia, and certain countries of the African continent [1-3].

The origins of Shilajit have been studied in detail and many hypotheses have been put forth. Shilajit mainly consists of organic humic matter along with inorganic soil minerals. Studies suggest that certain mosses (bryophytes) are responsible for the formation of Shilajit through humification [3-5].

Shilajit generally comprises organic acids such as fulvic acid and humic acid which act as the main physiologically active constituents. Shilajit also contains various inorganic minerals in their ionic states. Fulvic acid is mainly responsible for the transport of these minerals to various cells and tissues where they function as electric potential mediators thus preventing cell death and promoting rejuvenation [5-7].

It also regulates metabolism and helps in energy production. Shilajit is an immunomodulator that promotes

haematopoiesis and also possesses a significant detoxification potential. Shilajit has also shown considerable promise in the treatment and prevention of cardiovascular diseases mainly through its antioxidant and anti-inflammatory properties.

Humic acid present in Shilajit exhibits antioxidant properties against NO and OH radicals. Constituents present in Shilajit have also been known to promote longevity by preventing cell impairment and damage [7-8]. Despite having a considerable mineral content, Shilajit can be safely used in the long run as data collected from chronic toxicity studies of Shilajit and its formulations have indicated its safety [8-10].

Drugs of natural origin often show low or erratic absorption thus leading to decreased bioavailability. Shilajit has been used as a bioavailability enhancer. In the Ayurvedic system of medicine drugs like long pepper, ginger, and black pepper are commonly used as bio enhancing agents. Fulvic acid is majorly responsible for improving bioavailability as it acts as a carrier owing to its micropore-filled structure. Water-insoluble compounds can be incorporated into these voids in a proportion of 0.5-40% by weight of fulvic acid [11-14].

Many formulations of Shilajit are widely available in the market. However, the techniques adopted in the current study aim at improving the bioavailability of Shilajit by using modern formulation techniques to form zinc nanoparticles loaded with Shilajit.

Zinc is a vital component present in most antioxidant enzymes such as catalase and superoxide dismutase as well as metallothionein. An insufficiency of zinc may cause a disruption of their synthesis thereby leading to oxidative stress and decreased free radical scavenging which plays a major role in cardiovascular disease [15-18].

## MATERIALS AND METHODS

**Materials** Purified Indian Shilajit was obtained from Jairamdas Khushiram Company (Navi Mumbai, India). Fulvic acid standard was sourced from Hexon Laboratories Pvt. Ltd. Zinc acetate and sodium hydroxide were procured from Sigma-Aldrich and Merck, respectively. All other reagents used were of analytical grade.

**Preformulation Studies** The authenticity of Shilajit was confirmed using pharmacognostic parameters, including ash values, extractive values, and iron content. Phytochemical screening of aqueous extracts was performed to detect alkaloids, glycosides, tannins, saponins, flavonoids, proteins, carbohydrates,

amino acids, steroids, oils, mucilage, and starch.

## Chromatographic and Spectral Analysis

UV-Vis spectroscopy was used to evaluate the methanolic extract of Shilajit. A 100 µg/mL solution was scanned between 200–800 nm using a Thermo Scientific 300 UV-Vis spectrophotometer. For HPLC analysis, a C18 column was used with methanol:water as the mobile phase, increasing methanol from 1% to 90% over 15 minutes at a flow rate of 1 mL/min, with detection at 274 nm.

## Formulation and QbD Optimization

Nanoparticles were synthesized by mixing a specified volume of 1000 ppm aqueous Shilajit extract with zinc acetate solution, followed by dropwise addition of 0.1 M NaOH under constant stirring at 1000 rpm. The resulting mixture was homogenized and dried at 50°C for 8 hours. A Central Composite Design (CCD) was employed using Design-Expert software (Version 13) to optimize formulation variables (extract volume, zinc acetate concentration, NaOH volume) and process parameters (stirring time, temperature, homogenization speed). Responses measured were particle size and entrapment efficiency.

**Characterization** Particle size and zeta potential were analyzed using Malvern Nanosight Entrapment efficiency was

determined via indirect method by centrifuging nanoparticle suspension at 12,000 rpm for 90 minutes at 4°C, and analyzing the supernatant spectrophotometrically.

**In Vitro Drug Release** Drug release was assessed using a dialysis membrane method in a simulated gastrointestinal medium. Aliquots were withdrawn at predefined time intervals and analyzed to calculate cumulative drug release.

**CAM Assay** The angiogenic effect of Shilajit extract and nanoparticle formulation was evaluated using the CAM assay in fertilized chicken eggs. Gel foam sponges impregnated with test samples were placed on the chorioallantoic membrane. After 24 hours, the number of blood vessel branch points was counted, and an angiogenesis index was calculated.

**Trypan Blue Cell Viability Assay** H9c2 cardiomyocytes were seeded at 10,000 cells/well in a 96-well plate and incubated for 24 hours. Cells were treated with 50 ppm of test samples and incubated for another 24 hours. After washing with DPBS, 0.4% Trypan Blue was added, and cells were fixed with 4% paraformaldehyde. Viable and non-viable cells were counted under an inverted microscope, and cell viability percentage was calculated.

**XRD and EDAX Analysis** X-ray diffraction (XRD) was conducted to analyze the crystalline structure of the nanoparticles. Samples were scanned over a suitable 2θ range and step size.

Energy-dispersive X-ray spectroscopy (EDAX) was performed to identify elemental composition and confirm the presence of zinc.

#### **ICP-AES Analysis**

Inductively coupled plasma–atomic emission spectroscopy (ICP-AES) was used to quantify zinc content in the nanoparticle formulation. The total zinc concentration (ppm) and zinc loading (µg/mL) were determined

### **RESULTS AND DISCUSSION**

**Preformulation and Phytochemical Analysis** Shilajit was authenticated using standard parameters, and phytochemical screening confirmed the presence of tannins and saponin glycosides. The total polyphenol content was found to be  $129.82 \pm 0.38$  mg GAE/100 g extract. These polyphenolic compounds contribute to the antioxidant potential of shilajit which augments its cardioprotective potential<sup>19-20</sup>

**Spectral and Chromatographic Characterization** UV-Vis spectroscopy revealed a maximum absorbance at 274 nm for the Shilajit extract. HPLC analysis indicated the presence of 398.78 mg/g of fulvic acid in the extract. Shilajit being a drug

of herbomineral origin may vary in the content of fulvic acid depending upon the geographical source. HPLC was therefore performed using fulvic acid as a biomarker to standardise Shilajit [21-23].

**QbD-Based Optimization** Using CCD, optimized formulation parameters (extract volume, zinc acetate concentration, and NaOH volume) and process parameters (stirring time, temperature, and homogenization speed) were identified. Among the trials, the formulation with highest entrapment efficiency (64.62%) and smallest particle size (129 nm) was considered optimal. Additionally, all models were found to be significant ( $p < 0.05$ ) and having large F values. The predicted vs adjusted values of  $R^2$  were also in agreement. Utilisation of QbD ensures uniformity and reproducibility while providing a better understanding of the interactions between the factors [24-25].

**Particle Size and Zeta Potential** The particle size of the optimized batch was found to be 129 nm, with a zeta potential of -23.5 mV, indicating moderate electrostatic repulsion between particles thus allowing for sufficient dispersion in biological fluids [26-28].

**Entrapment Efficiency** The entrapment efficiency of the optimized Shilajit nanoparticle formulation was recorded as 64.62%. This suggests that the active

constituents such as fulvic acid drug are entrapped by capping and other mechanisms [29].

**In Vitro Drug Release** The cumulative drug release from the optimized nanoparticles reached 62.31% over 24 hours, compared to significantly lower release from the crude extract. The nanoparticle formulation demonstrated a sustained release beneficial for maintaining therapeutic drug levels [30, 31].

**CAM Assay** The number of new vessel branch points in the control group averaged 24, while the Shilajit extract group showed 32 branch points (a 33.33% increase). The nanoparticle formulation group exhibited an average of 54 branch points, reflecting a 127.08% increase in angiogenesis relative to the control. These results indicate that Shilajit nanoparticles significantly enhanced angiogenic activity compared to both the crude extract and control. Therapeutic angiogenesis may be a mechanism of cardioprotective action and may play a role in post-ichemic myocardial repair [32-35].

**Cell Viability Assay** Trypan Blue exclusion assay revealed cell viability of  $91.33 \pm 1.59\%$  for the nanoparticle-treated group, compared to  $80.98 \pm 1.49\%$  for the Shilajit extract-treated group, indicating improved cytoprotective activity [36-39].

### Structural and Elemental Characterization

**XRD Analysis:** X-ray diffraction analysis was performed to assess the crystalline structure of the synthesized nanoparticles. The diffractogram displayed characteristic peaks corresponding to crystalline zinc oxide, confirming successful nanoparticle formation. The  $2\theta$  peaks observed at  $32.15^\circ$ ,  $34.82^\circ$ , and  $36.66^\circ$  correspond to ZnO confirming a hexagonal wurtzite structure. The peak patterns suggested the nanoscale nature of the particles [40-44].

**EDAX Analysis:** Energy-dispersive X-ray spectroscopy (EDAX) confirmed the elemental composition of the nanoparticles. The spectrum revealed prominent peaks for zinc and oxygen, confirming the presence of zinc oxide, while minor peaks for carbon indicated the organic matrix contribution from Shilajit [45-47].

**ICP-AES Analysis:** Inductively coupled plasma-atomic emission spectroscopy (ICP-AES) was used to quantify zinc content in the nanoparticles. The concentration of zinc was

found to be 2.66ppm, with a calculated zinc loading of  $337.1 \mu\text{g/mL}$  in the final formulation. These findings corroborate the elemental analysis and support the successful incorporation of zinc within the nanoparticulate system.

**Stability Studies** Stability studies were conducted over a period of 3 months at three different storage conditions:  $4^\circ\text{C} \pm 2^\circ\text{C}$  (refrigerated),  $25^\circ\text{C} \pm 2^\circ\text{C}$  (room temperature), and  $45^\circ\text{C} \pm 2^\circ\text{C}$  (accelerated). No significant changes were observed in particle size or entrapment efficiency at  $4^\circ\text{C}$ , indicating excellent stability under refrigerated conditions. At  $25^\circ\text{C}$ , a slight increase in particle size and marginal decrease in entrapment efficiency were noted. However, at  $45^\circ\text{C}$ , a pronounced increase in particle size along with a noticeable decline in entrapment efficiency was observed, suggesting reduced formulation stability at elevated temperatures. These findings underscore the importance of controlled storage to maintain nanoparticle integrity.

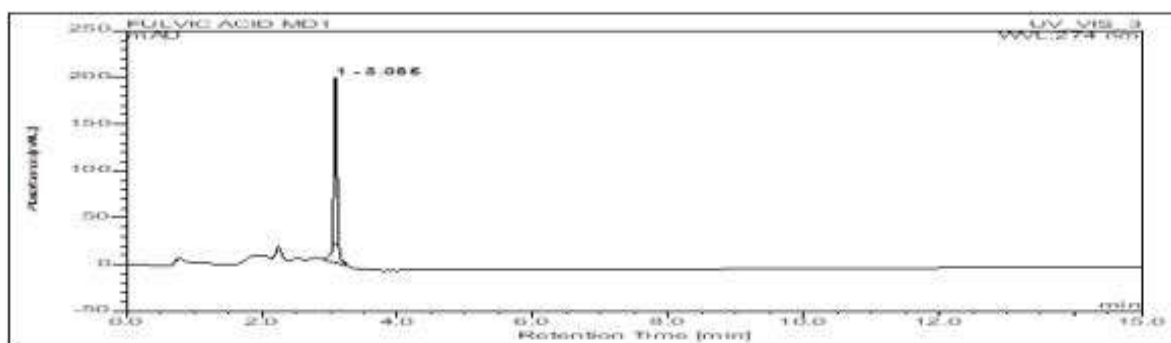


Figure 1a: HPLC chromatogram of Shilajit extract

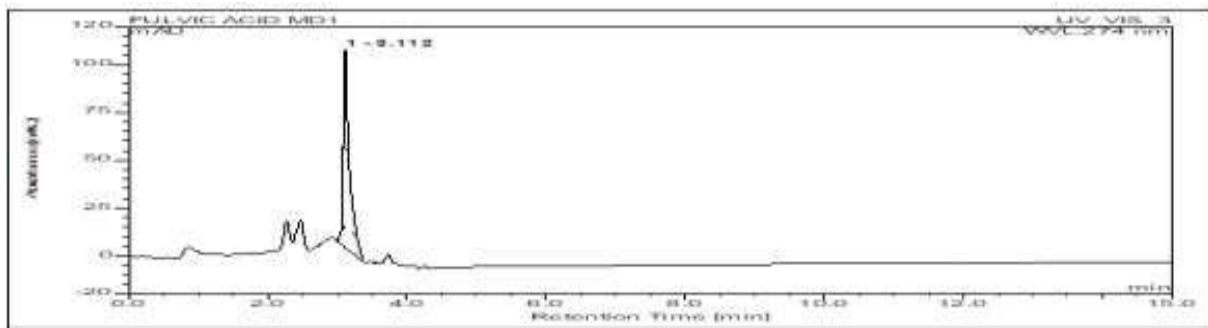


Figure 1b: HPLC chromatogram of standard Fulvic acid

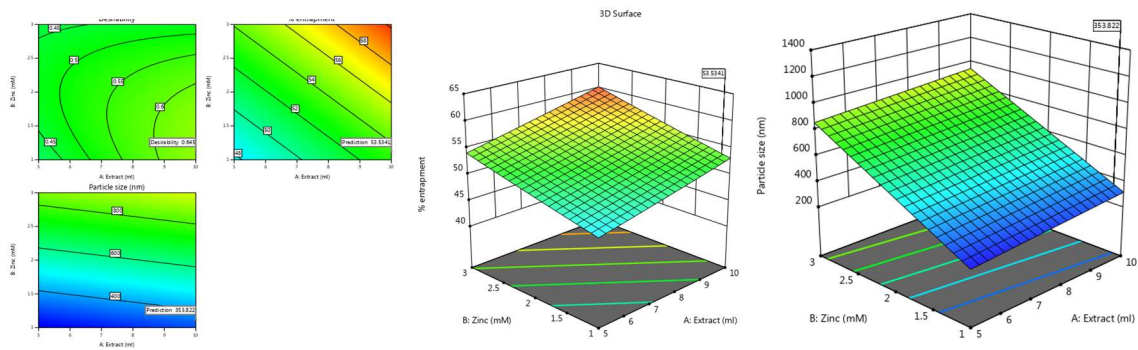


Figure 2a. Contour and 3D surface plot showing QbD optimization model results for formulation parameters

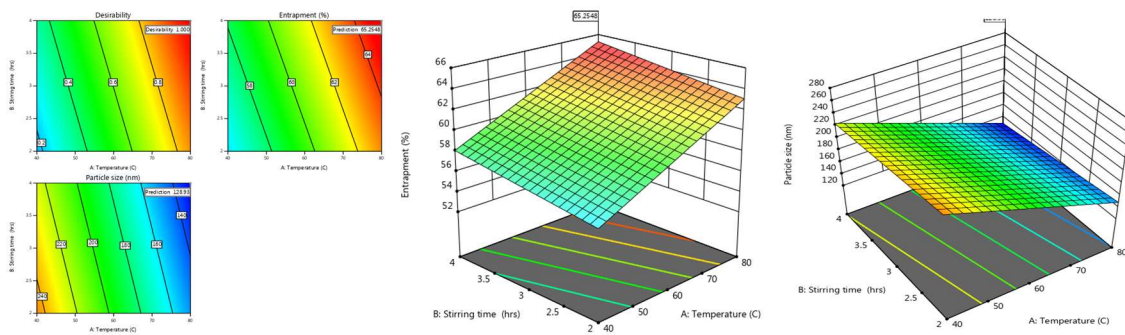


Figure 2b: Contour and 3D surface plot showing QbD optimization model results for process parameters



Figure 3: CAM assay comparative images for shilajit extract and nanoparticles

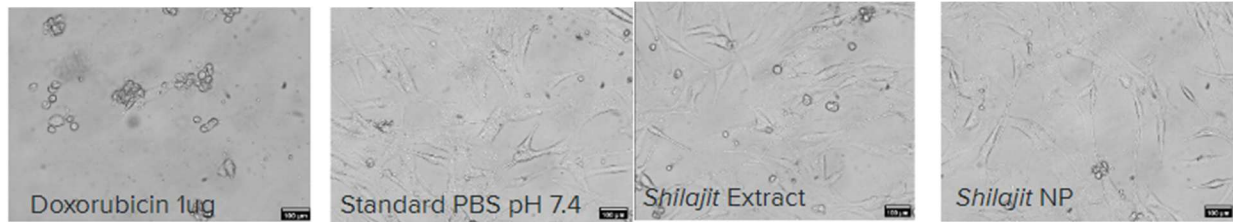


Figure 4: Trypan Blue cell viability images

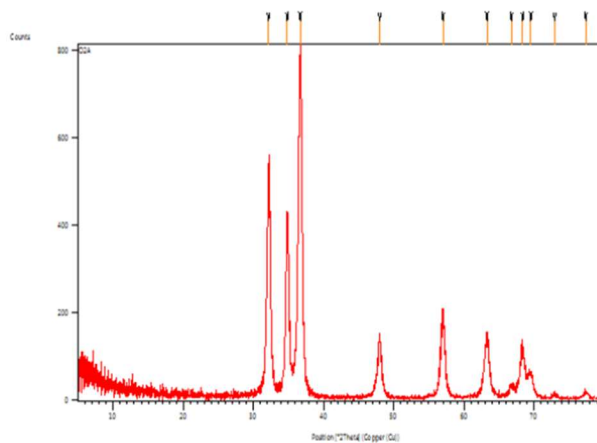
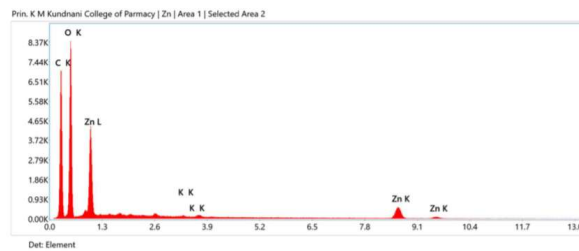


Figure 5: XRD diffractogram of synthesized nanoparticles



**eZAF Quant Result**

Element	Weight %	MDL	Atomic %	Net Int.	Error %	R	A	F
C K	54.7	0.14	63.9	1469.2	10.0	0.9280	0.1371	1.0000
O K	39.8	0.10	34.9	1778.8	10.5	0.9367	0.0893	1.0000
K K	0.1	0.04	0.0	17.3	15.3	0.9659	0.9348	1.0321
Zn K	5.4	0.13	1.2	295.5	4.2	0.9849	0.9966	1.1461

Figure 6: EDAX spectrum showing elemental analysis

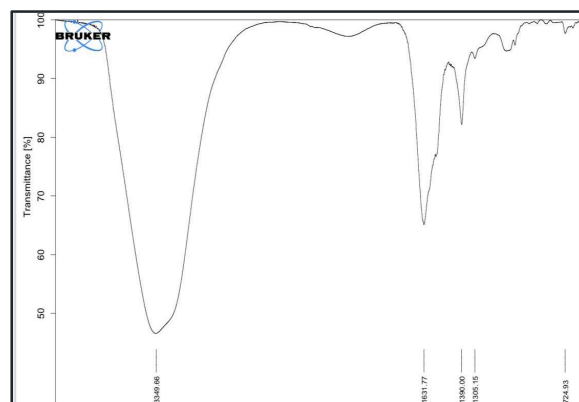


Figure 7a: FTIR Spectrum of Shilajit

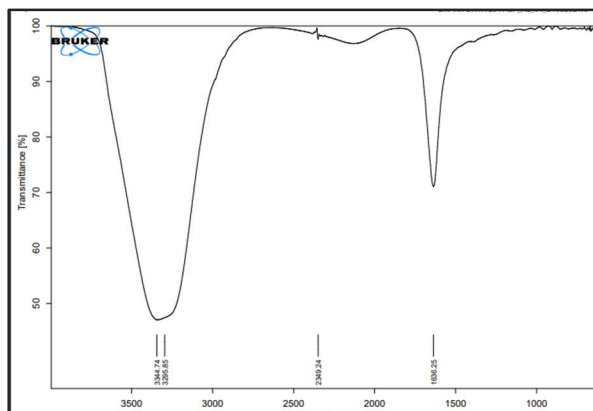


Figure 7b: FTIR Spectrum of Shilajit loaded zinc nanoparticles

Table 1a: CCD design matrix with response values (formulation parameters)

FACTOR 1: VOLUME OF EXTRACT (ml)	FACTOR 2: CONC. OF Zn (mM)	FACTOR 3: VOLUME OF 1 M NaOH (ml)	RESPONSE 1: PERCENT ENTRAPMENT	RESPONSE 2: PARTICLE SIZE (nm)
5	3	5	53.25	810
5	3	10	56.51	1135
5	2	7.5	57.62	603
5	1	5	43.96	241
5	1	10	49.44	538
7.5	2	7.5	56.01	663
7.5	1	7.5	52.37	405
7.5	2	10	55.29	685
7.5	3	7.5	57.83	1130
7.5	2	5	53.46	586
10	3	5	60.84	969
10	1	5	54.58	365
10	3	10	61.02	1222
10	2	7.5	57.03	644
10	1	10	55.67	568

Table 1b: CCD design matrix with response values (process parameters)

FACTOR 1: STIRRING TEMPERATURE (°C)	FACTOR 2: STIRRING TIME (hrs)	FACTOR 3: HOMOGENISATI ON SPEED (rpm)	RESPONSE 1: PERCENT ENTRAPMENT	RESPONSE 2: PARTICLE SIZE(nm)
40	2	500	54.7	263
40	4	500	57.74	244
40	2	1000	53.46	236
40	4	1000	55.94	226
40	3	750	56.36	215
60	3	750	60.66	173
60	3	1000	61.73	167
60	3	500	62.4	178
60	4	750	61.9	157
60	2	750	60.15	196
80	4	500	64.62	139
80	2	1000	60.26	143
80	4	1000	62.93	129
80	2	500	63.77	157
80	3	750	63.95	134

Table 2: Stability data: Particle size and entrapment efficiency over 3 months under different storage conditions

Time (months)	Percent Entrapment			Particle Size		
	5°C ± 3	30°C ±2°C / 65%RH± 5%RH	40°C ±2°C / 75%RH± 5%RH	5°C ± 3°C	30°C ±2°C / 65%RH± 5%RH	40°C ±2°C / 75%RH± 5%RH
0	63.95±0.05	63.95±0.05	63.95±0.05	134	134	134
1	62.91±0.02	58.91±0.9	50.63±0.21	145	156	307
3	61.1±0.09	55.91±3.7	48.91±1.19	159	187	498

Table 3: CAM Branching

Condition	Branching observed (1)	Branching observed (2)	Avg branching	% angiogenesis index
Control	22	26	24	
Shilajit extract	30	34	32	33.33%
Shilajit NP	51	58	54	127.08%

Table 4: Trypan blue viability data

Culture condition	% cell viability
Untreated	96.64±1.29
Doxorubicin (1ug/ml)	32.70±0.25
Shilajit Extract	80.98±1.49
Shilajit NP	91.33±1.59

Table 5: ICP-AES zinc quantification

Sample	Zinc concentration (ppm)	Zinc per ml of extract
Shilajit	2.66	337.1ug/ml

Table 6a: FTIR peaks of Shilajit extract

Peak	Interpretation
3349 cm	O-H stretching (COOH) / N-H stretching
1631 cm	C=C /C=O stretching /N-H bending
1305 cm	C-O/N-O stretching (Phenols or nitro)

Table 6b: FTIR peak interpretation of Shilajit-loaded nanoparticles

Peak	Interpretation
3344 cm	O-H stretching (COOH) / N-H stretching
1636 cm	C=C stretching /N-H bending

## CONCLUSION

The studies performed conclude that the overall therapeutic efficacy of containing purified Indian Shilajit surpasses that of the aqueous extract which can help further its use as a part of a holistic treatment regime targeted towards improving a patient's overall well-being.

Shilajit is one of the many natural agents which can be used safely in treatment of Shilajit is a powerful adaptogen and rejuvenator which can be effectively used to promote longevity and thus improve the overall quality of life. When traditional medicinal agents such as Shilajit are

combined with modern formulation techniques, a several fold improvement is seen in its activity. Hence there is great potential in the development of novel drug delivery systems for the traditional medicinal systems.

**Funding:** This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

**Conflict of Interest:** The authors declare no conflicts of interest.

**Author Contributions:**

**Aliefia Lehri** – Conceptualization, Methodology, Investigation, Data Analysis, Writing – Original Draft.

**Dr. Swati Patil** – Supervision, Validation, Writing – Review & Editing.

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