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RESEARCH ARTICLE

Evaluation of *Shilajit* as Putative HIV-Protease Inhibitor

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Manuscript Info *Abstract*

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Ethanol and aqueous extracts of *Shilajit* were included for the present *in vitro* study. Pepsin was used as a substitute for HIV-protease to evaluate inhibitory activity of *Shilajit* extracts, as pepsin has close resemblance with HIV-protease in proteolytic activity. Both the extracts;viz.,ethanol and aqueous,showed potent inhibitory activity with IC_{50} values of 9.61 and 193.80 µg/ml respectively. In our earlier study, these extracts exerted their anti-HIV activity via multiple mechanisms of action; viz., interference with the gp120 / CD4 interaction and inhibition of HIV-reverse transcriptase. In the present study, they showed potent inhibitory activity against pepsin enzyme, suggesting that *Shilajit* may be useful as a HIV protease inhibitor. The inhibitory activity could be due the high flavonoids content.

Introduction

Acquired Immuno Deficiency Syndrome (AIDS) is a devastating human disease caused by Human Immunodeficiency Virus (HIV). HIV protease plays a vital role in viral replication cycle (Castro *et al.*, 2011). Blockage of HIV protease leads to formation of immature non-infectious virions (Kohl *et al.*, 1988). Hence it has become an important target in HIV drug development. Several natural products have been shown to possess HIV-protease inhibitory activity (Xu*et al.*, 1996; Tewtrakul*et al.*, 2003a;Magadula and Tewtrakul, 2010; Chaitra Narayan *et al.*, 2011).Although, a 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). Numerous traditional uses of *Shilajit* have been reported previously(Agarwal*et al.*, 2007).

In the present study, ethanol and aqueous extracts were prepared from *Shilajit.* Pepsin has a close resemblance with HIV-protease in proteolytic activity as both of them belong to same aspartate enzyme family(Maria *et al.*, 2004). Hence in the present study, pepsin was used as a substitute for HIV-protease(Govindappa*et al.*, 2011).

The aims and objectives of present study were to prepare ethanol and aqueous extracts of *Shilajit* and to evaluate their inhibitory effect on pepsin enzyme.

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 was extracted in a Soxhlet apparatus with ethanol to obtain ethanol extract. Aqueous extract was obtained by plain decoction method (Indian Herbal Pharmacopoeia, 1998) using material with distilled water. All the extracts were made free from solvents using boiling water bath and percentage yield of individual extract was calculated (Table 1).

Assessment of pepsin enzyme inhibitory activity

a) Preparation of hemoglobin

Hemoglobin was prepared as stated earlier (www.worthington-biochem.com/pm/assay.html, 2011). Briefly, 2.5 gm hemoglobin powder (HiMedia) was dissolved in 100 ml distilled water. It was blended at maximum speed for 5 min and then filtered through gauze. Eighty ml of filtrate was diluted with 20 ml of 0.3N HCl and stored at 4^0C until further use.

b) Pepsin assay

Pepsin assay was carried out as described by Singh *et al*(Singh *et al.*, 2010). Briefly, 50 µg pepsin (HiMedia), 800 µg hemoglobin (HiMedia) and different concentrations of each extract were taken in 500 µl of reaction mixture. The mixture was allowed to incubate at 37^0C for 20 min. After incubation, 700 µl of 5% trichloro acetic acid (TCA) (Himedia) was added to stop the reaction. It was then centrifuged (Rotina 38R) at 14000 rpm for 5 min and the supernatant was collected. Optical density was recorded spectrophotometrically (Cary 50 Bio UV-Visible spectrophotometer) at 280nm. Pepstatin-A (Sigma) was included as a standard. Negative control without extract(s) was set up in parallel. Separate blanks were used for extracts. All the determinations were done in triplicate. Percent Inhibition was calculated as, Inhibition (%) = $(A_{\text{Negative control}} - A_{\text{Test}})/A_{\text{Negative control}}$ X 100, where A is absorbance. The result is expressed as IC_{50}.

Flavonoids content estimation

The method of Oyedemi*et al* (Oyedemi*et al.*, 2011) was used to estimate total flavonoids content of the extract solutions based on formation of a complex flavonoids-aluminums. Briefly, a volume of 0.5 ml of 2% $AlCl_3$ ethanol solution was added to 0.5 ml of each extract solution. After one hour of incubation at room temperature, the absorbance was measured at 420nm using Multimode Reader (Synergy HT, BioTek). Yellow color indicated presence of flavonoids. Quercetin at various concentrations (20 to 100 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 flavonoid content was calculated from the equation ($y= 0.008x$, $R^2= 0.975$) obtained from the Quercetin standard curve. Total flavonoids content was expressed as quercetin equivalent in milligrams per gram 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.

Table 1- Percentage yield of extracts

S. No.	Extracts	Yield (%)
1.	Ethanol	20.68
2.	Aqueous	55.26

Table 2- Effect of *Shilajit* on pepsin assay

S. No.	Extract	Conc. (µg/ml)	% Inhibition (Mean ± SD)	IC_{50} (µg/ml)
1.	**Ethanol**	1	39.75 ± 2.8	
		10	52.45 ± 1.2	9.61
		20	59.90 ± 1.3	
		30	74.23 ± 2.7	
		40	92.39 ± 0.8	
2.	**Aqueous**	1	13.57 ± 0.9	
		100	36.24 ± 1.2	193.80
		200	54.20 ± 2.9	
		300	78.39 ± 2.4	
		400	99.04 ± 0.6	
3.	**Pepstatin-A** (Standard)	-	-	< 0.2

Table 3- Flavonoids content estimation of *Shilajit*

S. No.	Extracts	Quercetin equivalent(mg/gm)*
1.	Ethanol	350
2.	Aqueous	237.5

*Mean of triplicate determinations

Result and Discussion

The development of safe, effective and low-cost anti-HIV agents is among the top global priorities of drug development, since the long-term complications of this disease are multifactorial and can be related to the virus itself or to adverse effects of antiretroviral therapy(Reust, 2011). Traditional knowledge-driven drug development can follow a reverse pharmacology path and reduce time and cost of development(Patwardhan*et al.*, 2004). *Shilajit* is considered as a panacea in traditional medicine, as it is effective in a number of ailments (Agarwal*et al.*, 2007).

HIV proteasebelongs to class of asparticproteases and has similar structural features and mechanism to asparticprotease enzymes(Brik and Wong, 2003). Asparticproteasesincludepepsin, cathepcin D, renin, chymosin and the proteasesisolated fromnumerous fungi(Polgár, 1987). In the present study, pepsin wasused as a substitute of HIV protease for screening HIV proteaseinhibitory activity. Similarstudies have been carried out by Govindappa*et al* (Govindappa*et al.*, 2011) and Singh *et al*(Singh *et al.*, 2010). Both the extracts of *Shilajit;*viz.,ethanol and aqueous, showed potent inhibitory activitywith IC_{50} values of 9.61 and 193.80 µg/ml respectively. As variouspreviousstudiessuggestedstructural and functional similarity between pepsin and HIV protease (Pearl and Taylor, 1987; Seelmeier*et al.*, 1988; Navia*et al.*, 1989; Davis, 1990;Wlodawer and Vondrasek, 1998), *Shilajit* extracts that showed inhibitory activity of pepsin enzyme should also inhibit activity of HIV protease. Ethanol extract of *Shilajit* exhibited high enzyme inhibitory activity compared to aqueous extract (Table 2). This may be due to high flavonoids content of ethanol extract (Table 3). As per the literature survey, flavonoids have shown inhibitory activity against HIV protease(Xu*et al.*, 2000; Tewtrakul*et al.*, 2003b; Yu *et al.*, 2007). According to Ko*et al*(Ko*et al.*, 2009), flavonoids belonging to flavonol or flavone group simultaneously inhibit HIV reverse transcriptase and protease, whereas, flavonoids belonging to flavanol, isoflavone or flavanone group are less likely to inhibit reverse transcriptase or protease.

In our earlier study, these extracts exerted their anti-HIV activity via multiple mechanisms of action; viz., interference with the gp120 / CD4 interaction and inhibition of HIV-reverse transcriptase(Rege *et al.*, 2012). In the present study, they also showed potent inhibitory activity against pepsin enzyme, suggesting that *Shilajit* may be useful as a HIV protease inhibitor. The inhibitory activity could be attributed to high flavonoids content. Phytochemical investigation of *Shilajit* also revealed presence of tannins, saponins, carbohydrates and cardiac glycosides(Rege *et al.*, 2009). These phyto-constituents have shown anti-HIV potential in the past(Joshi, 2002; Cos *et al.*, 2004; Singh *et al.*, 2005). Because of such phytochemical diversity, *Shilajit* may be involved in the entire process, from virus adsorption to host cell, to formation, growth and activation of virus proteins and budding into mature virions.

Conclusion

The present study indicates that *Shilajit* might be of value as sources or leads for novel antiviral compounds which can be useful in the continuing fight against HIV.

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