Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 4, Issue 2, 2012

Research Article

# *IN VITRO* ANTIOXIDANT AND ANTI-ARTHRITIC ACTIVITIES OF *SHILAJIT*

**ANUYA REGE, PARIKSHIT JUVEKAR, ARCHANA JUVEKAR**

**Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, Matunga, Mumbai- 400019, Maharashtra, India.**

*Received: 23 Dec 2011, Revised and Accepted: 19 Mar 2012*

**ABSTRACT**

The aim of present *in vitro* study was to investigate antioxidant and anti-arthritic activities of *Shilajit*. The antioxidant activity of aqueous extract of *Shilajit* was determined by using 3 *in vitro* parameters, namely, DPPH radical-scavenging assay, lipid peroxidation inhibitory assay and reducing power assay, whereas, anti-arthritic activity was evaluated by proteinase inhibitory assay. Besides, phenolic content of the extract was estimated using Folin-Ciocalteu method. Aqueous extract of *Shilajit* exhibited DPPH radical-scavenging activity with $IC_{50}$ value of 11.9µg/ml, which was similar to that of standard ascorbic acid. Its reductive ability increased with increasing concentration which was comparable to that of standard. It also showed significant ($P<0.05$) lipid peroxidation inhibitory and anti-arthritic activities. High phenolic content of *Shilajit* can be correlated to its respective antioxidant and anti-arthritic activities.

**Keywords:** *Shilajit,* Anti-arthritic, Non-enzymatic lipid peroxidation, Rat liver mitochondria, Antioxidant.

## INTRODUCTION

All biological molecules are prone to oxidative damage by free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). This oxidative damage leads to various disease conditions, viz., heart disease[1], autism[2], cancer[3,4], diabetes[5], arthritis[6], Alzheimer's dementia[7], Parkinson's disease[8], cataracts[9] and aging[10]. Antioxidants are the compounds that prevent this oxidative damage by different mechanisms[11]. However synthetic antioxidants possess adverse effects[12]. Hence screening of safe, effective and economical antioxidants from natural sources is preferred. Although, majority of natural drugs are derived from plant and animal origins, a few of them, obtained from mineral sources, like *Shilajit*, are of paramount significance as pharmaceutical aids[13]. *Shilajit* is a pale-brown to blackish-brown exudation from rocks in Himalayan ranges of Indian subcontinent. It is also found in Nepal, Bhutan, Tibet and China. Numerous traditional uses of *Shilajit* have been reported previously[14]. The aim of the present study was to evaluate the antioxidant and anti-arthritic activities of *Shilajit* using different *in vitro* models.

## MATERIALS AND METHODS

### Collection of the material

*Shilajit* was purchased from Vaidya (Dr.) Rajeev K. Kanitkar, Ayurvedic Physician, Mumbai and it was identified and authenticated by Dr. J. M. Pathak, Research Director (Pharmacognosy), Zandu Pharmaceuticals, Mumbai.

### Preparation of aqueous extract

One gram of material was added to 20ml of distilled water and kept at RT for 24 hr. It was then filtered and evaporated to dryness on boiling water bath and the percentage yield of the extract was calculated which was found to be 69%.

### DPPH radical-scavenging assay

The free radical-scavenging activity of *Shilajit* was measured by 1,1 diphenyl-2-picryl hydrazyl (DPPH) assay[15]. For this, 1ml of DDPH solution (0.1mM) in methanol was added to different concentrations of extract. Thirty minutes later, the absorbance was measured at 517nm using a UV-Visible spectrophotometer (SHIMADZU UV-1650PC). A control was prepared without adding extract. Ascorbic acid at various concentrations (2-20µg/ml) was used as a standard. The percent DPPH-scavenging activity was calculated as,

$$\text{DPPH Scavenged (\%)} = A_{control} - A_{test} / A_{control} \times 100$$

Where, $A_{control}$ is absorbance of control reaction and $A_{test}$ is absorbance in presence of the extract. The antioxidant activity of *Shilajit* extract is also expressed as $IC_{50}$ and compared with the standard.

### Lipid peroxidation inhibitory activity

The protective effect of *Shilajit* was evaluated against $Fe^{2+}$-Ascorbic acid-induced lipid peroxidation using rat liver mitochondria as model system.

The rat liver mitochondria were isolated by differential centrifugation method[16]. Female Wistar rats (220-250gm) were used for the preparation of mitochondria. In brief, rat livers were excised, minced and homogenized in 0.25M sucrose solution. The homogenate was centrifuged at 2500 rpm for 10 min at $4^0$C to remove cell debris and nuclear fraction. The resultant supernatant was centrifuged at 10,000 rpm for 10 min at $4^0$C to sediment mitochondria. This pellet was washed thrice with 1M Tris-HCl buffer (pH 7.4) and suspended in same buffer. The protein was estimated by Lowry's method[17].

The effect of *Shilajit* on lipid peroxidation in mitochondrial samples was estimated by thiobarbituric acid reactive substances (TBARS) method. Protein concentration of mitochondrial samples was adjusted to 5mg/ml. Different concentrations of extract were incubated with reaction mixture that contained, 300µl basic medium (0.15M Tris-HCl buffer containing 1mM $KH_2PO_4$, pH 7.4), 100µl mitochondrial samples (500µg protein), 50µl $FeSO_4$ (0.05mM) and 50µl ascorbic acid (0.4mM). The mixture was incubated for 1 hr at $37^0$C. After incubation, the lipid peroxidation of mitochondrial samples was estimated as malonaldehyde (MDA) equivalents[18] by keeping the samples in boiling water bath with 1ml thiobarbituric acid (TBA) reagent for 15 min. After cooling, the absorbance was measured at 532nm using a UV-Visible spectrophotometer (SHIMADZU UV-1650PC). A control was run simultaneously without adding extract. Butylated Hydroxyl Toluene (BHT) was included as a standard. The lipid peroxidation inhibitory activity was calculated as,

$$\text{Inhibition (\%)} = \text{Control} - \text{Test} / \text{Control} \times 100.$$

### Reducing power assay

The reducing power of *Shilajit* was determined as described earlier[15]. Ascorbic acid at various concentrations (2-20µg/ml) was included as a standard. The antioxidant activity of *Shilajit* extract was calculated as percent increase in reducing power as, Increase in Reducing Power (%) = $[A_{Test}/A_{control} -1] \times 100$. The result is also expressed as $EC_{50}$ and compared with the standard.

### Proteinase inhibitory activity

Proteinase inhibitory activity of *Shilajit* was estimated as described by Chatterjee *et al*[19]. Acetyl salicylic acid was included as a standard.

The result is expressed as percent inhibition which was calculated as,

Inhibition (%) = Test – Control/Test X 100.

**Phenolic content determination**

The total phenolic compounds in *Shilajit* were determined using Folin-Ciocalteu reagent according to the method of Singleton *et al*[20] using gallic acid as a standard. Hundred µl of the extract was incubated with 1ml of diluted Folin-Ciocalteu reagent (1:2 with water) at RT for 5 min. One ml 7% $Na_2CO_3$ was added to the reaction mixture which was incubated at RT for 90 min and absorbance was read at 750nm using a UV-Visible spectrophotometer (SHIMADZU UV-1650PC). The total phenolic content is expressed as gallic acid equivalent (GAE) in milligrams per gram of dry sample. Statistical analysis of the data was done by Student's t-test and P<0.05 was regarded as significant.

**RESULTS AND DISCUSSION**

Natural compounds especially derived from Indian traditional medicine provide a large number of antioxidants[21-23] and anti-arthritic agents[24-26]. *Shilajit* is considered as a panacea in Indian traditional medicine because of its effectiveness in the treatment of various ailments[27]. In the present study, *Shilajit* was evaluated for its antioxidant and anti-arthritic activities using various *in vitro* parameters.

Free radical scavenging activity of *Shilajit* extract was evaluated by testing its ability to bleach (purple to yellow color) the stable DPPH radical which is a widely used rapid and simple method. It was observed that free radicals were scavenged by the test compound in a concentration dependent manner. $IC_{50}$ value (Table 1) of the extract was found to be 11.9µg/ml, which is more or less similar to that of the standard ascorbic acid (11.3µg/ml).

**Table 1: DPPH radical scavenging activity of aqueous extract of *Shilajit* and standard ascorbic acid**

| | Conc. (µg/ml) | % Inhibition (Mean ± SD) | $IC_{50}$ (µg/ml) |
|---|---|---|---|
| *Shilajit* | 2 | 2.71 ± 1.03* | |
| | 4 | 11.82 ± 0.25 | 11.9 |
| | 8 | 32.28 ± 2.06 | |
| | 12 | 50.76 ± 0.88 | |
| | 16 | 72.21 ± 0.20 | |
| | 20 | 84.16 ± 0.04 | |
| Ascorbic acid (Standard) | 2 | 1.66 ± 2.13* | |
| | 4 | 11.01 ± 1.27 | 11.3 |
| | 8 | 34.90 ± 3.98 | |
| | 12 | 52.15 ± 0.16 | |
| | 16 | 71.97 ± 0.82 | |
| | 20 | 95.18 ± 1.28 | |

*Non-Significant (P> 0.05), whereas, rest of the values Significant at P< 0.05 level

The ability to scavenge the DPPH radicals is related to the inhibition of lipid peroxidation[28]. Hence effect of *Shilajit* on lipid peroxidation was assessed in rat liver mitochondria by inducing lipid peroxidation with $Fe^{2+}$-ascorbate system. According to Conforti *et al*[29], ascorbic acid is a well-known antioxidant but it also has prooxidant properties in presence of certain transition metal ions like Fe or Cu. Iron can stimulate lipid peroxidation by the Fenton reaction and also accelerates peroxidation by different mechanisms[30]. Malondialdehyde (MDA) is one of the products of lipid peroxidation which is frequently used as an index to measure the extent of lipid peroxidation in biological samples[31]. In the present study, aqueous extract of *Shilajit* showed significant (P<0.05) decrease in MDA level and prevented lipid peroxidation (Table 2).

**Table 2: Lipid peroxidation inhibitory activity of aqueous extract of *Shilajit* and standard BHT**

| | Conc. (µg/ml) | % Inhibition (Mean ± SD) |
|---|---|---|
| *Shilajit* | 40 | 66.85 ± 2.93 |
| | 100 | 70.93 ± 7.21 |
| BHT (Standard) | 40 | 83.78 ± 1.45 |

Significant at P< 0.05 level

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Hence the reducing capability of *Shilajit* was measured by the transformation of $Fe^{3+}$ to $Fe^{2+}$ in the presence of the extract. The reduction ability was found to increase with the increasing concentration. $EC_{50}$ value of the extract was found to be 15.2µg/ml, which was comparable to that of the standard ascorbic acid (Table 3).

**Table 3: Effect of aqueous extract of *Shilajit* and standard ascorbic acid on reducing power assay**

| | Conc. (µg/ml) | % Increase (Mean ± SD) | $EC_{50}$ (µg/ml) |
|---|---|---|---|
| *Shilajit* | 2 | 5.30 ± 2.61* | |
| | 4 | 17.70 ± 11.33 | 15.2 |
| | 8 | 28.61 ± 9.04 | |
| | 12 | 47.06 ± 2.77 | |
| | 16 | 51.67 ± 2.35 | |
| | 20 | 61.11 ± 9.50 | |
| Ascorbic acid (Standard) | 2 | 9.82 ± 3.79* | |
| | 4 | 21.3 ± 15.50 | 13.7 |
| | 8 | 28.66 ± 1.51 | |
| | 12 | 48.26 ± 1.07 | |
| | 16 | 60.47 ± 6.41 | |
| | 20 | 66.58 ± 6.02 | |

*Non-Significant (P> 0.05), whereas, rest of the values Significant at P< 0.05 level

*Shilajit* extract was also evaluated for its anti-arthritic activity. Rheumatoid arthritis still remains a formidable disease capable of producing functional disabilities[32]. It was previously reported that proteinases have been implicated in arthritic reactions and significant level of protection was provided by proteinase inhibitors[33]. In the present study, aqueous extract of *Shilajit* showed significant (P<0.05) proteinase inhibitory activity when compared with control in dose dependent manner. At 800µg/ml, it also exhibited strong (P<0.05) inhibitory activity in comparison with the same dose of acetyl salicylic acid which was included as a standard (Table 4).

**Table 4: Anti-arthritic activity of aqueous extract of *Shilajit* and standard acetyl salicylic acid estimated by proteinase inhibition**

| | Conc. (µg/ml) | % Inhibition (Mean ± SD) |
|---|---|---|
| *Shilajit* | 100 | 14.07 ± 2.63 |
| | 200 | 22.11± 0.37 |
| | 400 | 34.42 ± 0.39 |
| | 800 | 54.41 ± 5.09 |
| Acetyl salicylic acid (Standard) | 800 | 16.19 ± 5.93 |

Significant at P< 0.05 level

As evidenced, phenolic compounds contribute to antioxidative action through various mechanisms, viz., scavenging free radicals, by stabilizing lipid peroxidation, through redox properties and by chelating metals[34-36]. Hence the total phenolic content of *Shilajit* was determined by Folin-Ciocalteu method and it was found to be 295mg Gallic acid Equivalent/gm (Table 5).

**Table 5: Phenolic content determination of aqueous extract of *Shilajit***

| | Gallic Acid Equivalent (mg/gm) (Mean ± SD) |
|---|---|
| *Shilajit* | 295 ± 9.90 |

The present study thus provides evidence that *Shilajit* is a valuable source of natural antioxidants. *Shilajit* also exhibited anti-HIV activity by 2 different mechanisms[37]. In the present study it showed strong antioxidant potential which would help in decreasing damage caused by oxidative stress in AIDS.

## CONCLUSION

On the basis of the results obtained in the present study it is concluded that, *Shilajit* exhibits profound antioxidant activity through reductive ability and by scavenging free radicals like DPPH and those that initiate or propagate lipid peroxidation. It also showed potent anti-arthritic activity. High phenolic compounds present may be responsible for these activities which need further investigation.

## ACKNOWLEDGEMENTS

We are grateful to Vaidya (Dr.) Rajeev K. Kanitkar, Ayurvedic Physician, Mumbai for providing *Shilajit* and to Dr. J. M. Pathak, Research Director (Pharmacognosy), Zandu Pharmaceuticals, Mumbai for the authentication of the same.

## REFERENCES

1. Singh U, Jialal I. Oxidative stress and atherosclerosis. Pathophysiology 2006; 13: 129-142.
2. Chauhan A, Chauhan V. Oxidative stress in autism. Pathophysiology 2006; 13: 171-181.
3. Babbs CF. Free radicals and the etiology of colon cancer. Free Radical Biol Med 1990; 8: 191-200.
4. Sun Y. Free radicals, antioxidant enzymes and carcinogenesis. Free Radical Biol Med 1990; 8: 583-599.
5. Jain SK, McVie R, Bocchini JA. Hyperketonemia (ketosis), oxidative stress and type 1 diabetes. Pathophysiology 2006; 13: 163-170.
6. Mahajan A, Tandon VR. Antioxidants and rheumatoid arthritis. J Indian Rheumatol Assoc 2004; 12: 139-142.
7. Chauhan V, Chauhan A. Oxidative stress in Alzheimer's disease. Pathophysiology 2006; 13: 195-208.
8. Sudha K, Rao AV, Rao S, Rao A. Free radical toxicity and antioxidants in Parkinson's disease. Neurol India 2003; 51(1): 60-62.
9. Vinson JA. Oxidative stress in cataracts. Pathophysiology 2006; 13: 151-162.
10. Dizdaroglu M, Jaruga P, Birincioglu M, Rodriguez H. Free radical-induced damage to DNA: Mechanisms and measurement. Free Radical Biol Med 2002; 32(11): 1102-1115.
11. Bergendi L, Benes L, Durackova Z, Ferencik M. Chemistry, Physiology and pathology of free radicals. Life Sci 1999; 65(18/19): 1865-1874.
12. Chanda S, Dave R. *In vitro* models for antioxidant activity evaluation and some medicinal plants possessing antioxidant properties: An overview. Afr J Microbiol Res 2009; 3(13): 981-996.
13. Kokate CK, Purohit AP, Gokhale SB. Drugs of mineral origin. In: Pharmacognosy. Pune: Nirali Prakashan; 2002. p. 560-568.
14. Meena H, Pandey HK, Arya MC, Ahmed Z. *Shilajit*: A panacea for high-altitude problems. Int J Ayurveda Res 2010; 1(1): 37-40.
15. Nikhat F, Satyanarayana D, Subhramanyam EVS. Isolation characterization and screening of antioxidant activity of the roots of *Syzygiumcuminii* (L) Skeel. Asian J Research Chem 2009; 2(2): 218-221.
16. Choliparambil KP, Mylvaganan S, Thomas PAD, Singh BB. Study on lipid peroxidation potential in different tissues induced by ascorbate-$Fe^{2+}$: Possible factors involved in their differential susceptibility. Mol Cell Biochem 1998; 178(1-2): 197-202.
17. Lowry OH, Rosebrough HJ, Farr Al, Randall RJ. Protein measurements with the Folin phenol reagent. J Biol Chem 1951; 193(1): 265-275.
18. Hammer CT, Wills ED. The role of lipid components of the diet in the regulation of the fatty acid composition of the rat liver endoplasmic reticulum and lipid peroxidation. Biochem J 1978; 174: 585-593.
19. Chatterjee S, Das SN. Anti-arthritic and Anti-inflammatory effect of a poly-herbal drug (EASE@): Its mechanism of action. Indian J Pharmacol 1996; 28: 116-119.
20. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Vitic 1965; 16: 144-158.
21. Suresh Kumar P, Sucheta S, Sudarshana Deepa V, Selvamani P, Latha S. Antioxidant activity in some selected Indian medicinal plants. Afr J Biotechnol 2008; 7(6): 1826-1828.
22. Zahin M, Aqil F, Ahmed I. The *in vitro* antioxidant activity and total phenolic content of four Indian medicinal plants. Int J Pharm Pharm Sci 2009; 1 Suppl 1: 88-95.
23. Sharma RK, Chatterji S, Rai DK, Mehta S, Rai PK, Singh RK, *et al*. Antioxidant activities and phenolic contents of the aqueous extracts of some Indian medicinal plants. J Med Plant Res 2009; 3(11): 944-948.
24. Patwardhan SK, Bodas KS, Gundewar SS. Coping with arthritis using safer herbal options. Int J Pharm Pharm Sci 2010; 2(1): 1-11.
25. Babaria P, Mute V, Awari D, Ghodasara J. *Invivo* evaluation of antiarthritic activity of seed coat of *Tamarindus indica* Linn. Int J Pharm Pharm Sci 2011; 3(4): 204-207.

26. Bothara SB, Marya BH, Saluja AK. Antiarthritic activity of root extracts of *Cocculus hirsutus*. Int J Pharm Pharm Sci 2011; 3 Suppl 4: 175-177.
27. Agarwal SP, Khanna R, Karmarkar R, Anwer MK, Khar RK. *Shilajit*: A Review. Phytother Res 2007; 21: 401-405.
28. Rekka E, Kourounakis PN. Effect of hydroxyethyl rutenosides and related compounds on lipid peroxidation and free radical scavenging activity, some structural aspects. J Pharm Pharmacol 1991; 43: 486-491.
29. Conforti F, Loizzo MR, Statti GA, Menichini F. Comparative radical scavenging and antidiabetic activities of methanolic extract and fractions from *Achillea ligustica* All. Biol Pharm Bull 2005; 28(9): 1791-1794.
30. Arulmozhi S, Mazumder PM, Ashok P, Narayanan LS. *In vitro* antioxidant and free radical scavenging activity of *Alstonia scholaris* Linn. R.Br. Iran J Pharmacol Ther 2007; 6(2): 191-196.
31. Devasagayam TPA, Boloor KK, Ramasarma T. Methods for estimating lipid peroxidation: An analysis of merits and demerits. Indian J Biochem Biophys 2003; 40: 300-308.
32. Yende SR, Sannapuri VD, Vyawahare NS, Harle UN. Antirheumatoid activity of aqueous extract of *Piper longum* on freunds adjuvant-induced arthritis in rats. Int J Pharm Sci Res 2010; 1(9): 129-133.
33. Deshpande V, Jadhav VM, Kadam VJ. *In vitro* anti-arthritic activity of *Abutilon indicum* (Linn.) Sweet. J Pharm Res 2009; 2(4): 644-645.
34. Lakshmi B, Tilak JC, Adhikari S, Devasagayam TPA, Janardhanan KK. Inhibition of lipid peroxidation induced by γ-radiation and AAPH in rat liver and brain mitochondria by mushrooms. Curr Sci 2005; 88(3): 484-488.
35. Banerjee D, Chakrabarti S, Hazra AK, Banerjee S, Ray J, Mukherjee B. Antioxidant activity and total phenolics of some mangroves in Sudarbans. Afr J Biotechnol 2008; 7(6): 805-810.
36. Basniwal PK, Suthar M, Rathore GS, Gupta R, Kumar V, Pareek A, *et al*. *In-vitro* antioxidant activity of hot aqueous extract of *Helicteres isora* Linn. Fruits. Nat Prod Rad 2009; 8(5): 483-487.
37. Rege AA, Ambaye RY, Deshmukh RA. *In vitro* testing of *Shilajit* for anti-HIV activity. Int J Pharmacol Biol Sci 2009; 3(2): 57-64.