Original Research Article (Experimental)

Mercury based drug in ancient India: The red sulfide of mercury in nanoscale

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A R T I C L E   I N F O

A B S T R A C T

Mercury is one of the elements which had attracted the attention of the chemists and physicians of ancient India and China. Among the various metal based drugs which utilize mercury, we became interested in the red sulfide of mercury which is known in ancient Indian literature as rasasindur (alias rasasindura, rasasindoor, rasasinduram, sindur, or sindoor) and is used extensively in various ailments and diseases. Following various physico-chemical characterizations it is concluded that rasasindur is chemically pure α-HgS with Hg:S ratio as 1:1. Analysis of rasasindur vide Transmission Electron Microscopy (TEM) showed that the particles are in nanoscale. Bio-chemical studies of rasasindur were also demonstrated. It interacts with Bovine Serum Albumin (BSA) with an association constant of $(9.76 \pm 0.56) \times 10^3 \, \mathrm{M}^{-1}$ and behaves as a protease inhibitor by inhibiting the proteolysis of BSA by trypsin. It also showed mild antioxidant properties.

1. Introduction

Ayurveda, meaning the science of life, is one of the ancient medical systems of the Indian subcontinent. The principles and practices of the subject are documented in a large number of age-old texts; Charaka Samhita and Sushruta Samhita being two main Ayurvedic classics [1,2]. Ayurvedic material medica is dominated by substances of vegetable, animal and mineral origin. Metals and minerals used include mercury, gold, silver, copper, iron, tin, zinc etc. An extensive range of chemical and physical processing of these metals and their compounds has been elaborated in texts which are generally known as Rasashastra [3–7].

Mercury is one of the metals which attracted wide attention of ayurvedic chemists and physicians [8]. Indeed the documentation of chemical and physical processes involving mercury is truly enormous in ancient texts of which classics by Vagabhatta and Nagarjuna are noteworthy. Among the various procedures which utilize mercury, we became interested in the one that involves mercury and sulfur. The process is divided in three distinct steps, namely (i) pre-treatment of mercury and sulfur with herbal and milk products, (ii) mixing of mercury and sulfur along with other herbal ingredients resulting in the formation of black sulfide of mercury, (iii) thermal treatment of black sulfide of mercury at 600–650 °C [9]. The sublimed red sulfide of mercury is termed as rasasindur (alias rasasindura, rasasindoor, rasasinduram, sindur, or sindoor) in Rasashastra and is used extensively in various ailments and diseases [10].

The context of toxicity in metal based drugs in general, and of mercurial preparation in particular is an important issue [11]. It needs to be emphasized that the Rasashastra texts elaborately emphasize the concept of specific attributes in starting materials, intermediates, and products which lead to toxicity and adverse

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effects in a patient [12]. However, such narrations are at times difficult to interpret in equivalent modern scientific terms. The elaborate preparative protocol in the synthesis of rasasindur prompted us to study the physico-chemical properties of rasasindur. As presented below, we are delighted to find that the synthesis protocol described in the ancient text is indeed a case of bottom-up synthesis of red sulfide of mercury in nanoscale [13]. In light of the results of the present study and those by others [14,15] the context of nanotransformation (and its plausible implication on bio-efficacy/bio-availability/in-vivo toxicity) in metal-based ayurvedic drugs warrants a relook.

2. Experimental

2.1. Materials and methods

X-ray powder diffraction (XRD) patterns were obtained in a Bruker D8 Advance powder diffractometer using Cu Kα radiation ($\lambda = 1.5406$ A$^\circ$). The optical absorption spectra of the sample were measured in the range of (500–800 nm) using PerkinElmer UV WinLab 5.2.0.0646/1.61.00 Lambda 900. Raman spectra were recorded with LabRAM HR800 micro-Raman spectrometer (Manufacturer HORIBA JobinYvon) using 632.8 nm laser excitation wave length. All measurements were made in a backscattering geometry, using a 50$\times$ microscope objective lens with a numerical aperture of 0.7. Typical laser power at the sample surface was 2.0 mW with spot size of 2 μm. The acquisition time for all the spectra was 2 s. Emission measurements were performed (excited at 270 nm) at room temperature with a Fluorolog-3 (HORIBA JOBINYVON) Spectrofluorimeter. Transmission electron microscopy (TEM) studies were carried out with a Jeol, Ultra high resolution Transmission Electron Microscope (PP resolution: 0.19 nm), at 200 KeV. Rasasindur powder was first dispersed in isopropanol to prepare a 0.1 mg/mL suspension, which was sonicated for 1 h. One drop of the suspension was taken on a copper coated grid, dried at room temperature and submitted for TEM. X-ray photoemission spectra (XPS) were recorded on a KRATOS AXIS 165 with a dual anode (Mg and Al) apparatus using the Mg Kα anode. Lens Mode: Electrostatic; Resolution: pass energy 80, Anode: A1 (150 W), step size is 100.0 meV. The pressure in the spectrometer was about 10^{-9} Torr. For energy calibration, we have used the carbon 1s photoelectron line. The carbon 1s binding energy was taken to be 285.0 eV. Spectra were deconvoluted using the Sun Solaris based Vision 2 curve resolver. The location and the full width at half-maximum (FWHM) for a species were first determined using the spectrum of a pure sample. The location and FWHM of the products, which were not obtained as pure species, were adjusted until the best fit was obtained. Symmetric Gaussian shapes were used in all cases. SEM analysis was carried out in a Zeiss Merlin Compact Oxford instrument. EDS analysis was performed at 20 kV. The working distance was set in the range 4–5 mm. The image was analyzed with secondary 1 detector. Energy of X-ray is characteristic of the difference in energy between the two shells or the atomic structure of the element.

2.2. Preparation of rasasindur

Rasasindur was prepared at Arya Vaidya Sala, Kottakkal, Kerala, India following validated standard operating procedure according to rasatarangini, which is one of the classics of Rasashastra [9]. The same procedure has been also cited in other reports [10,14]. Briefly, the preparation involves the following major steps each of which takes days to complete. Initially 350 g mercury and 350 g lime were ground on a mortar after which the mercury was filtered through a muslin cloth. The mercury thus obtained was again ground on a mortar with garlic and rock salt. Finally it was washed with water and kept ready for the next step. The processing of sulfur involved melting it, pouring the liquid sulfur in milk and in the juice of *Eclipta alba* in stages. After washing with water the sulfur was dried. 310 g of detoxified mercury and 310 g of detoxified sulfur, obtained as above, were ground together to a fine paste in the presence of the juice of *Ficus benghalensis*. The resulting black sulfide of mercury (called Kajjali in traditional texts) was then sun-dried. 250 g of Kajjali was taken in a porcelain reactor and the lid was closed. The reactor was smeared with five layers of clay and dried. Finally the reactor was heated in an open-hearth furnace at 650 °C for 33 h 15 min. The sublimed red crystals were milled on a mortar for such duration till the powder of Rasasindur passed through the standard quality control (QC) parameters as in Ayurvedic texts.

3. Results & discussion

3.1. Structure and morphological study

The crystalline behavior of rasasindur was established through Powder X-Ray Diffraction. The PXRD pattern was shown in Fig. 1. On comparison with standard JCPDS (Card No. 00-006-0256) data base values of all peaks, it is indexed to be a pure hexagonal lattice (cinnabar phase) and space group P3221. The lattice parameters are a = 0.415 ± 0.005 nm, c = 0.949 ± 0.005 nm. There are no impurities detected from the PXRD pattern, suggesting the high purity of rasasindur.

A complete analysis of elemental composition of rasasindur was possible by SEM-EDX (Fig. 2 (e)). EDX analysis concludes that the presence of only two elements e.g. Hg and S in the red HgS where Hg (80%) is present as a major element. Presence of other elements C and O are due to use of carbon tape for support and oxygen due to adsorption of atmospheric oxygen on the surface of the sample. Atom balance shows that both Hg and S are present ~50%. Thus from the EDX results we can interpret that the atomic ratio of Hg to S is 1:1.

HRTEM analysis indicated that the particles of rasasindur have spherical shape nanostructures. Particle size distribution shows that most of the particles are in the range of 8–16 nm range. From the high resolution HRTEM image (Fig. 2 (b)), the lattice spacing have been determined to be 0.337 ± 0.005 nm, 0.332 ± 0.005 nm which corresponds to (101) plane of HgS. The lattice parameters are a = 0.415 ± 0.005 nm and c = 0.949 ± 0.005 nm corresponding to Hexagonal crystal system, Space group-P3221 (PCPDF No. 060256).

From the diffraction pattern (Fig. 2 (c)), the interplanar distance has been calculated to be 0.281 nm, 0.231 nm and 0.171 nm which
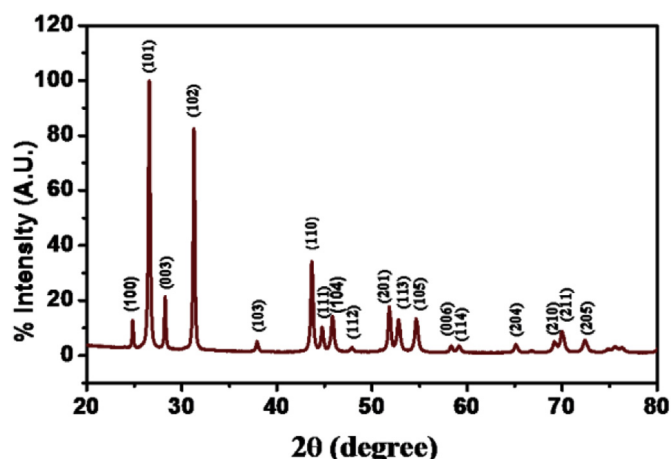

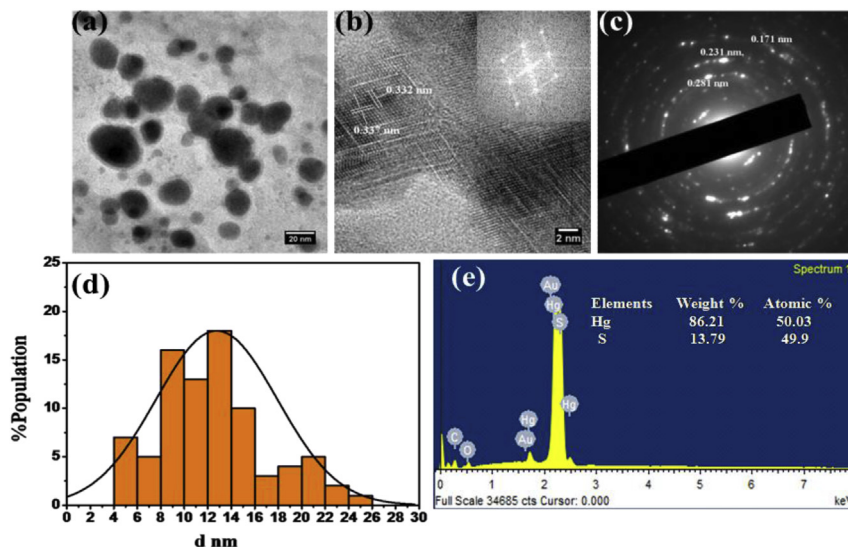**Fig. 1.** Powder X-Ray Diffraction patterns of rasasindur.

Fig. 2. (a) Low magnification image revealing the formation of nanoparticles; (b) HRTEM micrograph (inset shows the FFT of the HRTEM region); (c) Diffraction pattern taken on the nanoparticles; (d) Particle size distribution graph (e) EDX spectrum of rasasindur.

corresponds to (102), (103) and (113) planes of HgS (Crystal system-Hexagonal, Space group-P3221). The lattice parameters are a = 0.415 nm and c = 0.949 (error bars are similar to those described for Fig. 2(b)).

The binding energies obtained in the XPS analysis (Fig. 3) are corrected for specimen charging by referencing the C 1s to 285.0 eV. The two peaks obtained at 100.89 and 104.91 eV are for Hg (4f). Peaks at 161.89 and 163.29 eV are for S (2p). These values are well matched with the reported data of binding energies of HgS [16,17]. Hence from XPS it is attributed that it is a chemically pure material containing Hg and S.

Raman spectroscopy is an important technique to predict structural properties of nano materials. Raman spectrum of rasasindur (Fig. 4) gives the characteristic bands at 82 nm, 100 nm, 140 nm, 250 nm, 280 nm and 340 nm. Raman active modes are given as E mode at 82 nm, 100 nm, 280 nm, 340 nm; A1 mode at 250 nm and A2 mode at 141 nm which is well matched with the literature data [18].
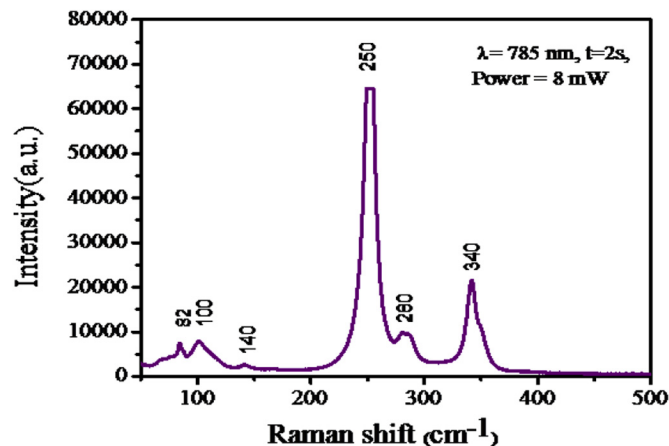
3.2. Optical properties

In order to investigate the optical property of the rasasindur, the UV−vis adsorption spectrum of the product was measured. Fig. 5 (b) illustrates the DRS spectrum of rasasindur.
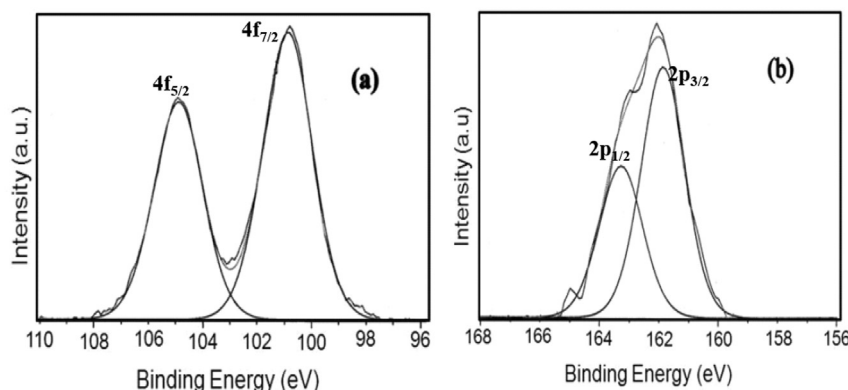

Fig. 4. Raman scattering spectrum.

Band Gap was calculated using the formula

$$\alpha h\nu = B(h\nu - Eg)^{1/2}$$

Where α is the absorption coefficient, $h\nu$ is the energy of the incident radiation Eg is the band gap, and B is a Constant [19]. Fig. 5

Fig. 3. X-Ray Photoelectronic Spectra (a) Hg 4f binding energy spectrum and (b) S 2p binding energy spectrum.
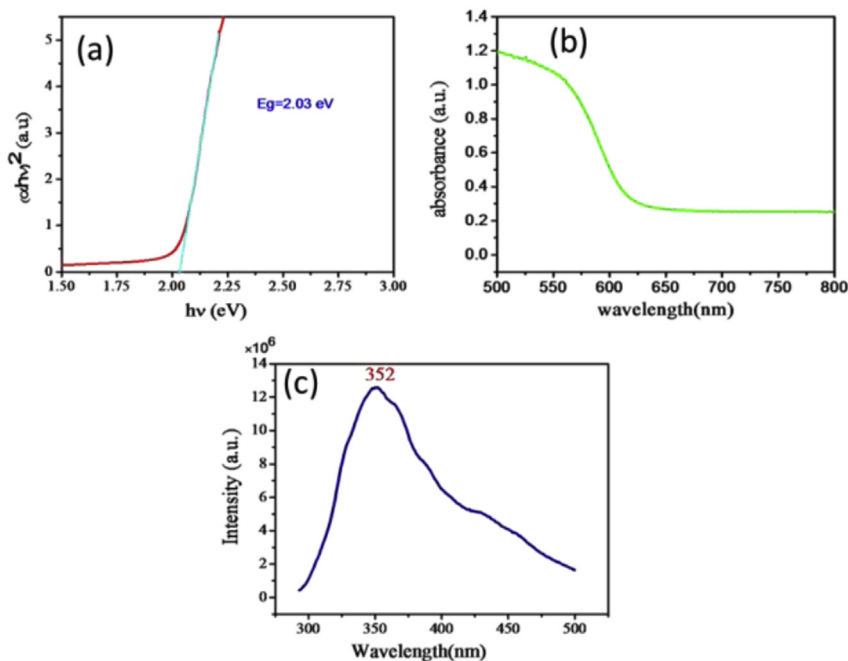
Fig. 5. (a) Band gap calculation graph, (b) UV−Vis DRS spectrum of rasasindur, (c) photoluminescence spectrum.

(a) shows the plot of $(\alpha h\nu)^2$ vs. $h\nu$ which is linear over a wide range of photon energies indicating a direct type of transitions. The intercepts (extrapolations) of these plots (straight lines) on the energy axis reflect the energy band gaps. Calculated band gap is 2.03 eV. Photoluminescence spectrum is shown in Fig. 5 (c). It was carried out at excitation wavelength 270 nm and the emission maximum was found to be 352 nm.

3.3. Biochemical studies

DPPH is a stable free radical which when dissolved in ethanol exhibits violet coloration. In the presence of compounds which are able to donate hydrogen or electrons, the color of DPPH changes from violet to yellow. Such class of compounds which are able to quench the violet coloration of DPPH are known as antioxidants [20]. To evaluate whether rasasindur has potential antioxidant activity, we carried out DPPH scavenging activity with rasasindur in a range of concentration from 10 to 1000 μg/ml. It was observed that the free radical scavenging activity or percent (%) inhibition of the rasasindur increased in a concentration dependent manner which saturated at 800 μg/ml (Fig. 6). As depicted from Fig. 6, the highest antioxidant property obtained for rasasindur at 800 μg/ml was ∼24%. Under similar condition, a standard sample of ascorbic acid showed ∼82% free radical scavenging activity at a concentration of 200 μg/ml. Therefore as compared to ascorbic acid, rasasindur exhibited a lower antioxidant property.

Binding capacity of serum albumins has a great impact on the pharmaco kinetic properties of therapeutic drugs [21]. Since rasasindur is a traditional drug used in the Indian system of medicine [9] therefore we attempted to study its interaction with bovine serum albumin (BSA). BSA is highly homologous to human serum albumin (HSA) and is often chosen as a model protein to study small molecule albumin interactions [22,23]. Tryptophan fluorescence quenching experiment was carried out to study the interaction of the BSA with rasasindur. The intrinsic fluorescence of the protein is mainly due to the amino acids tryptophan, tyrosine and phenylalanine. BSA has two tryptophan residues and exhibits tryptophan fluorescence at an excitation of 295 nm with an

emission maximum at 344 nm. The interaction of rasasindur with BSA was studied by monitoring the quenching of tryptophan fluorescence upon the addition of the rasasindur (Fig. 7 (a)). The intrinsic tryptophan fluorescence of BSA decreased gradually on increasing rasasindur concentration. The Stern−Volmer quenching constant (Ksv) as calculated from Eq. (1) was found to be $(3.21 \pm 0.19) \times 10^3 \ M^{-1}$.

$$F_0/F = 1 + K_{SV} [Q] \tag{1}$$

where F_0 and F are the emission intensity of BSA in absence and in presence of rasasindur, [Q] is the concentration of rasasindur and K_{SV} is Stern−Volmer quenching constant.

The association of the rasasindur with BSA is directly correlated with the extent of fluorescence quenching [24]. Small molecules often bind to a set of sites in a protein molecule. The equilibrium
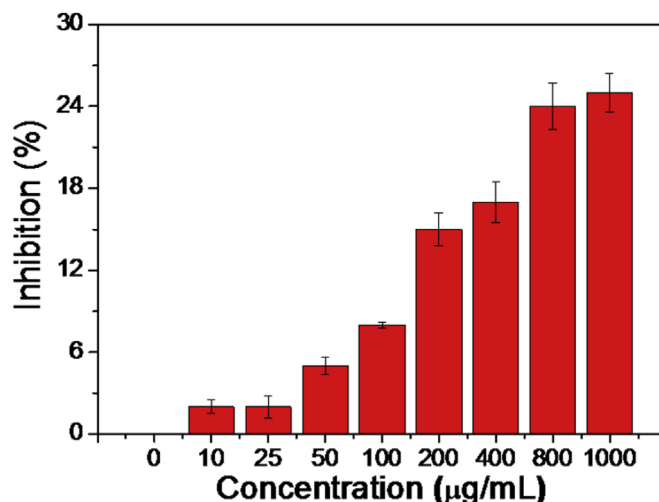

Fig. 6. Concentration dependent DPPH radical scavenging activity by rasasindur.
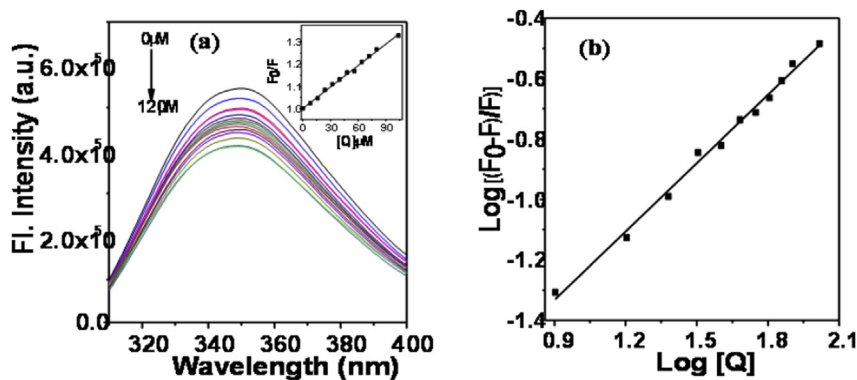
Fig. 7. Rasasindur interaction with bovine serum albumin (BSA). (a) Intrinsic tryptophan fluorescence spectra of BSA (2 μM) in the presence and absence of rasasindur [0−120 μM in 50 mM phosphate buffer (pH 7.5)]. The inset shows the linear fit of the F0/F vs. [complex] and Stern−Volmerquenching constant (KSV). (b) The plot represents the linear fit of log [(F$_0$−F)/F] vs log [Q] for rasasindur and the association binding constant (KBSA).
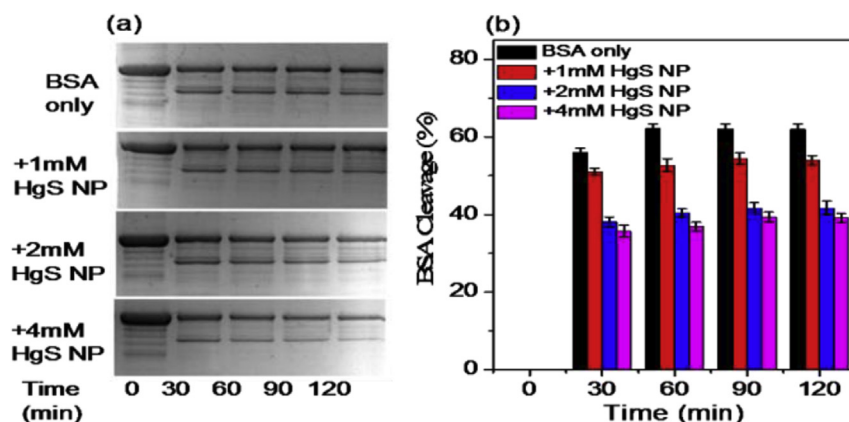

Fig. 8. Proteolysis of BSA in the presence and absence of rasasindur. (a) SDS-PAGE profile of the trypsin digest of BSA in presence and absence of rasasindur. The trypsin to BSA ratio was 1:1 (w/w). (b) Bar diagram representation of the net cleavage percent of BSA by trypsin at different time points in presence and absence of rasasindur.

between the free and the bound small molecule is given by the Scatchard analysis. The number of binding sites (n) and the association binding constant (KBSA) as calculated from Scatchard equation was found to be $(0.85 \pm 0.08) \times 10^3$ and $(9.76 \pm 0.56) \times 10^3$ M^{-1} respectively (Fig. 7 (b)).

Trypsin is a serine protease which is found in pancreas and helps in the proteolysis of the proteins. Inside the pancreas trypsin activity is inhibited by pancreatic secretory trypsin inhibitor (PSTI). But failure to inhibit trypsin activity can lead to damage of pancreatic cells. This can lead to pancreatitis, which may eventually develop into pancreatic cancer [25]. Therefore an imbalance between inhibition and activation of proteolytic enzymes may result in the damage of the extracellular matrix, which may lead to cancer, heart disease, neurodegenerative disease etc [26]. Thus, small molecules which can act as a potential protease inhibitor may prevent the unwanted cell damage and act as a drug against the disease caused by excessive proteolysis. Since rasasindur is used in traditional Indian medicinal system, therefore we attempted to explore its proteolytic inhibition activity by taking trypsin as the proteolytic enzyme and BSA as the substrate.

We carried out trypsin digestion of BSA in presence and absence of rasasindur at 37 °C at various concentration of the rasasindur i.e 1 mM, 2 mM and 4 mM. The SDS-PAGE profiles of the trypsin digested products of BSA in presence and absence of rasasindur is shown in Fig. 8 (a). In absence of rasasindur the proteolytic cleavage of BSA by trypsin after 2 h was ~61%. Interestingly, in presence of 1 mM rasasindur, the BSA cleavage decreased from ~61% to ~54%,

which eventually saturated at ~39% when the rasasindur concentration was 4 mM (Fig. 8 (b)). Control experiments performed with BSA incubated with various concentrations of rasasindur at 37 °C for 2 h showed that rasasindur itself was cleavage inactive in absence of trypsin. These results clearly revealed that, rasasindur inhibited the proteolytic cleavage of BSA by trypsin.

4. Conclusion

Aided by a battery of experimental tools namely PXRD, HRTEM, SEM-EDS, XPS, Raman, UV−Vis-DRS, and PL-spectroscopy we conclude that:

(a) The Ayurvedic drug Rasasindur is nano-crystalline α-HgS with most of the particles in the range of 8−16 nm size.
(b) Rasasindur belongs to pure hexagonal lattice (cinnabar phase) and space group P3221.
(c) Rasasindur is a wide-band gap material having optical band-gap of 2.03 eV.
(d) Raman spectroscopy, SEM and XPS confirms the chemical purity of Rasasindur with Hg:S ratio as 1:1.

In addition to the above results, the following information from bio-chemical studies is noteworthy:

(a) Rasasindur interacts with Bovine Serum Albumin (BSA) with an association constant of $(9.76 \pm 0.56) \times 10^3$ M^{-1}.

(b) Rasasindur behaves as a protease inhibitor by inhibiting the proteolysis of BSA by trypsin.

(c) This Ayurvedic drug also shows mild anti-oxidant property.

In light of the results of the present study and those by others, the context of nanotransformation and its plausible implication on bio-efficacy in metal-based ayurvedic drugs warrants a relook.

Conflict of interest

None declared.

Acknowledgement

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