CHAPTER V

ASSESSMENT OF QUALITY AND SAFETY OF COMMERCIAL ANTI-SCORPION (*Mesobuthus tamulus)* ANTIVENOMS

5.1 Results

5.1.1 Physicochemical characterization of anti-scorpion antivenoms (ASAs)

Some of the parameters to be used for determining the physicochemical properties of commercial ASA are shown in Table 5.1 [1]. The lyophilized commercial ASAs (PSVPL and HBC) were white and homogenous (Fig. 5.1a,b). They were entirely dissolved in de-ionized sterile water (provided with the ASA package) within 6-7 min. They did not contain insoluble material because no precipitate material was observed post-centrifugation of the solution. The PSVPL and HBC ASAs turbidity was determined at 19.3±0.95 and 23.7±1.18 nephelometric turbidity units (NTUs). The pH of aqueous solutions of PSVPL and HBC ASA was slightly acidic, whereas the percent moisture contents of PSVPL and HBC ASA were below 3% (Table 5.2).

Table 5.1. Parameters that should be assessed in the routine quality control of commercial antivenoms, according to the WHO Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins, 2017) [2].

Parameters	**Testing methods**	**Requirements**
Appearance	Observation of colour, cloudiness, and appearance of the powder (in case of freeze-dried preparations)	Compliance with the description of the marketing dossier
Solubility (freeze-dried preparations)	Addition of solvent and observation of time to reach solubility and appearance	The product should be completely dissolved within 10 min. The solution should not be cloudy.
Extractable volume	Extraction of the total volume from the vial or ampoule	Compliance with the indications of the label
Venom neutralizing potency	Determination of the Median Effective Dose (ED_{50}), i.e., the ability to neutralize the lethal effect of the venom	The value was expressed as the mg venom neutralized per mL antivenom Compliance with the specifications of ED_{50} for the product or with the requirements of national regulatory agencies.

Osmolality	Determination of osmolality by accepted methods	At least 240 mOsm/kg
Identity test	Biological, physicochemical, or immunological tests	The identity of antivenom confirmed
Protein concentration	Kjeldahl, colourimetric or spectrophotometric procedures	Not more than 10 g/dl
Purity and integrity of immunoglobulin	SDS-PAGE under reducing and non-reducing conditions	IgG or IgG fragments should comprise more than 90% of the proteins
Molecular size distribution	Size exclusion chromatography (FPLC or HPLC)	The product should consist mainly of monomeric IgG or IgG fragments.
Test for pyrogen substances	▪ Rabbit pyrogen test ▪ Limulus Amebocyte Lysate (LAL) test when validated and approved by the national regulatory agency	Accepted limits by the Pharmacopeia in use
Abnormal toxicity test	Intraperitoneal injection and observation of signs of toxicity (not required by some regulatory agencies)	No evidence of toxicity
Sterility test	Filtration of commercial ASAs through membranes, neutralization (when preservatives are used), and additionto culture media (trypticase soy broth and thioglycolate).	Absence of microbial growth
The concentration of sodium chloride and other excipients	Standard chemical methods	Specifications of Pharmacopeias and regulatory agencies.
Determination of pH	By using a potentiometer (pH meter)	Specifications of Pharmacopeias and regulatory agencies (generally neutral

		pH).
Concentration of preservatives	Spectrophotometric or HPLC-based methods	Phenol: maximum 2.5 g/l Cresol: maximum 3.5 g/l
Chemical agents used in plasma fractionation	Several methods depending on the chemical agent to be quantified	Specifications of Pharmacopeias, national regulatory agencies, or manufacturers.
Residual moisture (freeze-dried preparations)	Karl-Fischer titration, gravimetric or thermogravimetric methods	Less than 3%

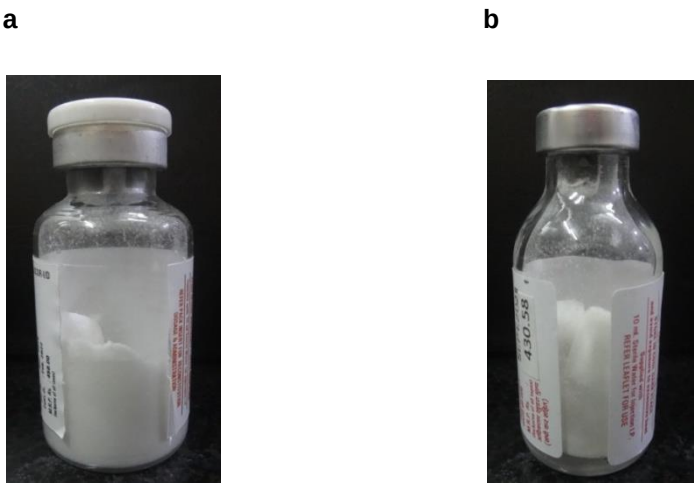

Fig. 5.1. Photograph of lyophilized ASA manufactured by **(a)** PSVPL and **(b)** HBC (Camera: Sony Cybershot)

Table 5.2. A summary of the physicochemical properties of commercial ASAs against *M. tamulus* venom (MTV) manufactured in India.

Parameter unit	**Testing method**	**Results**		**Regulatory requirement (WHO, 2016)**
		PSVPL ASA	**HBC ASA**	
Appearance	Visual observation of the colour and physical appearance of the powder (in case of freeze-dried preparations)	White and homogenous appearance	White and homogenous appearance	Compliance with the description of the marketing dossier
Residual moisture (freeze-dried-preparations) (%)	Heat drying method	<3	<3	Less than 3%
Solubility freeze-dried-preparations)(min)	Addition of solvent and observation of time to reach solubility and of appearance	~6	~7	The product should be completely dissolved within 10 min
Turbidity (NTU)	By using a turbidimeter	19.3±0.95	23.7±1.18	The solution should not be cloudy, but the threshold level is not mentioned in the guidelines
pH of solution	By using a potentiometer (pH meter)	6.22± 0.31	6.35±0.32	Specifications of Pharmacopeia and regulatory agencies (generally neutral pH)
Total protein concentration (g/dL)	Lowry method	81.68	85.94	Not more than 10 g/dL
Serum albumin	LC-MS/MS	1.81	7.66	Ideally should be

content (%)				within 1%
The concentration of preservative (g/L)	RP-HPLC-based method (cresol)	0.34	0.23	Phenol: maximum 2.5 g/l Cresol: maximum 3.5 g/l
Complement activation (CH_{50}/ mL and AP_{50}/mL)	Biochemical assay to determine CH_{50} and AP_{50} values for classical and alternative pathways, respectively	CH_{50}/mL (%)-54.79% AP_{50}/mL (%)-62.18	CH_{50}/mL (%)-51.19% AP_{50}/mL (%)-59.67	The threshold level is not mentioned
Fc content of IgG (%)	Immunological assay (ELISA and immunoblotting) by using anti-Fc specifc antibody	6.52	9.84	The threshold level is not mentioned

5.1.2 Electron microscopic analysis of ASAs

The FESEM analysis showed the existence of smooth plates and channel-like structures in ASAs, which act as the primary way of vapour transport through the product [3; 4] (Fig. 5.2.a-d). On the other hand, TEM analysis characterized the morphological and biophysical properties of the particles present in ASAs. The images show the presence of Y-shaped individual immunoglobulin (white arrow). In contrast, the black arrows indicate the presence of other non-IgG proteins/oligomers (Fig. 5.2.e-h), which appeared as a dark spot or cluster-like structure.

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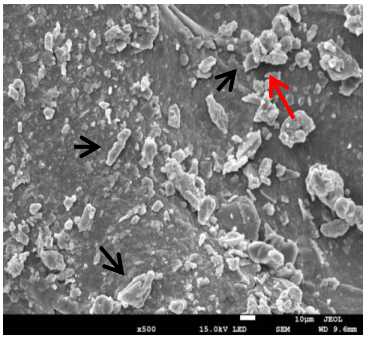

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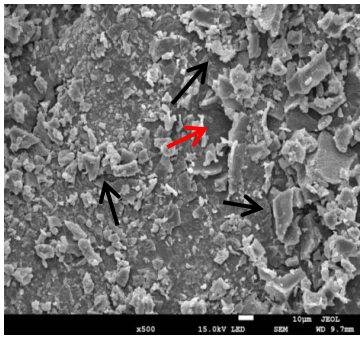

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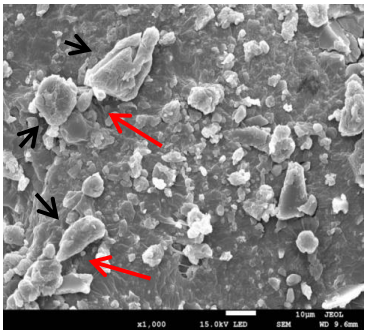

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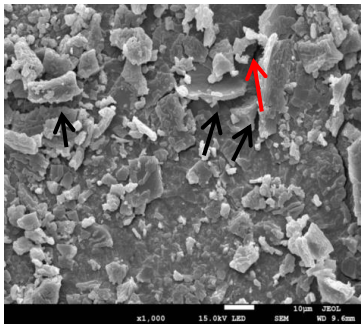

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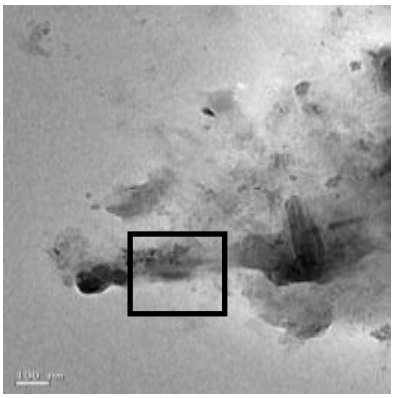

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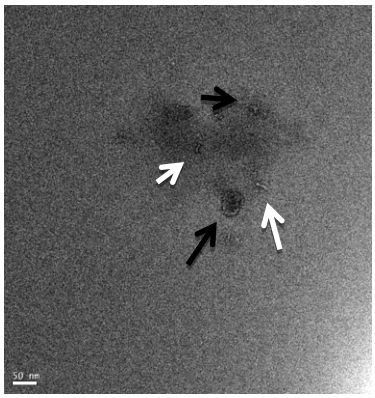


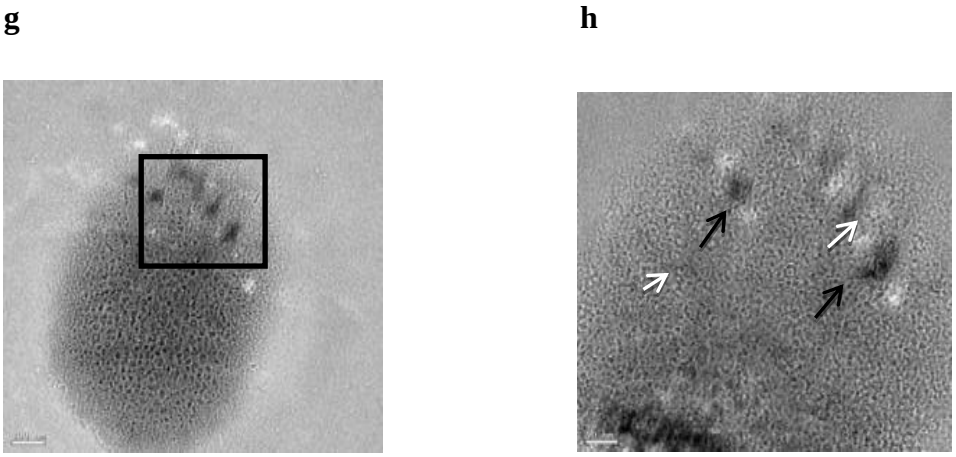


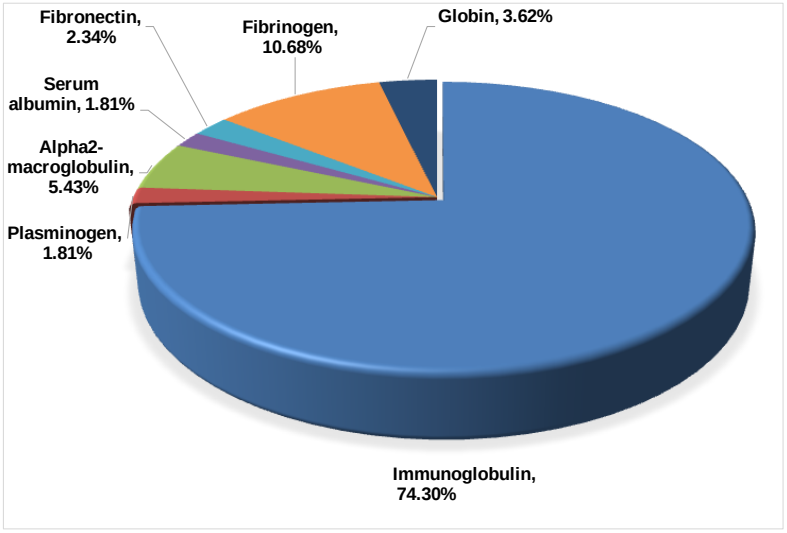
Fig. 5.2. Field emission scanning electron microscope (FESEM) image analysis of PSVPL and HBC ASA shows the smooth plates and channel-like structures. FESEM images of PSVPL ASA with **(a).** 500X, and **(b).** 1000X magnification. FESEM images of HBC ASA with **(c).** 500X, and **(d).** 1000X magnification. The black arrow indicates the smooth plates, and the red arrow indicates the channel-like structure in commercial ASA. Transmission electron microscopy (TEM) images of PSVPL and HBC ASA showing Immunoglobins/dimers. TEM images of PSVPL ASA at **(e).** 100 nm, and **(f).** 50nm view. TEM images of HBC ASA at **(g).** 100 nm, and **(h).** 50 nm microscopic view. The white arrow shows the individual immunoglobulin molecules, and the black indicatorshows other non-IgG proteins/oligomers. The black box in a1 and a2 indicate the selected field for high magnification. The images were captured with an FEI TECNAI G2 camera with magnifications of 9900X, 17000X, 38000X, and 71000X at 200kV.

5.1.3 Determination of the purity of active substance in commercial ASAs by mass spectrometry, SDS-PAGE, and size exclusion chromatography (SEC)

The dry weight of PSVPL and HBC ASA in each vial was determined at 750 ± 22.5 mg and 1510 ± 30.3 mg. This study determined 81.68% and 85.94% protein content in each vial of PSVPL and HBC ASAs, respectively. The LC/MS-MS analyses showed that approximately 74.30% of PSVPL and 67.16% of HBC ASA were immunoglobulins, whereas the total percent composition of other contaminating serum proteins was 25.69% and 32.83%, respectively. The latter group of proteins were identified as plasminogen, fibrinogen, fibronectin, alpha2- macroglobulin, serum albumin, globin, and haptoglobin

(Fig. 5.3.a,b, Fig. 5.4.a,b, Appendix table A2). Among them, the fibrinogen content of PSVPL and HBC ASAs was determined at 10.68% and 12.84%, respectively, of the total protein content. In contrast, the albumin content in PSVPL and HBC ASA was determined at 1.81% and 7.66%, respectively.

a



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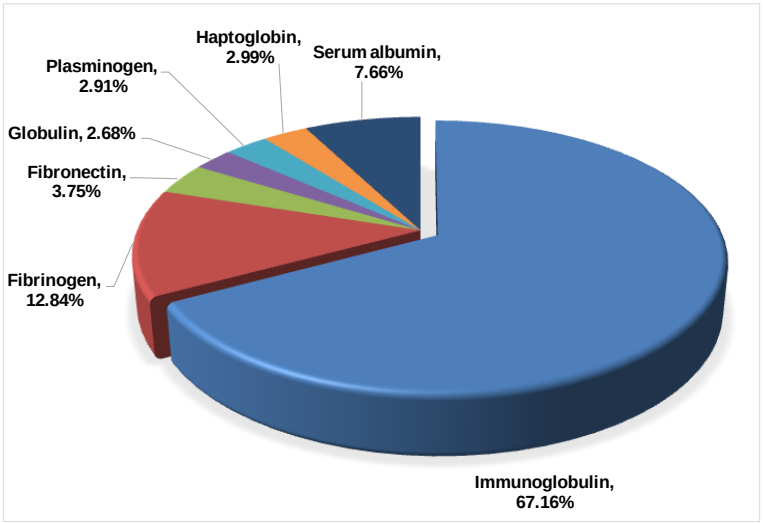

Fig. 5.3. Composition of PSVPL and HBC ASA determined by LC-MS/MS analysis. **(a).** %Relative abundance of different protein families in PSVPL ASA. (**b).** %Relative abundance of diverse protein families in HBC ASA.

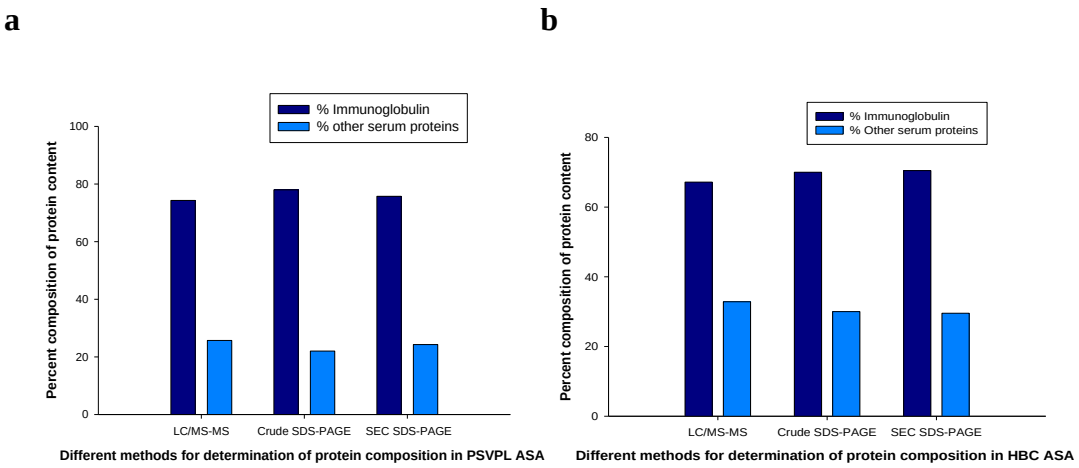

Fig. 5.4. Comparison of % immunoglobulin content and other contaminating proteins determined by different biophysical techniques. **a.**% Protein composition of PSVPL ASA determined by different methods. **b.** % Protein composition of HBC ASA determined by different methods.

The size exclusion chromatograms of the commercial ASAs (PSVPL and HBC) were resolved into one prominent peak (P1) and two minor peaks (P2 and P3) (Fig. 5.5.a). The major peak (P1) of the PSVPL SEC chromatogram was eluted in 13 fractions (26 mL elution volume). In contrast, the HBC SEC chromatogram's prominent peak (P1) was eluted in 12 fractions (24 mL elution volume). The percent protein content of the major SEC peak (P1) of PSVPL and HBC ASA was determined as 75.10% and 73.59%, respectively, whereas the minor peak (P2) comprised of 24.90% and 26.41% protein content, respectively (Fig. 5.5.a). The reference chromatograms of horse IgG / purified $F(ab')_2$ (Fig. 5.5.b) showed that each major peak (P1) of both the commercial ASAs contained mostly $F(ab')_2$ and a minor quantity of undigested IgG. Nevertheless, SEC showed that commercial ASAs lacked aggregate content (Fig. 5.5.a).

The SEC peaks of PSVPL and HBC ASA and the crude ASA, when subjected to 12.5% SDS-PAGE analysis, showed an aggregated/ smeared band at a 100 to 150 kDa molecular mass range under non-reduced conditions. But a faint band of ~35 kDa was also observed under non-reduced conditions, which may correspond to residual pepsin. Moreover, a distinct protein band of ~50 kDa under non-reduced conditions represented the heavy chain of the undigested IgG molecule (Fig. 5.5.c,e). However, under the reduced conditions, the ASAs

showed a distinct protein band at ~25 kDa and ~30 kDa as light and heavy chains of pepsin-digested IgG (Fig. 5.5.c,d).

The densitometry analysis of SDS-PAGE protein bands (SEC prominentpeak) revealed the presence of 75.73% and 70.45% immunoglobulin content (both IgG and $F(ab')_2$) in PSVPL and HBC ASA, respectively. In contrast, they showed 24.27% and 29.55% of other serum proteins, respectively (Fig. 5.5.f, g).

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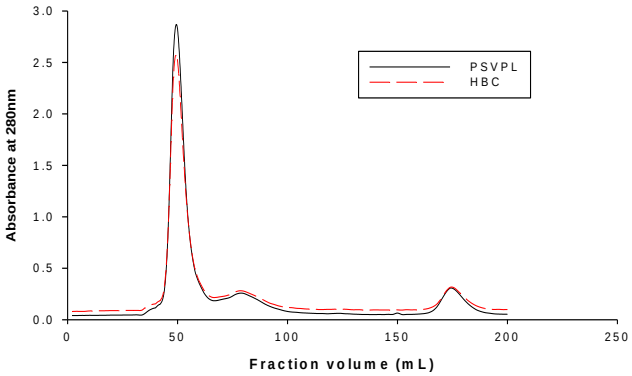

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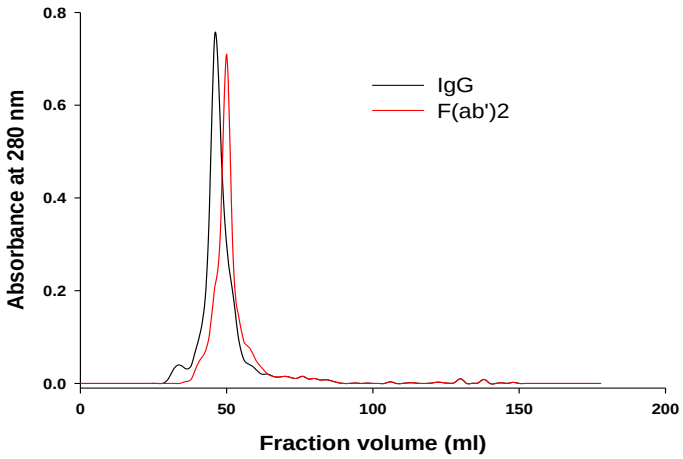

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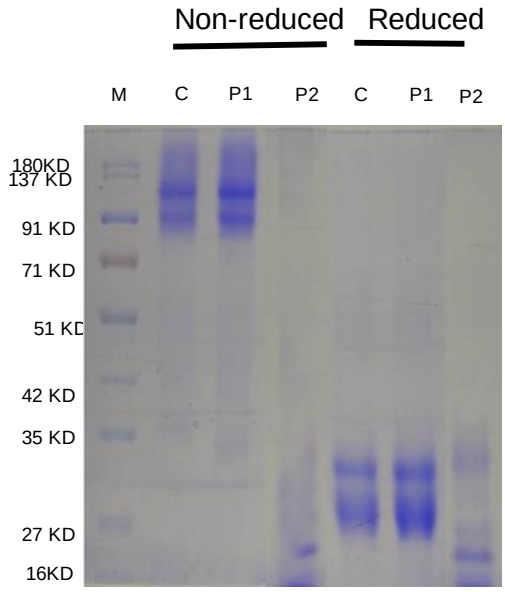
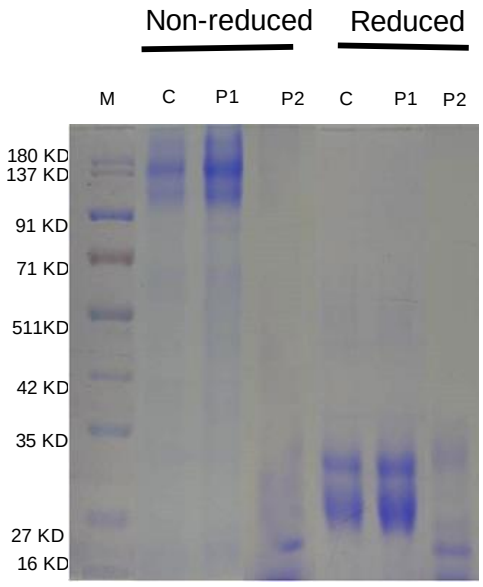


PSVPL ASA



HBC ASA

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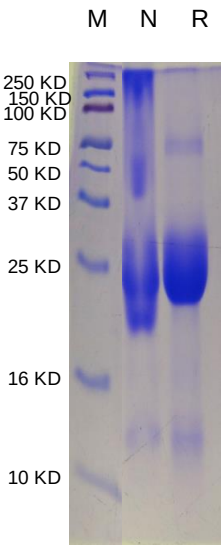

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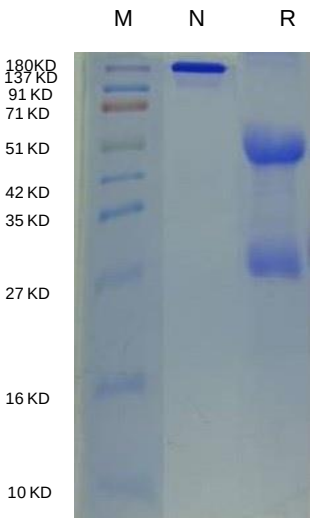


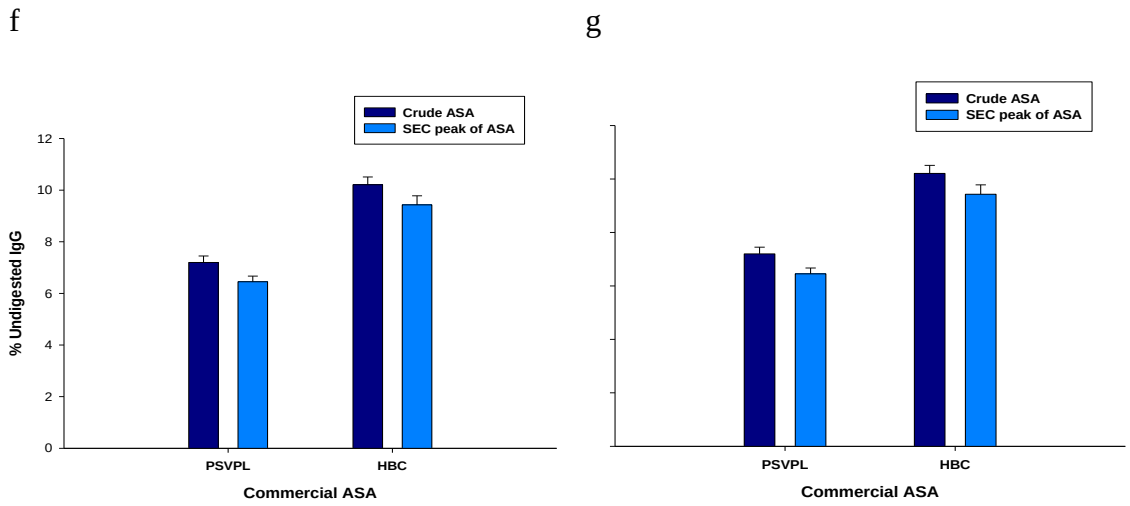


Fig. 5.5. Size exclusion chromatography of anti-scorpion antivenom (ASA) on an FPLC Sephacryl S-200 size-exclusion column. **(a).** Fractionation of PSVPL and HBC ASA (60mg dry weight). **(b).** Fractionation of purified horse IgG and $F(ab')_2$ (5mg dry weight) in identical experimental conditions. **(c).** 12.5% SDS-PAGE analysis of size exclusion chromatography (SEC) and crude PSVPL and HBC ASAs under reduced and non-reduced conditions. Lane M contains a protein molecular marker. Lane C contains crude PSVPL and HBC ASA (20µg). P1 and P2 contain SEC peak1 and peak2 PSVPL and HBC ASA (20 µg), respectively. **(d-e).** SDS-PAGE analysis of purified $F(ab')_2$ and IgG (20µg) under non-reduced (lane N) and reduced (lane R) conditions. **(f).** Percent of undigested IgG / IgG aggregates present in PSVPL and HBC ASA. **(g).** % Protein content present in size exclusion chromatogram (SEC) PSVPL and HBC ASA peaks. Values are mean ± SD of triplicate determinations. The ASAs did not show a significant difference ($p > 0.05$) in their presence of undigested IgG content.

5.1.4 Particle distribution (aggregate formation) in scorpion ASA determined by DLS analysis

Immunoglobulin aggregate in commercial ASAs, if any, was investigated by DLS analysis. In our study, IgG aggregate that was generated by 48h of edge-to-edge agitation (which was confirmed by SDS-PAGE analysis) was used as a positive control for aggregation determination in ASA preparations (Fig. 5.6). The DLS analysis determined the hydrodynamic size of IgG aggregate (positive control) at 5755nm. In contrast, the

hydrodynamic diameter of PSVPL and HBC ASAs was determined at 803.6 nm and 335.4 nm, respectively, which was far less than the positive control (IgG aggregate) (Fig. 5.7. a,b,c).

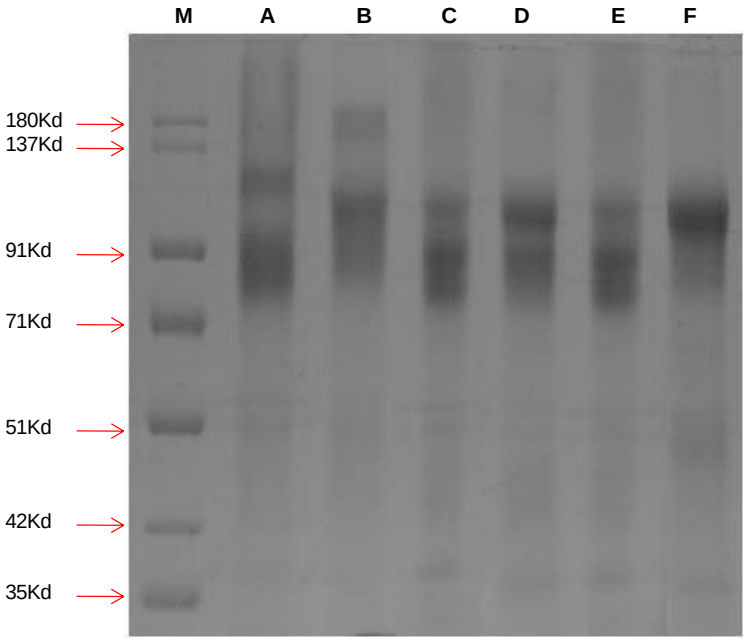


Fig. 5.6. 7.5% SDS-PAGE analysis of ASA with and without agitation for 48 h. Lane M contains protein molecular markers, lanes A and B contain IgG (20 µg) without agitation and with agitation, respectively. Lanes C and D contain PSVPL ASA (20 µg) without agitation and with agitation, respectively. Lanes E and F contain HBC ASA (20 µg) without agitation and with agitation, respectively.

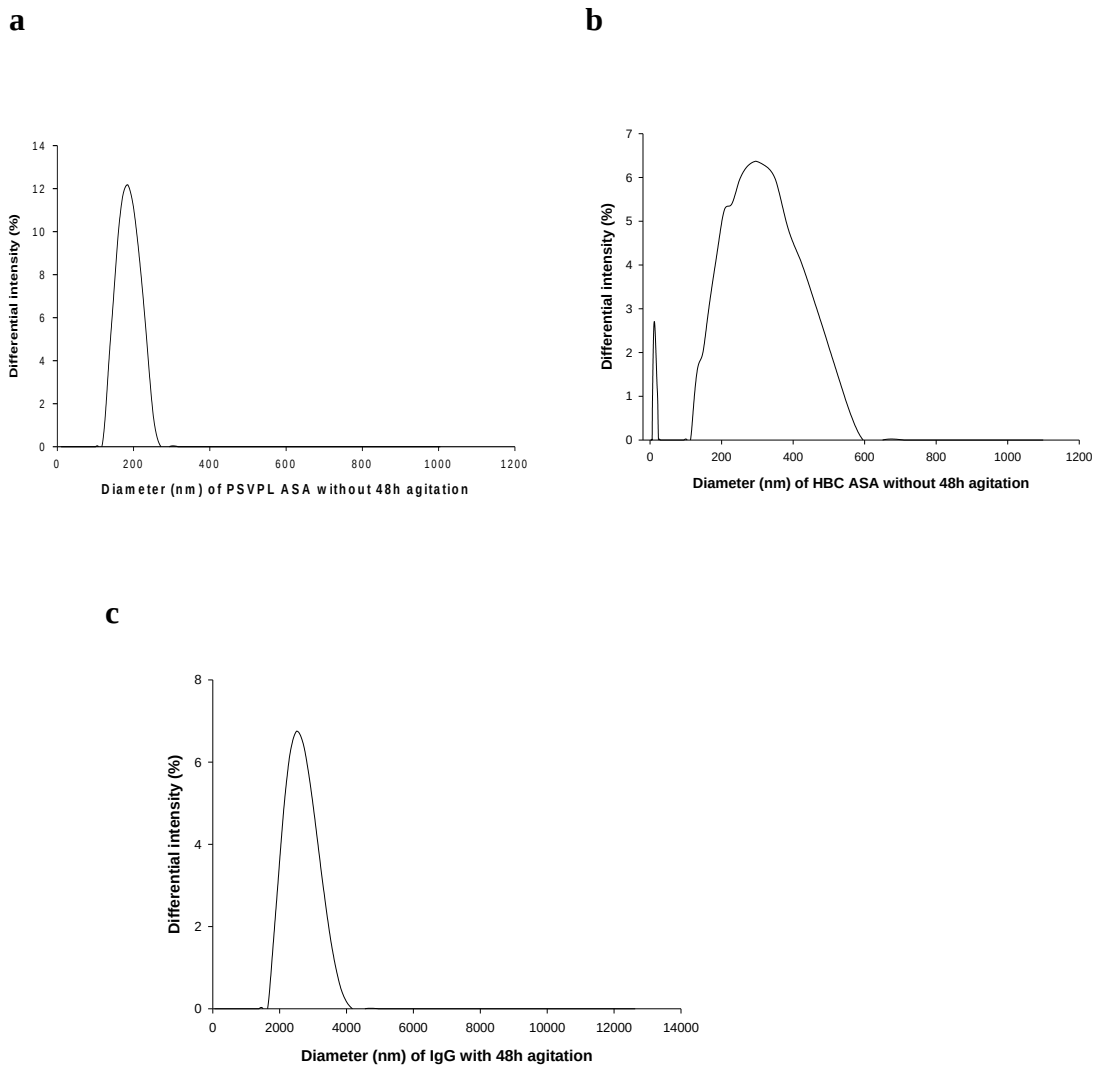


Fig. 5.7. Dynamic light scattering (DLS) analysis anti-scorpion antivenom (ASA) and native IgG. **a.** DLS of PSVPL ASA (1mg/mL). **b.** DLS of HBC ASA (1mg/mL). **c.** DLS of native IgG (1mg/mL).

5.1.5 Commercial ASAs showed a trace amount of Fc content and moderate complement activation property

From the results of ELISA and immunoblot analysis (non-reduced condition), the Fc content in both the commercial ASAs was found between 6-10%, whereas, under the reduced states of immunoblot analysis, a very faint band of the heavy chain was observed, Fc content of which was found as 3-5% for the incompletely digested heavy chain of IgG in both the ASAs (Fig. 5.8, Fig. 5.9. a1,b1,a2,b2, Table 5.2). Some molecules of IgG in the solution were

digested, and some were not digested, thus generating an antivenom with a mixture of IgG and $F(ab')_2$. For classical and alternative pathways of complement activation, $\%CH_{50}$ and $\%AP_{50}$ value showed at 54.79% and 62.18%, respectively, for PSVPL ASA, whereas the same parameters for HBC ASA were determined at 51.19% and 59.67%, respectively. The commercial ASAs showed moderate complement activation by classical and alternative pathways, but a significant difference in this property between these two ASAs was not observed (Fig. 5.10. a,b)

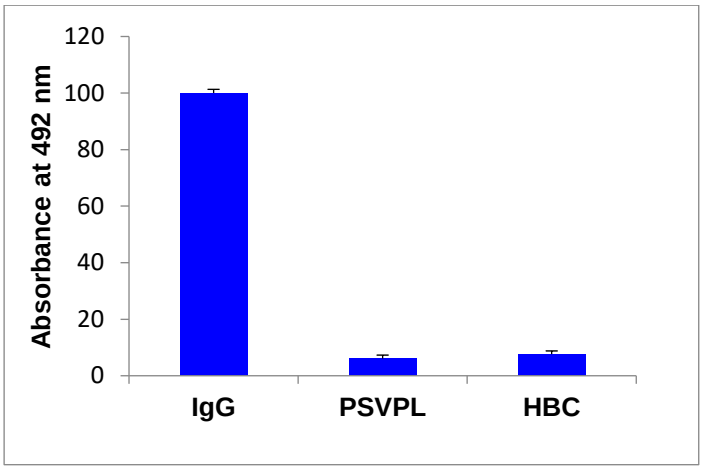


Fig. 5.8. ELISA to determine thepercent Fc content in commercial ASAs. The percent content of Fc in the purified horse IgG was considered 100%, and other values were measured relative to that. Values are mean ± SD of triplicate determinations.

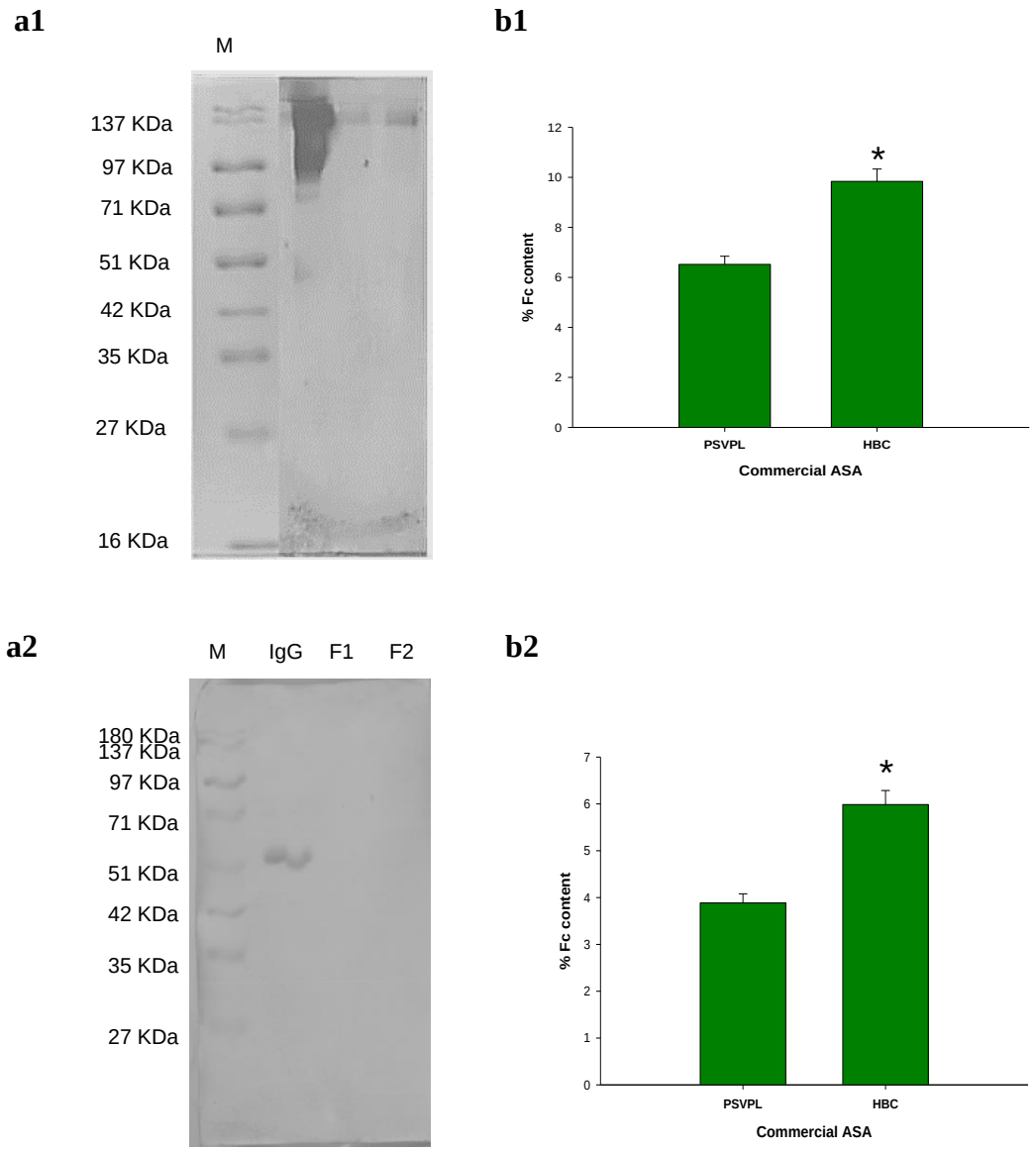


Fig. 5.9. (a1, a2). Immuno-blot analysis to determine the Fc content in anti-scorpion antivenom (ASA) IgG in non-reduced and reduced conditions, respectively. Lane M contains a protein molecular marker. Lanes IgG, F1 and F2 represent immunoblot of IgG (positive control), PSVPL and HBC ASA, respectively. Immunoblot detected by HRP conjugated Fc-specific antibody. **(b1, b2).** Densitometry analysis of SDS-PAGE images in non-reduced and reduced conditions, respectively, to quantify IgG's per cent Fc content in PSVPL and HBC ASAs. Values are mean ± SD with triplicate determinations. The ASAs did not significantly differ ($p > 0.05$) in their Fc content.

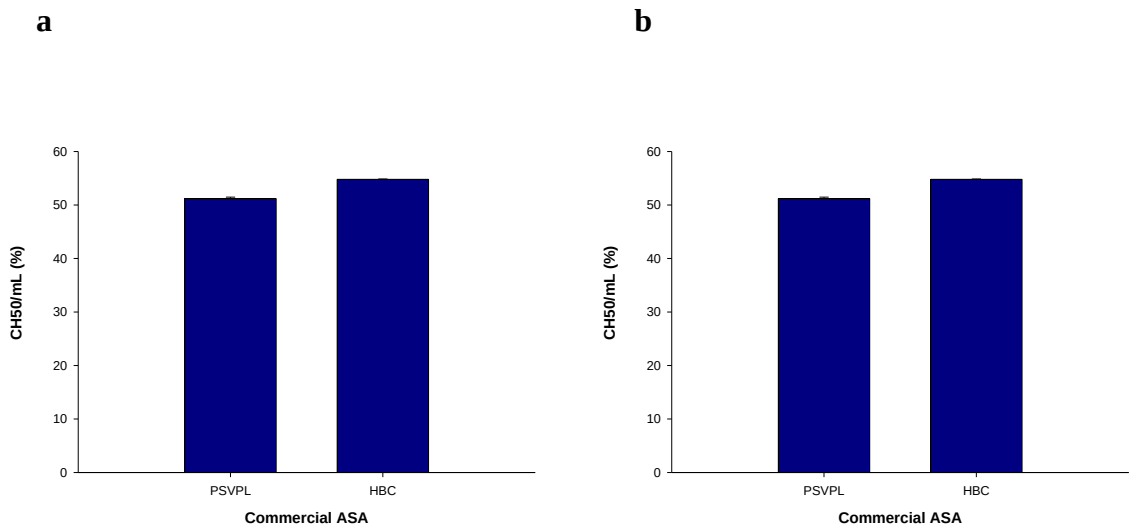


Fig. 5.10. Determination of the complement activation pathways ASAs. **a.** Classical pathway of PSVPL and HBC ASA (%CH_{50}/mL). **b.** Alternative pathway of PSVPL and HBC ASA (%AP_{50}/mL). The experimental details are described in the text. Values are mean ± SD for triplicate determinations. The ASAs did not show a significant difference, $p>0.05$.

5.1.6 The ASAs contained IgA, devoid of IgE, and contained endotoxin and preservative contents within the threshold limit

The commercial ASAs (PSVPL and HBC) were not contaminated with IgE antibodies, confirmed by ELISA and immunoblot analysis (Fig. 5.11.a,b). However, ELISA and immunoblot studies revealed the presence of IgA in both the ASA (Fig. 5.12. a,b,c).

The endotoxin level in PSVPL and HBC ASA (0.52 and 0.96 EU/mL, respectively) was found below the threshold limit indicating their safety to administer to scorpion sting victims. Moreover, the percent cresol content determined in PSVPL and HBC ASA samples was 0.023g/L and 0.034g/L, respectively, within the recommended limit (3.5g/L) of WHO (Fig. 5.13, Table 5.2).

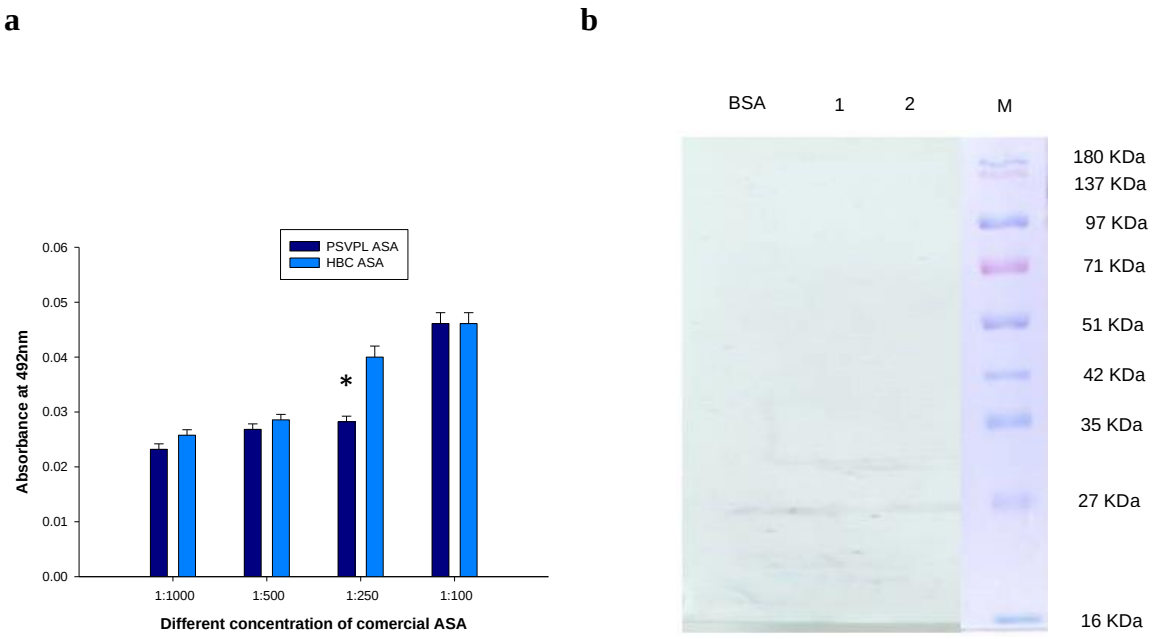
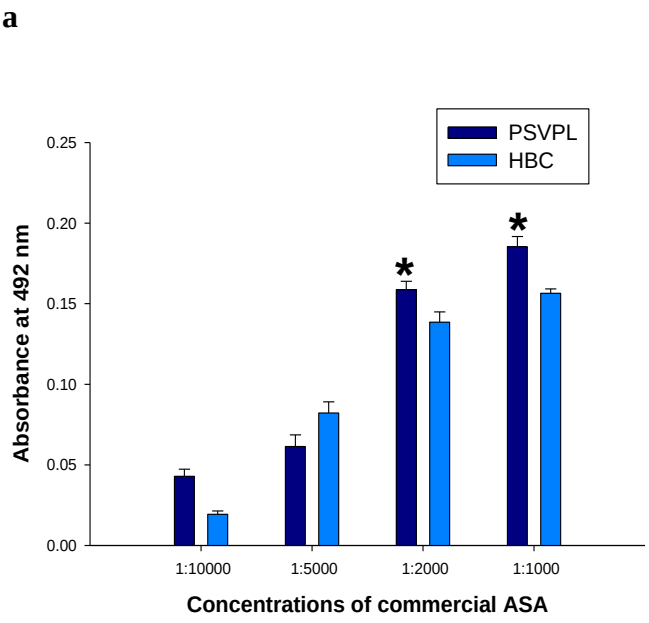


Fig. 5.11. a. Immunological cross-reactivity of PSVPL and HBC ASAs against anti-horse IgE antibodies (HRP-conjugated) determined by ELISA. Values are mean ± SD of triplicate determinations. **b.** Immuno-blot analysis of IgE contamination in ASA. Lanes BSA 1 and 2 represent immuno-blot of BSA (negative control), PSVPL and HBC ASA, respectively. The proteins were immuno-detected by HRP-conjugated anti-horse IgE antibodies. Lane M contains protein markers. Values are mean ± SD for triplicate determinations. The ASAs did not show IgE content.



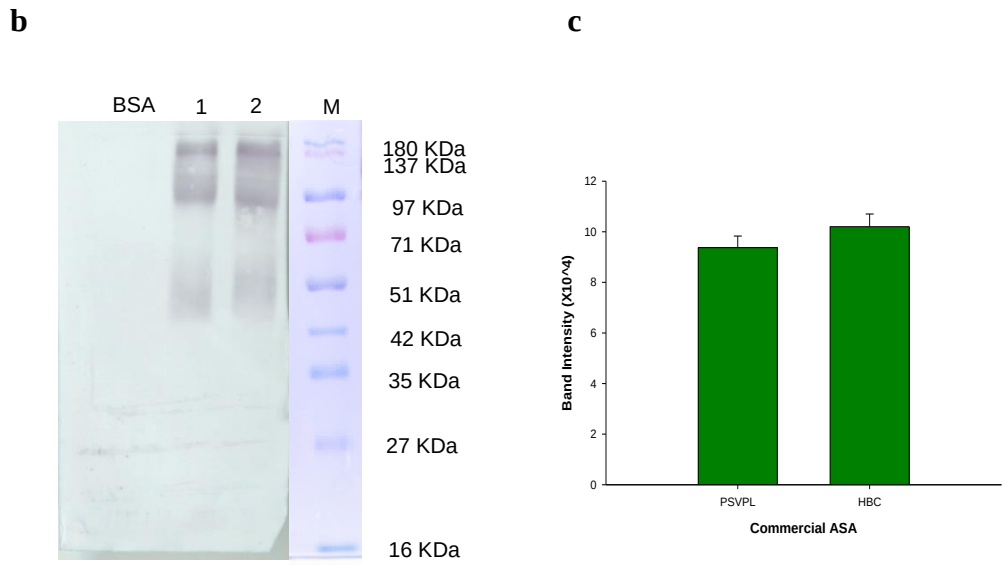


Fig. 5.12. (a) ELISA to determine the immunological cross-reactivity between anti-scorpion antivenom (ASA) and anti-horse IgA antibodies (HRP-conjugated). Values are mean ± SD with triplicate determinations. Significance of difference with respect to HBC ASA, *p<0.05. **(b)** Immuno-blot analysis to determine the IgA contamination /co-precipitationin ASAs. Lanes BSA, A, and B represent immuno-blot of BSA (negative control), PSVPL, and HBC ASA, respectively. The immuno-blots were detected by HRP-conjugated anti-horse IgA antibody. **(c)** Densitometry analysis to quantitate the IgA content in PSVPL and HBC ASAs. Values are mean ± SD of triplicate determinations. There was no significant difference between the values, p>0.05.

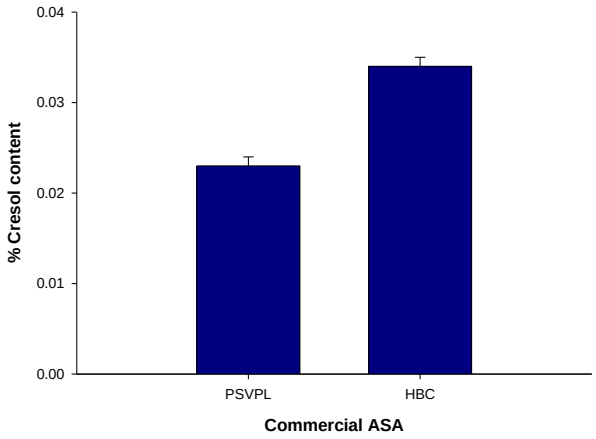
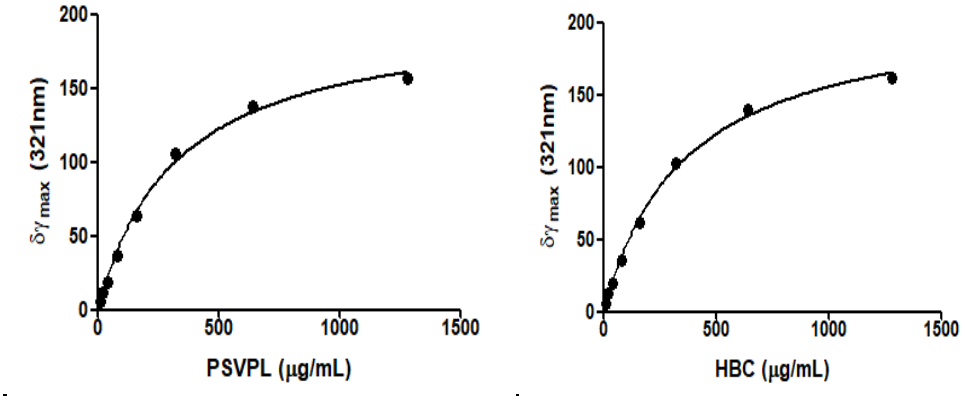


Fig. 5.13. Determination of % cresol content present in PSVPL and HBC ASAs by RP-UHPLC analysis. One mg of the dry weight of ASA (PSVPL and HBC) on an Acclaim300 C18 RP-UHPLC column (2.1× 150mm,3.0 µm) was separated for cresol determination. Values are mean ± SD for triplicate determinations. The ASAs did not show significant differences in cresol content, $p>0.05$.

5.1.7. Spectrofluorometric titration and AFM analyses showed weak binding affinity of scorpion venom with commercial ASAs, and they contained less proportion of venom toxins-specific antibodies

By spectrofluorometric titration, the kd value determined for PSVPL and HBC ASA was 321.5 ± 16 µg/mL and 358.7 ± 18 µg/mL, respectively (Fig. 5.14.a,b), indicating these ASAs did not show a significant difference ($p>0.05$) in their binding affinity towards *M. tamulus* venom (MTV). Further, from the spectrofluorometric titration curve, the venom-specific antibodies in each vial of PSVPL and HBC ASA were determined at 6.29% and 5.36%, respectively (Table 5.3). By AFM analysis, the kd value for PSVPL and HBC was determined at 984±0.52 µg/mL and 1257±0.69 µg/mL, respectively (Fig. 5.14.c, d, and Fig.5.15.a,b).

a **b**



c **d**

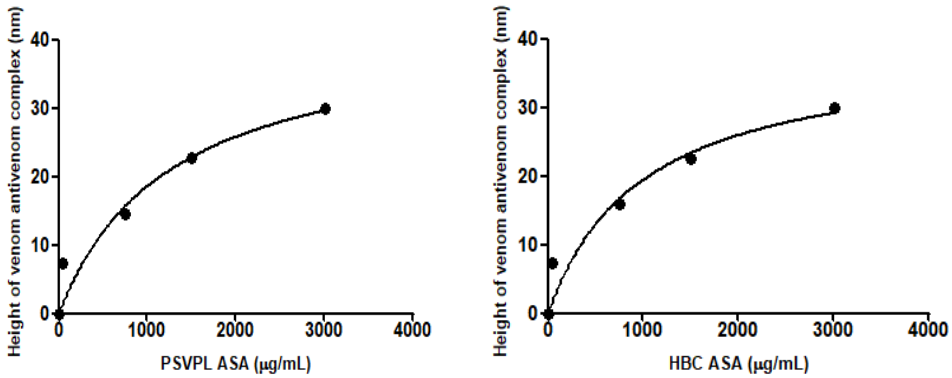
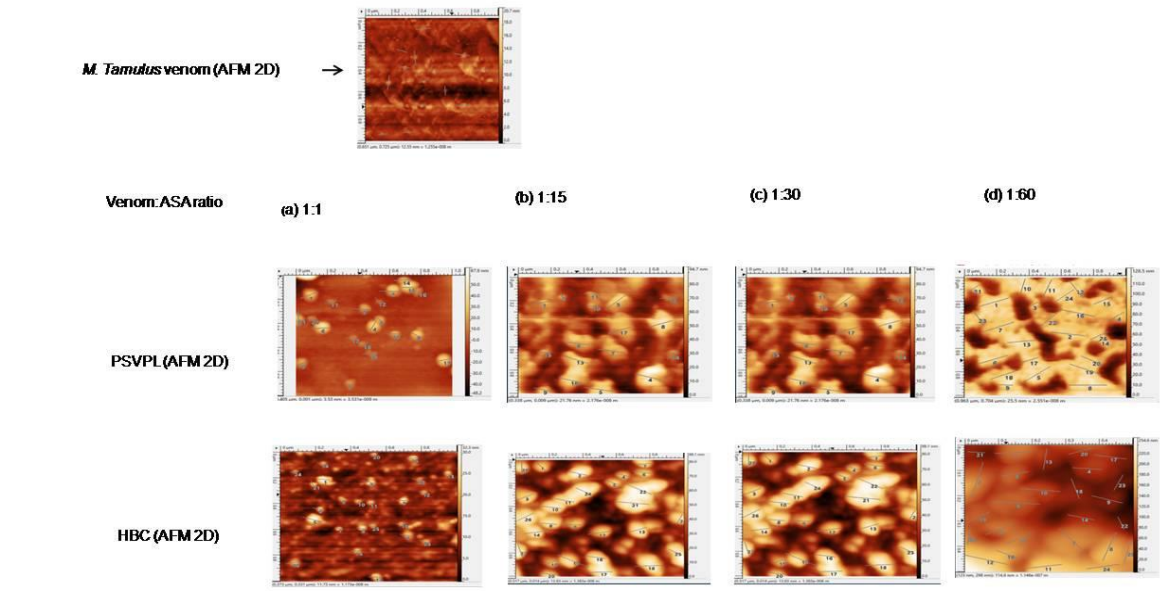


Fig. 5.14. One-site specific binding curves to determine the kd value of interaction between MTV and commercial ASA. **(a-b).** Spectrofluorometric study to determine the interaction between MTV (10µg/mL) and different concentrations of PSVPL / HBC ASA (10 µg/mL to 1280 µg/mL) **(c-d).** Atomic force microscope analysis to determine the interaction between MTV (50 µg/mL) and different concentrations of PSVPL / HBC ASA (50 µg/mLto 3000 µg/mL). Values are the mean of triplicate determinations. The graphs were plotted using GraphPad Prism 5.0 software.

Table 5.3. Determination of MTV toxins-specific antibodies in commercial ASAs by spectrofluorometric interaction study. Values are mean ±SD of triplicate determinations. PSVPL: Premium Serum and Vaccines Pvt. Ltd, HBC: Haffikine Biopharmaceuticals.

Antivenom manufacturing company	**Saturated antivenom concentration (µg/mL)**	**Protein content (%)**	**Total protein content (mg)**	**Immunoglobulin content determined by LC/MS-MS analysis(%)**	**Total immunoglobulin in vial (mg)**	**Wholevenom toxins-specific antibodies in a vial (mg)**	**Venom-specific antibody in the vial (%)**
PSVPL	643.0±26.52	81.68	612.60±27.56	74.30	455.16±21.23	47.17±2.35	6.29
HBC	717.4±30.02	85.94	1297.69±35.12	67.16	871.53±25.36	81.00±4.05	5.36

a



b

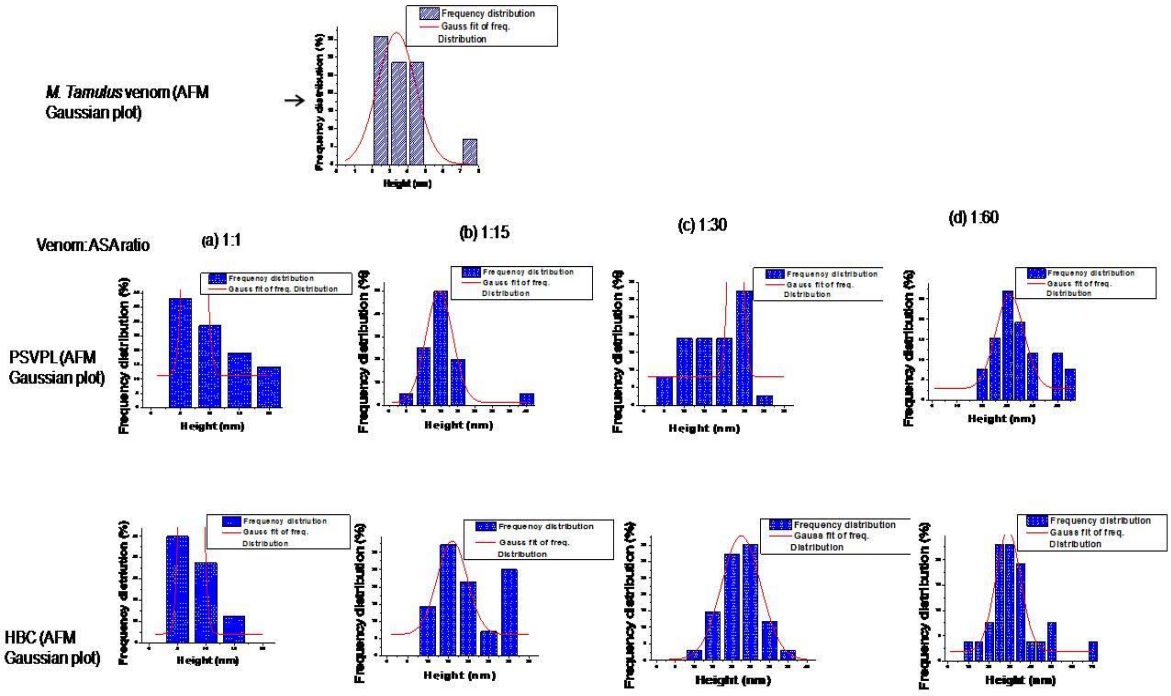


Fig. 5.15. Atomic force microscope (AFM) analysis of venom-antivenom interaction by two-dimensional (2D) images and Gaussian plot. The venom and antivenom were incubated at different ratios from 1: 1 to 1:60. **(a)** PSVPL ASA, **(b)** HBC ASA.

5.2 Discussion

Laboratory-based quality assessment of commercial antivenom is essential for efficient treatment against envenomation/sting and minimizes adverse reactions [5]. Freeze-drying is necessary for stabilizing the antivenom during transportation and storage (preferably without refrigeration in rural health centres). Following the WHO guidelines, the freeze-dried ASAs were dissolved in water within the recommended time limit (10 min), and no dispersed material was present. However, mechanical damage perhaps occurred in HBC ASA during product transportation [3]. The fragmented cake-like structures may induce poor rehydration and low permeability properties that could affect the reconstitution time, turbidity, and particle formation [6]; however, these structures do not affect the biological activity (venom neutralization potency) of lyophilized products [7]. Accordingly, it has been suggested that a quantitative measurement of the turbidity of antivenoms solution be determined if any dispersed material is present; however, no standard turbidity value is fixed [2].

A neutral pH (7.0 ± 0.5) is suitable for antivenom formulation because the higher pH (> 7.5) decreases the stability of IgG or $F(ab')_2$ and thus causes the formation of aggregates [2]. Therefore, to improve stability and prevent protein aggregation during the freeze-drying process, commercial ASAs in this study were formulated at acidic pH. The moisture content may affect the stability of the lyophilized ASA by denaturation of the $(Fab')_2$ [2] and is one of the factors for causing early adverse reactions noted post antivenom treatment [8]. The recommended limit for freeze-dried immunoglobulin's residual moisture content is 3%, and our tested ASAs contain permissible moisture content. Concisely, the commercial ASAs under study demonstrated good solubility, shallow moisture content, and slightly acidic to neutral pH in a solution, indicating they were prepared according to the WHO guidelines.

An electron microscopic study can obtain a high-resolution image of size, shape, and aggregate formation in a sample (protein and non-protein), characterizing the porosity and local composition. This instrument has a wide application for particle counting and size determination and is often used for industrial quality control and failure analysis [9; 10; 5]. In both TEM and FESEM, electrons are used as an excitation source for image formation [11; 12]. TEM provides direct high-resolution images and detailed qualitative/quantitative information to characterize components of commercial ASAs, as it allows the electrons to

penetrate through the samples [13]. On the other hand, FESEM is an advanced technology used to characterize surface morphology and microstructure in commercial ASAs [11]. The FESEM images of commercial ASAs showed some smooth plates and channel-like structures that define superior drying of antivenom [3] and low humidity [14]. Further, the idealdrying of the antivenom is also well corroborated by its low moisture content (<3%).

The TEM at a higher resolution (38000X, 71000X) is also used to visualize the shape and structure of the particles, for example, the Y shaped structure of IgG molecule. Besides, using TEM, the presence of viruses in serum products [15]. However, the TEM study did not show the presence of horse virus particles in commercial ASAs, indicating they are safe to administer. This observation may be plausible because, during pepsin digestion at a low pH, the virus particles are degraded and lose their structural integrity [16].

Pharmacokinetics study in drug development is a vital prerequisite for characterizing the drug's therapeutics and mode of action [17]. Although $F(ab')_2$ and IgG differ in their pharmacokinetics properties, they did not show a difference in potency and efficacy in snake venom neutralization [18; 2]. The $F(ab')_2$ reduces the possibility of immune-complex formation due to lacking Fc fragments, thus showing fewer common adverse serum reactions. They are distributed easily in the extravascular tissue compartment [19]. Nevertheless, the whole IgG purified from horse serum is responsible for late antivenom reactions such as serum sickness and precludes complement activation, and they have a longer shelf life. Thus the former can serve as a preferred molecule during envenomation therapy, according to WHO (2019) [2]. The size exclusion chromatography of both the commercial ASAs (PSVPL and HBC) revealed that their prominent peak (P1) contains mainly $F(ab')_2$ and some amount of partially/undigested IgG. This data correlates well with SDS-PAGE and LC-MS/MS analyses of commercial ASAs. Besides, the peak aggregate content for both commercial ASAs was not observed in the SEC chromatogram, which was also supported by SDS-PAGE and DLS analyses as no such aggregate content was observed. However, recent studies have reported aggregate formation in Indian and Sri Lankan polyvalent antivenoms [20; 1].

From LC-MS/MS analysis, the albumin content in ASAs was determined to be far higher than the recommended range of this protein (1%) [2], which induces adverse serum sickness [2]. It thus may compromise the safety of the product [2]. Aggregate formation in therapeutic antibodies causes adverse serum reactions in the treated patients [2], and it may

occur during the product's production, storage, and shipping. The DLS study revealed that commercial ASAs (PSVPL and HBC) are devoid of protein aggregate, and the size exclusion chromatography and SDS-PAGE analyses of ASAs also supported this data. Further, the LC-MS/MS analysis indicated that the commercial ASAs (PSVPL and HBC) were contaminated with non-IgG proteins (25.69 to 32.83%).

Improper pepsin digestion of IgG will reduce the $F(ab')_2$ content and increase the IgG-containing Fc portion responsible for complement activation and early adverse reactions [21; 22; 23]. The Fc content of the commercial ASAs was found to be marginal, and this result is well correlated with the densitometry analysis of SDS-PAGE bands of crude PSVPL and HBC ASAs, which too suggested a trace amount of undigested IgG in these ASAs.

Antivenom, during its production, may be contaminated with endotoxin-producing Gram-negative bacteria; therefore, the use of preservatives, such as cresol and phenol, is recommended to prevent bacterial contamination [2]. Depending upon the volume administered to the patients, the level of endotoxin concentration in ASA should not exceed 17.5 EU/mL for approximately 20–120 mL of antivenom solution to prevent pyrogenic reactions in patients [24], and the safe amount of cresol should be below 3.5g/L [25; 2] otherwise it may lead to liver and kidney damage or even multi-organ failure [26]. The PSVPL and HBC-manufactured ASAs are formulated with m-cresol, so we determined the cresol content in the tested ASAs. The endotoxin level and preservative content in PSVPL and HBC ASA were confined within the threshold limit of WHO, and they are safe to administer to scorpion sting victims. Further, IgE in ASA causes hypersensitivity reactions whereas no adverse reaction is reported for IgA. Although ASAs in this study were devoid of IgE content, IgA content was detected. Notably, IgA has no toxin neutralization value and, therefore, may reduce the therapeutic potency of ASA.

Apart from the analysis of venom-antivenom cross-reactivity by immunological techniques, spectrofluorometric titration and AFM study can also shed light on the quality of ASA [1]. The spectrofluorometric titration and AFM analysis showed poor recognition of commercial ASAs by MTV, which correlated well with our previous study [27]. The kd value of interaction determined from the above biophysical analyses could also help assess ASA efficacy; the smaller the kd value denotes higher binding affinity between venom and antivenom [20; 1]. However, in absence of similar data with other scorpion venom, it

could not be compared. The kd value suggests that ASAs under study possesses a slightly different binding affinity towards the MTV. One reason for the low relationship between scorpion venom and ASA is the low proportion of venom toxins-specific antibodies in ASAs, a significant concern for effective therapy against Indian red scorpion sting. Therefore, it is essential to enhance the proportion of venom toxins-specific antibodies in commercial ASA to improve their venom neutralization potency, resulting in in-patient treatment of Indian red scorpion sting [20; 1]. However, the adjuvant may be an excellent option to enhance the antigenicity of scorpion venom toxins of low molecular mass [28; 29]. Moreover, scorpion venom composition should be warranted with additional studies for producing antivenom of high efficacy, which can target a large variety of species.

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