ISSN 2320-5407 International Journal of Advanced Research (2014), Volume 2, Issue 2, 735-740

Journal homepage:http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Evaluation of Alpha-Amylase and Alpha-Glucosidase Inhibitory Activities of *Shilajit*

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Manuscript Info

Manuscript History:

Received: 10 December 2013
Final Accepted: 25 January 2014
Published Online: February 2014

Key words:
Alpha-amylase, Alpha-glucosidase, *Shilajit*,Antioxidant, Phenolic content, Antidiabetic

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Abstract

Shilajit is considered as a panacea in Ayurveda, as it is effective in number of ailments including diabetes. In the present study, 2 extracts namely, aqueous and ethanol of *Shialjit*were evaluated for their effect on alpha-amylase and alpha-glucosidase enzymes using *in vitro* assays. Alpha-Amylase inhibitory activity of *Shilajit* extracts was evaluated using Porcine pancreatic alpha-amylase (PPA) with starch as a substrate, whereas, alpha-glucosidase inhibitory activity was evaluated using p-nitrophenyl-α-D-Glucopyranoside (PNPG) as a substrate. Further, the antioxidant activity of extracts was monitored using DPPH assay, whereas, phenolic content was estimated using Folin-Ciocalteu reagent. Ethanol extract showed the highest inhibitory activity against alpha-amylase (IC_{50}= 95.05 µg/ml) as well as alpha-glucosidase (IC_{50}= 1.87 µg/ml) than aqueous extract. Both the extracts were strong inhibitors of alpha-glucosidase than alpha-amylase and inhibited the enzyme more potently than the standard acarbose. The alpha-amylase and alpha-glucosidase inhibitory activities of *Shilajit* correlated to antioxidant activity and phenolic content of extracts. Thus *Shilajit* can be effective in treatment of diabetes not only through inhibition of alpha-amylase and alpha-glucosidase enzymes, but also by its antioxidant effect.

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Introduction

Diabetes mellitus is a major endocrine disorder characterized by high blood sugar (hyperglycemia). In the list of nations having maximum number of diabetics, India stands first followed by China and America and the global prevalence of diabetes is estimated to be 4.4% by the year 2030 (Wild et al., 2004). Type 2 diabetes is the commonest form of diabetes constituting 90% of the diabetic population (Ramachandran et al., 2002) and it has increased morbidity and mortality because of its chronic complications (Kadhe and Arasan, 2002). Postprandial glucose control has been proven to be important for prevention of diabetic complications (Basak and Candan, 2010). Currently, there are 5 classes of conventional anti-diabetic drugs; however, these drugs are associated with various side-effects (Chakrabarti and Rajagopalan, 2002). Hence there is urgent need to identify and explore natural sources with fewer side-effects for such inhibitors.

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 (Kokate et al., 2002). *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 (Meena et al., 2010). There are four different varieties of *Shilajit*, namely, savarna (Gold), rajat (Silver), tamra (Copper) and lauha (Iron-containing). Lauha*Shilajit* is supposed to be the most effective according to the therapeutic point of view (Mirza et al., 2010). Hence it was included for the present *in vitro* study. Numerous traditional uses of *Shilajit* have been reported and it is said to be efficacious against diabetes (Mittal et al., 2009). It is given along with milk to treat diabetes in traditional medicine. Also, *Shilajit* is considered as a panacea in Ayurveda, as it is effective in number of ailments (Agarwal et al., 2007). Hence in present *in vitro* study, aqueous and ethanol extracts were prepared from *Shilajit* and were evaluated for their effect on alpha-amylase and alpha-glucosidase enzymes.

Materials and Methods

Collection of the material

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

Preparation of extracts

The material (41.33gm) was extracted in a Soxhlet apparatus with ethanol (230ml) to obtain ethanol extract. Aqueous extract was obtained by plain decoction method using material (10gm) with distilled water (100ml) (Rege et al., 2009). All the extracts were made free from solvents and percentage yield of individual extract was calculated, which was found to be 20.86% and 55.26% for ethanol and aqueous extracts respectively. Extracts were kept at 4^0C until further use. Ethanol extract was reconstituted in 20% ethanol as 20% ethanol was not inactivating the enzymes, whereas, aqueous extract was reconstituted in distilled water.

Alpha-Amylase inhibitory assay

Alpha-amylase inhibitory activity of extracts was carried out according to the method of Sudha et al. (2011) with slight modification. In a 96-well plate, reaction mixture containing 50µl phosphate buffer (50mM, pH= 6.8), 10µl alpha-amylase (10U/ml) [SRL] and 20µl of varying concentrations of extracts was pre-incubated at 37^0C for 10 min. Then 20µl soluble starch (0.05%) [HiMedia] was added as a substrate and incubated further at 37^0C for 15 min. The reaction was stopped by adding 20µl 1N HCl, followed by addition of 100µl iodine reagent (5mM I_2 and 5mM KI, stored in amber colored bottle). The absorbance was read at 620nm using Multimode Reader (Synergy HT, BioTek). Each experiment was performed in triplicates, along with appropriate blanks. Acarbose at various concentrations (10-100 µg/ml) was included as a standard. Acarbose was provided by Mr. DnyaneshwarNagmoti, Institute of Chemical Technology, Mumbai. Negative control without extracts was set up in parallel. The result is expressed as percentage inhibition, which was calculated as,

Inhibition (%)=$A_{Test} - A_{Negativecontrol} / A_{Test}$ X 100, where, A is absorbance. The result is also expressed as IC_{50} value.

Alpha-Glucosidase inhibitory assay

Alpha-glucosidase inhibitory activity of extracts was carried out according to the method of Bachhawat et al. (2011) with slight modification. In a 96-well plate, reaction mixture containing 50µl phosphate buffer (50mM, pH= 6.8), 10µl alpha-glucosidase (1U/ml) [SRL] and 20µl of varying concentrations of extracts was pre-incubated at 37^0C for 15 min. Then 20µl p-nitrophenyl-α-D-Glucopyranoside (PNPG) (1mM) [SRL] was added as a substrate and incubated further at 37^0C for 30 min. The reaction was stopped by adding 50µl sodium carbonate (0.1M). The yellow color produced was read at 405nm using Multimode Reader (Synergy HT, BioTek). Each experiment was performed in triplicates, along with appropriate blanks. Acarbose at various concentrations (200-1000 µg/ml) was included as a standard. Negative control without extracts was set up in parallel. The result is expressed as percentage inhibition, which was calculated as,

Inhibition (%)=$A_{Negativecontrol} - A_{Test}/A_{Negativecontrol}$ X 100, where, A is absorbance. The result is also expressed as IC_{50} value.

DPPH radical-scavenging assay

The free radical scavenging activity of *Shilajit* was measured by 1,1-diphenyl-2-picryl hydrazyl (DPPH) assay (Nikhat et al., 2009). For this, 1 ml of DPPH solution (0.1mM) in methanol was added to different concentrations of extracts. After incubating for 30 minutes in dark, the absorbance was measured at 517nm using Multimode Reader (Synergy HT, BioTek). Ascorbic acid at various concentrations (3-7 µg/ml) was included as a standard. Negative control without extracts was set up in parallel. The percent DPPH-scavenging activity was calculated as,

DPPH scavenged (%)=$A_{Negative\ control} - A_{Test}/A_{Negative\ control}$ X 100. Where, Ais absorbance. The antioxidant activity of *Shilajit* is also expressed as IC_{50} value.

Phenolic content estimation

The total phenolic content of *Shilajit*was determined using Folin-Ciocalteu reagent according to the method of Pandima Devi et al. (2008). Gallic acid at various concentrations (4-20 µg/ml) was included as a standard. All the determinations were done in triplicate. Mean values of triplicate determinations were used to plot the graph. Total phenolic content was calculated from the equation (y= 0.045x, R^2= 0.997) obtained from the Gallic acid standard curve. The total phenolic content was expressed as Gallic acid equivalent (GAE) in mg/g of dry sample.

Statistical Analysis

All the determinations were done in triplicate. Means, standard deviations and IC_{50} values were calculated using a Microsoft Excel program.

Result and Discussion

One of the anti-diabetic therapeutic strategies is inhibition of carbohydrate digesting enzymes such as alpha-amylase and alpha-glucosidase (Narkhede et al., 2011). Alpha-amylase hydrolyzes complex starches to oligosaccharides, while, alpha-glucosidase hydrolyzes oligosaccharides to glucose and other monosaccharides. Inhibition of these enzymes produces postprandial anti-hyperglycemic effect by reducing the rate and extent of glucose absorption from small intestine (Okoli et al., 2011). Many natural products have shown inhibitory activity against these enzymes (Tripathi et al., 2011; Kumar et al., 2012; Srivastava et al., 2012). In the present study, 2 extracts of *Shilajit* were evaluated for their effect on alpha-amyalse and alpha-glucosidase enzymes using *in vitro* assays.

Porcine pancreatic alpha-amylase (PPA) is closely related to human alpha-amylase (Sudha et al., 2011). Hence PPA was used to evaluate inhibitory activity of *Shilajit* extracts with starch as a substrate. The assay was based on starch-iodine color complex formation, whereas, alpha-glucosidase inhibitory activity was evaluated using p-nitrophenyl-α-D-Glucopyranoside (PNPG) as a substrate which was based on development of yellow color of p-nitro phenol. Ethanol extract showed the highest alpha-amylase and alpha-glucosidase inhibitory activities (Table 1 and 2) with IC_{50} values of 95.05 µg/ml and 1.87 µg/ml respectively. Both the extracts were strong inhibitors of alpha-glucosidase than alpha-amylase and inhibited the enzyme more potently than the standard acarbose. According to Ani and Naidu (2008), effective strategy for type-2 diabetes management is mild inhibition of alpha-amylase and strong inhibition of alpha-glucosidase and our study supports this finding. *Shilajit* has shown reduction of blood glucose levels in diabetic rats and in hyperglycemic subjects (Trivedi et al., 2004; Kamat et al., 2012). Inhibition of carbohydrate digesting enzymes such as alpha-amylase and alpha-glucosidase could be one of the mechanisms.

The role of oxidative stress in diabetes and diabetic complications has been reported (Giacco and Brownlee, 2010). Antioxidants can scavenge free radicals and play important role in prevention of diabetes. Hence in present study, antioxidant effects of *Shilajit* extracts were evaluated by testing their ability to bleach (purple to yellow color) the stable DPPH radical which is a widely used rapid and simple method. Ethanol extract showed potent free radical-scavenging activity than aqueous extract with IC_{50} value of 4.92 µg/ml (Table 3). *Shilajit* has also exhibited profound antioxidant activity through reductive ability and by scavenging free radicals that initiate or propagate lipid peroxidation in previous study (Rege et al., 2012a). The antioxidant activity of *Shilajit* is believed to be due to presence of iron-containing quinone-semiquinone-hydroquinone complex structures in the core of *Shilajit*(Anonymous, 2007).

The alpha-amylase inhibitory, alpha-glucosidase inhibitory and antioxidant activities of *Shilajit* can be attributed to its phenolic content. Phenolic components have shown effective inhibition of alpha-amylase and alpha-glucosidase enzymes as well as antioxidant effect (Hara and Honda, 1990; Toda et al., 2000; Basniwal et al., 2009). Hence total phenolic content of *Shilajit* extracts was determined by Folin-Ciocalteu method. It has been observed that these activities were proportional to phenolic content of *Shilajit*, as ethanol extract showed the highest phenolic content (Table 4).

According to Basak and Candan (2010), lipid peroxidation increases in plasma lipoproteins, erythrocyte membrane lipids and various tissues in diabetes. *Shilajit* was found to potently inhibit lipid peroxidation in rat liver mitochondria (Rege et al., 2012a). Besides, it has shown anti-HIV activity by inhibiting HIV-reverse transcriptase enzyme and interfering with the gp120/CD4 interaction in our earlier study (Rege et al., 2012b). It has also revealed putative HIV-protease inhibitory activity (Rege and Chowdhary, 2014). The use of combination antiretroviral therapy consisting of reverse transcriptase and protease inhibitors, has yielded clinical benefits for HIV-infected patients, however, it has also led to adverse metabolic effects such as diabetes (Dagogo-Jack, 2008) and when HIV and diabetes intersect, the treatment regimens required for both diseases can be overwhelming for patients (Spollett, 2006). Thus Lipid peroxidation and anti-HIV drugs are associated with diabetes. Our current and previous studies have shown anti-diabetic, antioxidant, anti-lipid peroxidation and anti-HIV activities of *Shilajit*. Hence it can be a good candidate for further investigations.

Table 1- Effect of *Shilajit* on alpha-Amylase

Extract	Concentration (µg/ml)	% Inhibition [Mean±SD]	IC_{50} (µg/ml)
	1200	23.27 ± 6.9	
	1400	36.18 ± 2.6	
Aqueous	1600	43.03 ± 5.3	1776.2
	1800	54.48 ± 3.6	
	2000	59.94 ± 1.5	
	90	38.22 ± 3.0	
	100	55.94 ± 3.8	
Ethanol	110	61.98 ± 2.3	95.05
	120	70.35 ± 5.6	
	160	81.53 ± 1.3	
	10	15.96 ± 14.6	
	20	43.46 ± 9.2	
Acarbose	40	59.04 ± 5.7	34
(Standard)	60	66.74 ± 2.1	
	100	72.73 ± 1.9	

Table 2- Effect of *Shilajit* on alpha-Glucosidase

Extract	Concentration (µg/ml)	% Inhibition [Mean±SD]	IC_{50} (µg/ml)
	4	12.73 ± 10.9	
	8	27.19 ± 14.4	
Aqueous	12	58.23 ± 4.0	11.65
	16	72.73 ± 2.1	
	20	82.34 ± 3.3	
	1.5	28.57 ± 13.9	
	2	54.85 ± 5.7	
Ethanol	2.5	78.54 ± 5.7	1.87
	3	86.95 ± 5.6	
	4	98.02 ± 0.4	
	200	34.21 ± 3.1	
	400	47.07 ± 1.9	
Acarbose	600	57.98 ± 7.2	468.92
(Standard)	800	63.14 ± 0.7	
	1000	78.76 ± 5.5	

Table 3- Effect of *Shilajit* on DPPH

Extract	Concentration (µg/ml)	% DPPH Scavenged [Mean±SD]	IC_{50} (µg/ml)
Aqueous	2	23.08 ± 5.4	11.49
	8	41.57 ± 3.1	
	12	51.19 ± 3.2	
	16	65.77 ± 1.9	
	20	69.95 ± 1.8	
Ethanol	1	18.33 ± 2.9	4.92
	2	29.41 ± 0.8	
	4	46.14 ± 2.5	
	6	58.30 ± 2.9	
	8	71.21 ± 2.2	
Ascorbic Acid (Standard)	3	24.84 ± 0.9	5.61
	4	34.13 ± 3.4	
	5	43.26 ± 1.7	
	6	54.82 ± 1.9	
	7	63.08 ± 1.8	

Table 4- Phenolic content Estimation of *Shilajit*

Extract	Gallic acid equivalent (mg/gm)*
Aqueous	322
Ethanol	500

*Mean of Triplicate determinations

Conclusion

Shilajit can be effective in treatment of diabetes not only by inhibition of alpha-amylase and alpha-glucosidase enzymes, but also through its antioxidant effect by scavenging free radicals.

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