

Antioxidant, cytotoxic and hyperalgesia-suppressing activity of a native Shilajit obtained from Bahr Aseman mountains

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Abstract: Shilajit, a blackish-brown exudation obtained from steep rocks of different mountains, has been longly used as a therapeutic agent in traditional medicine. The present study was designed to evaluate the antioxidant, cytotoxic and hyperalgesia-suppressing activity of the aqueous and DMSO extracts of a native Shilajit. The antioxidant and cytotoxic effects of Shilajit extracts was determined using DPPH scavenging activity and MTT assay methods, respectively. In order to examine the hyperalgesia-suppressing activity of the Shilajit aqueous extract the STZ-induced diabetic animals were subjected to oral administration of the extract (50, 100 and 200 mg/kg daily) for six weeks followed by evaluating the behavioral examination (hot plate and tail flick tests) compared to those of diabetic control (Sham) and vehicle groups. The obtained results of antioxidant evaluation of Shilajit represented scavenging activity of 50% at concentration of 2500 µg/mL and 6000 µg/mL in the case of aqueous and DMSO extracts, respectively. Cytotoxic study of water extract of Shilajit revealed IC₅₀ of 727.5±1.9 µg/mL and 1103±3.2 µg/mL on cell lines of MCF-7 (breast cancer) and A549 (lung cancer), respectively. Thermal pain response examination of diabetic rats treated with aqueous extract of Shilajit (100 mg/kg and 200 mg/kg) for six weeks reduced hyperalgesia compared to vehicle and Sham groups. To sum up, considering the moderate antioxidant and hyperalgesia-suppressing activity of this native Shilajit make it as a suitable candidate for further investigation after isolation and characterization of the active compounds.

Keywords: Shilajit, antioxidant, cytotoxic, hyperalgesia, streptozotocin

INTRODUCTION

Shilajit, also known as Salajit, Shilajatu, Mumie Or Mummyo, is a semi-solid mineral pitch with brown to black color exuded mainly from steep rocks in Himalayas (especially in altitudes of 1000 to 5000 meters) and mountains of other countries like Afghanistan, Pakistan, Iran, Nepal, China and Tibet (Wilson *et al.*, 2011; Agarwal *et al.*, 2007). There are different hypotheses about the origin of Shilajit (Agarwal *et al.*, 2007). However, it seems that Shilajit formed when the latex juice of plants exudates as a gummy from the rocks of mountains following of strong sun's heat (Wilson *et al.*, 2011). Chemical composition of Shilajits are found to be varied depend on the plant species, geological nature of rocks, humidity and local temperature (Ghosal *et al.*, 1991) but presence of organic compounds such as benzoic acid, hippuric acid, resin, fulvic acid, ellagic acid, 3,4-benzocoumarins, and fatty acids in addition to alkaloids and amino acids as the main components of Shilajits obtained from different sources has been confirmed by many investigations (Ghosal, 2006; Wilson *et al.*, 2011). A wide range of therapeutic applications like treatment of

gallstone, digestive disorders, diabetes mellitus, epilepsy, chronic bronchitis, osteoarthritis and anemia has been described for Shilajit in the literature and traditional medicines (Ghosal *et al.*, 1993; Jaiswal and Bhattacharya, 1992). Anti stress and anxiolytic activity as well as immunomodulatory and antiallergic effect of Shilajit have been also reported (Wilson *et al.*, 2011).

According to the literature, cancer and malignancies are the second main cause of death in the world after cardiovascular diseases among which breast and lung cancers are the most common and dangerous types (Althuis *et al.*, 2005; Imaizumi, 2005; Schaaaj-Visser *et al.*, 2013). Disadvantages of free radicals and their carcinogenic characteristic due to their destructive effect on the biological macromolecules like DNA and proteins have been reported by many investigations (Pardakhty *et al.*, 2012; Rani *et al.*, 2016). Furthermore, the detrimental effects of reactive oxygen species (ROS) in development of diabetic neuropathy have been previously well documented (Zangiabadi *et al.*, 2011; Sytze *et al.*, 2013). In addition, oxidative stress plays an important role in emergence of most neurologic and behavioral changes in diabetic patients (Amin & Hosseinzadeh, 2012; Shakeel, 2015). Such mentioned conditions encouraged

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investigators to find novel antioxidant compounds to protect cells against dangerous effect of the mentioned radical intermediates (Zangiabadi *et al.*, 2011; Rajanandh *et al.*, 2014).

The main aim of the present study was to evaluate the antioxidant (using DPPH scavenging and reducing power assay) and cytotoxic activity [on two cell lines of MCF-7 (breast cancer) and A549 (lung cancer)] of a native Shilajit obtained from Bahraseman mountains (Jiroft, Iran). In addition, the hyperalgesia-suppressing effect of the aqueous extract of Shilajit on streptozotocin-induced diabetic rats was also determined using hot plate and tail flick tests.

MATERIALS AND METHODS

Chemicals

Shilajit was collected from Bahraseman mountains (Jiroft 28°40'41"N and 57°44'26"E, Iran) and confirmed by native people. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Merck Chemicals (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Fetal bovine serum (FBS), DMEM culture media and antibiotics were provided by Gibco (Life Sciences Inc. USA). All other applied chemicals were of analytical grade.

Preparation of Shilajit extract

In order to prepare the aqueous extract of Shilajit 1 gram of Shilajit, previously grinded by a mortar and pestle, was dissolved in sterile warm PBS (100 mL) and shaken vigorously followed by centrifugation (6000 rpm for 5 min) to remove undissolved materials (Shakibaie *et al.*, 2015). Extraction was performed three times to dissolve more and more undissolved compounds. Thereafter, the aqueous extract was subjected to lyophilisation followed by preparation of different concentration of 100µg/mL to 3000µg/mL. DMSO extract of Shilajit was prepared by dissolving of it in DMSO to reach the concentration of 100µg/mL to 3000µg/mL.

Determination of antioxidant activity

DPPH scavenging activity

DPPH scavenging activity was determined according to the method described by Kim and Kim (2010) with some modifications. 1 mL of various concentrations of Shilajit extracts (either DMSO or water extract) was mixed with 1 mL of the freshly prepared DPPH solution in methanol (0.15 mM). After addition of methanol (3 mL), the mixture was incubated at room temperature in dark for 30 minutes and the absorbance of the mixture was then measured at 517 nm with a UV-visible double beam PC scanning spectrophotometer (UV-1800, Shimadzu CO, USA). The negative control was prepared by replacing the Shilajit extract with sterile PBS or DMSO. All

experiments were carried out in triplicate. Same experiment was also repeated for BHT and ascorbic acid as positive controls at the same concentration. Scavenging percentage of DPPH was calculated as followed:

DPPH radical scavenging ability (%) = $[1 - (A_a - A_b)/A_c] \times 100$

Where A_a is the absorbance of the sample mixed with DPPH solution, A_b is the absorbance of the sample without DPPH solution and A_c is the absorbance of the control solution. Required concentration to inhibit 50% of DPPH (IC_{50}) calculated by linear regression.

Reducing power assay

The reducing power of Shilajit extract was accomplished by modified protocol of Oyaizu *et al.* (1986) based on the reduction of Fe^{3+} to Fe^{2+} in the presence of antioxidants. Briefly, one mL from different concentrations of Shilajit extracts (either aqueous or DMSO extract) was mixed with 0.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 0.5 mL of potassium ferricyanide (30mM). The mixture was then incubated at 50°C while shake slowly (100 rpm for 20 min). Thereafter, 2 mL of trichloro acetic acid (TCA, 0.6 M) was added to the mixture, followed by centrifugation at 3000 rpm for 10 min. The obtained supernatant (0.5 mL) was then mixed with deionized water (0.5 mL) and 0.1 mL of the $FeCl_3$ solution (6 mM) and the absorbance was measured at 700 nm. The blank was designed by incubating of the mentioned reaction mixture in the absence of Shilajit extract. BHT and ascorbic acid was used as positive control. All experiments were done in triplicate and mean of three responses was presented.

MTT-based cell viability assay

Two cell lines of MCF-7 and A549 was purchased from the Iranian Biological Resource Center (IBRC, Tehran, Iran). The cells were grown in DMEM culture media supplemented with 10% FBS (Gibco), and antibiotics (100 U/mL of penicillin and 100 µg/mL of streptomycin) at 37°C in a CO₂ incubator (5% CO₂ and 95% relative humidity). In order to determine the cytotoxic activity of Shilajit extracts 10⁴ mentioned cells were seeded in a 96-well tissue culture plate and incubated for 24h for attachment followed by addition of desired concentration of Shilajit extracts to each well and further incubation for another 24 h. Subsequently, the culture media of each well was replaced by 20µL of MTT solution (5 mg/mL) and plate was incubated for 4 h. Cell viability was then determined by addition DMSO (100µL) to each well to dissolve formazane crystal followed by measuring of absorbances at 570 nm. Each experiment was performed three times and mean of the obtained results was reported.

Hyperalgesia investigation

Adult male Sprague-Dawley rats (weight, 250-300 g; Pasture Institute of Iran) were applied for the hyperalgesia experiments. Laboratory chow and water were available

Table 1: Metabolic characteristics of animals of each group applied in the present study.

Animal group (8)	Body weight (g)		Blood glucose (mg/dL)	
	Before STZ injection	End of experiment	Before STZ injection	End of experiment
Control	271.42±5.08	275.71±4.42	118.28±3.22	121.57±3.25
Sham	269.28±5.16	236.57±6.76***	440.14±29.17****	497.85±15.92***
Vehicle (Solvent)	265.55±6.65	225.52±5.21***	437.12±21.45****	485±11.21***
Shilajit (50 mg/kg)	268.57±4.46	241.48±8.88	453.85±13.60****	436±16.58†
Shilajit (100 mg/kg)	272.14±5.65	261.42±5.8	447.85±18.71****	415.71±19.43†
Shilajit (200 mg/kg)	273.27±3.61	259.42±7.29	442.71±8.50****	417.42±12.51†

Data are the mean ± SEM ***: $p < 0.001$, ****: $p < 0.0001$, compared with the control group; †: $p < 0.05$, compared with the Sham and vehicle group

to the rats at all times. Rats were individually housed at ambient temperature of 22 ± 2 °C with 12-h light and 12-h dark cycle. All animals were habituated to the apparatus before the onset of the experiments. All performed procedures were approved by the regional ethics committee of Kerman Neuroscience Research Center (EC/KNRC/91/433). Except for control group (group 1, received normal saline solution) all applied rats were induced to be diabetic via injection a single dose of Streptozotocin (65 mg/kg in 100 mM sodium citrate buffer, pH 4.5, i.p.). Measuring of blood sugar one week after STZ injection was performed and animals with blood glucose level >200 mg/dL selected for further investigations (Usuki *et al.*, 2007; Sigauco-Roussel *et al.*, 2007). These rats were further divided into five groups (at least 8 rats in each group) of diabetic control group (the second group as Sham group), the vehicle group (the third group), and the treated group which received three different doses of Shilajit (50, 100 and 200 mg/kg/day, respectively) by gavage. Treatment was continued for 6 weeks and body weight and blood glucose level of animals were measured at the end of experiment. Thereafter, hot plate and tail flick tests were performed in order to evaluate the treated and untreated groups.

In hot plate test, pain sensitivity was determined by an apparatus (LE710 model, Lsi LETICA, Spain) contained a plate with the diameter of 19 cm and a Plexiglas wall with height of 30 cm. Rats were placed on the hot plate which its temperature previously adjusted to 52 ± 2 °C. Response time to thermal pain was considered as the time between test onset and licking front paw or jumping. The cut-off time was set at 60 seconds to avoid damage to the paw (Sharma *et al.*, 2007).

In the tail flick experiment, thermal light was shown to the tail end of rats with intensity of 5 using tail flick machine (LE7406 model, Lsi LETICA, Spain). Mean of triplicate experiments with five minutes intervals was reported as tail flick latency. To prevent tissue damage, maximum light emission to the tail was considered 10 seconds (Liepinsh *et al.*, 2009).

STATISTICAL ANALYSIS

All above mentioned experiments were performed in triplicate and mean ± standard deviation of obtained data was reported. The statistical significance between mean values was calculated using one-way analysis of variance (ANOVA) with Tukey post hoc test (SPSS 15.0, SPSS Inc). Probability values <0.05 were considered significant.

RESULTS

Antioxidant activity

Two methods of DPPH scavenging and reducing power assay were used in order to determine the antioxidant activity of Shilajit extracts. As illustrated in fig. 1. ascorbic acid and BHT (as positive controls) represented the highest scavenging activities. In the case of Shilajit the scavenging percent of both water and DMSO extracts were gradually increased to reach the scavenging percent of 50% at the concentration of 2500 µg/mL and 6000 µg/mL, respectively. The reducing power of Shilajit extracts exhibited a dose-dependent manner in concentration range of 0–6000 µg/mL (fig. 1). At all concentrations, reducing power of water and DMSO extracts of Shilajit were not significantly different ($p < 0.05$). The reducing power (OD_{700}) of BHT and ascorbic acid reached to 1.64 and 1.93, respectively after increasing concentration to 6000 µg/mL while water and DMSO extract of represented OD_{700} of 0.72 and 0.65, respectively, at the same concentration.

Cytotoxicity assay

In order to evaluate the cytotoxic effect of Shilajit on two cell lines of MCF-7 and A549 MTT assay was applied. As shown in fig. 2., in the case of both investigated extracts (water and DMSO) the viability of applied cell lines was significantly decreased by increasing of extract concentration. The necessary concentration causing 50% cell death (IC_{50}) was 727.5 ± 1.9 µg/mL and 1103 ± 3.2 µg/mL in the case of MCF-7 and A549, respectively in the presence of aqueous extract of Shilajit (fig. 2).

However, the DMSO extract of Shilajit produced the same effect (i.e. 50% cell death) at the concentration of $2363.4 \pm 3.8 \mu\text{g/mL}$ and $2020.8 \pm 1.7 \mu\text{g/mL}$ on the cell line of MCF-7 and A549, respectively (fig. 2).

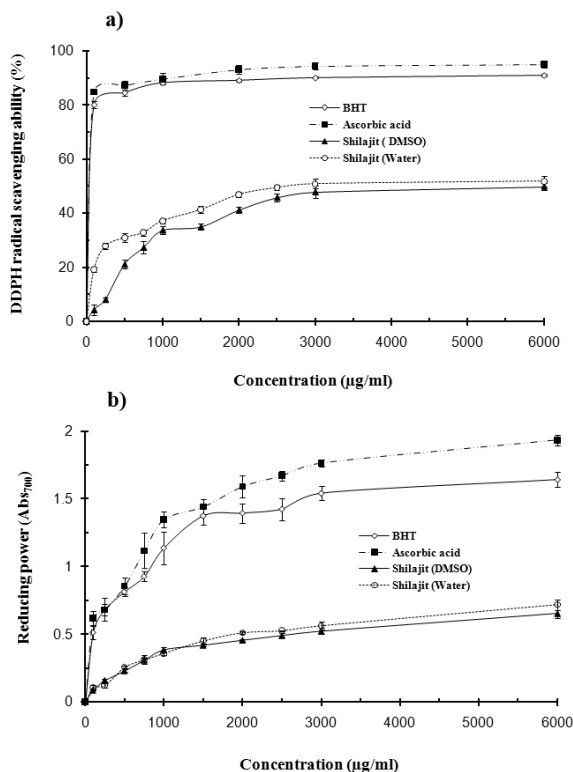


Fig. 1: Scavenging effects of DMSO and aqueous extracts of Shilajit on DPPH free radicals and b reducing power of DMSO and aqueous extracts of Shilajit compared to BHT and ascorbic acid as a standard control. Data are mean of triplicate experiments.

Hyperalgesia investigation

Metabolic characteristics

As summarized in the table 1 the plasma glucose concentration of all STZ-induced diabetic animals (including Sham, vehicle and Shilajit-treated groups) was significantly increased compared to that of control group ($p < 0.0001$). Although, a significant ($p < 0.05$) decrease of plasma glucose level was determined after treatment of diabetic animals by Shilajit at all applied concentration (50, 100 and 200 mg/kg) table 1 the glucose concentration of Sham and vehicle groups was found to enhance following the sixth week. The body weight of diabetic rats was significantly ($p < 0.001$) lower compared to that of control group at the end of 6th week (table 1). While the body weight of control group was found to increase during the period of experiment the mentioned index was reduced in the case of STZ-induced diabetic groups including Sham, vehicle and Shilajit-treated ones table 1.

The influence of shilajit on hot plate test

According to the obtained results of hot plate experiments Fig. 3. a significant decrease of response time to thermal pain in the 6th week following diabetes induction was determined in Sham, vehicle and Shilajit-treated (50 mg/kg) groups compared to control group ($p < 0.01$). However, in the animals of Shilajit-treated groups (received 100 and 200 mg/kg of aqueous extract) the mentioned index (response time to thermal pain) was significantly increased in comparison to vehicle and Sham groups ($p < 0.01$). In the case of Shilajit-treated group (received 50 mg/kg) the response time was not significantly changed fig. 3.

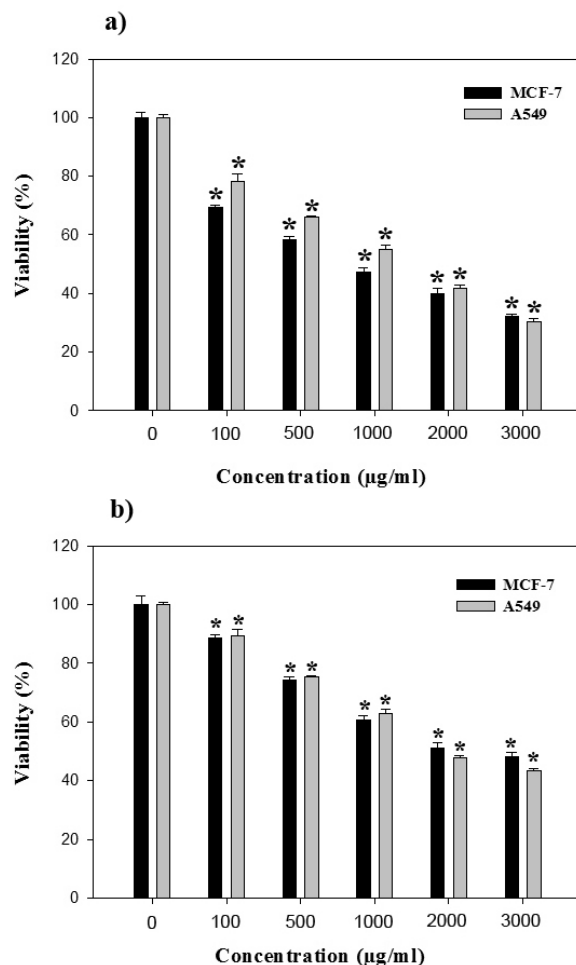


Fig. 2: Effect of a aqueous and b DMSO extract of Shilajit on cell viability of MCF-7 and A-549 cell lines examined by MTT assay. Each value is represented as mean \pm SD of three independent experiments.

The effect of Shilajit on tail flick test

As illustrated in fig. 3. Diabetic neuropathy caused significant decrease in reaction to pain and tail flick latency time in Sham, and vehicle groups compared to the control group ($p < 0.01$). Treatment of diabetic animals using Shilajit (100 mg/kg) lead to a significant increase of the response time in comparison to the Sham and vehicle

groups) $p < 0.05$), fig. 3. There was no significant difference between the applied concentrations of Shilajit ($p < 0.05$), fig. 3.

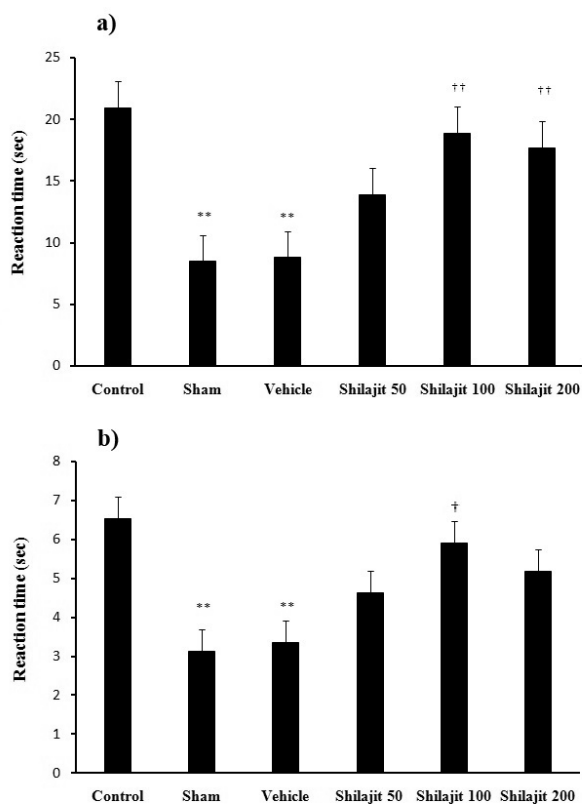


Fig. 3: Effect of aqueous Shilajit extract on explorative behavior of rats in a hot plate and b tail flick tests. ** $p < 0.01$ Significant difference of the vehicle and Sham groups with the controls. †† $p < 0.01$ Significant difference of the 100 mg/kg and 200 mg/kg Shilajit group with the vehicle and Sham groups.

DISCUSSION

In the present study, the effects of Shilajit extract at dose of 50, 100 and 200 mg/kg on diabetic neuropathy in streptozotocin induced diabetic male rats were investigated through behavioral and cytotoxic activities. According to the obtained results of the present study the hyperalgesia-suppressing effect of Shilajit on the studied animals was revealed which might be ascribed to the blood sugar lowering activity of the Shilajit. Similar results were previously reported by Trivedi *et al* (2004) who observed that blood sugar level was decreased after injection of Shilajit (100mg/kg/day) for 4 weeks. Bhattacharya (1995) was also determined that oral administration of Shilajit (100mg/kg) decreased the blood sugar of treated animals compared to untreated group. The critical role of reactive oxygen species in development of diabetic neuropathy has been well

documented (Trivedi *et al.*, 2004; Unger, 2008). It seems that the protective role of Shilajit components to neutralize the hazardous influence of free radicals improved the induced diabetic neuropathy in the present study. The obtained results of Bhattacharya (1995) revealed the free radical scavenging activity of Shilajit which decreased the destructive effect of accumulated free radicals on pancreatic β -cells.

Application of Shilajit extracts (as a safe natural biominerals) and/or their related fractions harboring antioxidant compounds to protect animal cells from oxidative damage have been widely described in both *in vitro* and *in vivo* studies (Stohs, 2014; Schepetkin *et al.*, 2002). Phaechamud *et al* (2008) prepared the aqueous, dichloromethane, and butanol extracts of a locally prepared mineral pitch and evaluated their antioxidant properties. They reported the IC_{50} of 297.9 μ g, 394.8 μ g, and 733.6 μ g for water, dichloromethane, and butanol extracts, respectively (Phaechamud *et al.*, 2008). Determination of oxidative parameters in the *in vivo* study performed by El-Sayed *et al.* (2012) revealed the antioxidant properties of Shilajit obtained from the mountain region of Yemen (Al-Jouf and Rayma), Russia (Tien-Shan) and India (Kumoan).

According to the announced results, it seems that Shilajit at a dose of 100 mg/kg for 4 weeks can prohibits of expansion of diabetic hyperalgesia and increased the latency time of response to thermal pain in hot plate and tail flick tests so that the reaction time to pain in these groups approached to the control group.

Cytotoxic study of water extract of Shilajit revealed IC_{50} of 727.5 ± 1.9 μ g/mL and 1103 ± 3.2 μ g/mL on cell lines of MCF-7 (breast cancer) and A549 (lung cancer), respectively. In a similar study conducted by Phaechamud *et al* (2008), the ability of mineral pitch (at concentration range of 25-250 μ g/mL) for inhibition of four cancer cell lines was evaluated. They found ED_{50} (50% effective concentration) of 89 μ g/mL, 96 μ g/mL, 225 μ g/mL, and 286 μ g/mL for A549 (lung cancer), HepG2 (liver cancer), MDA-MB-231 (human breast cancer), and SKOV-3 (ovarian carcinoma), respectively (Phaechamud *et al.*, 2008).

CONCLUSION

To sum up, the native Shilajit obtained from Bahr Aseman mountains represented moderate antioxidant activity together with hyperalgesia-suppressing activity in diabetic rats which launched it as a suitable candidate for further *in vivo* and *in vitro* investigations after isolation and identification of active compounds. So, it merits additional works to find about bioactive ingredients of this native Shilajit and investigate the molecular mechanisms involved in such biological effects.

ACKNOWLEDGEMENTS

This work was supported by research deputy of research Kerman University of Medical Sciences, Kerman, Iran.

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