A proteomic analysis of Pakistan Daboia russelii russelii venom and

were determined by densitometry scanning of SDS-PAGE bands (Supplementary Fig. S1). The published reports describing mass of RVV proteins were used as references for the identi cation of reduced SDS-PAGE protein bands as shown in Supplementary Fig. S1 [8..13,30..32]. The relative abundance of RVV proteins/peptides determined by SDS-PAGE and LC-MS/MS analysis was found to be nearly identical. For example, SDS-PAGE analysis demonstrates relative abundance of PLA_2 enzymes was ~34.0% of RVV protein (Supplementary Fig. S1) whereas LC-MS/MS analysis shows that PLA_2 enzymes constitute ~33.0% of the RVV proteome (see below). Similarly, by SDS-PAGE analysis the relative abundances of KSPIs and disintegrins were determined to be 30.5% (Supplementary Fig. S1) and this value correlates well with the relative abundances (~29.0%) of the above proteins determined by LC-MS/MS

>40 kDa, 14..40 kDa, and <14 kDa contribute approximately 15.5%, 19.8%, and 64.6% (reduced) (Fig. 1B) and 28.6%, 48.6%, and 24.9% (non-reduced), respectively of RVV proteins (Fig. 1C). By SDS-PAGE,

Table 3
Summary of different venom proteins identi ed in Pakistan RVV by ESI-LC-MS/MS analysis of the gel ltration peaks. The distribution of the proteins in different GF peaks are shown in the last column of the table.

Sl. no.	Protein	Accession no.	−10logP	% COVERAGE	Homology with protein from	GF peak(s)
		Enzymatic proteins				
Phospholipase A_2 (PLA_2)						
1	Chain A, Crystal Structure Of The Complex Of A Group Iia Phospholipase A2	gi|71042571	333.56	61	Daboia russellii pulchella	GF-1,4,5,6,8
2	Basic phospholipase A2 Drk-b1	gi|408407668	159.95	18	Daboia russelii russelii	GF-5
3	Basic phospholipase A2 VRV-PL-V	gi|81174981	147.00	60	Daboia russellii russellii	GF-1
4	Acidic phospholipase A2 Drk-a2	gi|408407660	143.78	57	Daboia russellii russellii	GF-1,3,4,5,6,7,8,10
5	Acidic phospholipase A2 RV-7	gi|400714	141.57	72	Daboia siamensis	GF-1
6	phospholipase A2-IV	gi|87130860	129.65	37	Daboia russelii russelii	GF-1,4,5,9
7	Chain A, anticoagulant class II phospholipase A2	gi|157834128	125.63	61	Daboia russelii russelii	GF-1,4,5,6,7,8,9,10
8	Chain H, structure of daboiatoxin	gi|149241831	119.64	47	Daboia siamensis	GF-1
9	Ammodytin I1(C) isoform	gi|50874274	111.95	38	Vipera aspis zinnikeri	GF-1
10	Basic phospholipase A2	gi|71912227	108.07	40	Daboia russelii limitis	GF-1,4,5,8
11	Ammodytin I1(B) isoform	gi|50874252	103.38	40	Vipera ammodytes ruffoi	GF-1
12	Basic phospholipase A2 Drk-b2	gi|408407671	97.27	26	Daboia russelii russelii	GF-1,4,5,6,8,10
13	Chain A, rst crystal structure of A C49 monomer Pla2	gi|48,425,253	94.72	10	Daboia russelii pulchella	GF-1,4
14	Ammodytin I2(A) variant	gi|50874438	91.11	29	Vipera aspis aspis	GF-1
15	Phospholipase A2/PLA2	gi|1839638	87.56	39	Daboia russelii	GF-4,6,8,9,10
16	phospholipase A2-II	gi|87130856	73.80	12	Daboia russelii russelii	GF-4
17	phospholipase A2	gi|478779	42.84	9	Daboia russelii russelii	GF-8
Snake venom metalloprotease (SVMP)						
1	Factor X activator heavy chain	gi|300079900	230.89	38	Daboia russelii russelii	GF-1,2,3,4,5,6,7,8,10
2	Chain A, crystal structure of Russell's viper venom metalloproteinase heavy chain	gi|162329887	216.39	49	Daboia russelii russelii	GF-1
3	coagulation factor X activating enzyme light chain	gi|251205	203.13	54	Daboia russelii russelii	GF-1
4	Factor X activator light chain 2	gi|300079896	177.15	68	Daboia russelii russelii	GF-1
5	Factor X activator light chain 2	gi|380765752	136.84	49	Daboia russelii russelii	GF-1
6	Chain B, crystal structure of Russell's viper venom metalloproteinase light chain	gi|162329888	136.84	57	Daboia siamensis	GF-1
7	Factor X activator light chain 2	gi|50980285	134.38	24	Macrovipera lebetina	GF-1
8	Coagulation factor X activating enzyme heavy chain	gi|251204	124.28	14	Daboia russelii	GF-3,4
9	Zinc metalloproteinase-disintegrin-like VLAIP-A	gi|82228619	115.74	11	Macrovipera lebetina	GF-1
10	Metalloproteinase, partial	gi|297593790	111.64	7	Echis carinatus sochureki	GF-1
11	Metalloproteinase, partial	gi|297593990	109.05	11	Echis pyramidum leakeyi	GF-1
12	Metalloproteinase MP-2, partial	gi|62547937	80.48	5	Bitis arietans	GF-1
13	Metalloproteinase, partial	gi|297593820	53.15	11	Echis carinatus sochureki	GF-1
Snake venom serine protease (SVSP)						
1	Serine beta- brinogenase precursor	gi|306756036	134.93	21	Macrovipera lebetina	GF-1
2	Factor V activator RVV-V gamma	gi|134130	129.7	30	Macrovipera lebetina	GF-1
3	Serine beta- brinogenase-like protein precursor	gi|311223824	120.14	30	Daboia siamensis	GF-1,2
4	Serine protease VLSP-3	gi|380875417	111.86	12	Macrovipera lebetina	GF-1
5	Venom serine proteinase-like protein 2	gi|13959655	102.88	14	Macrovipera lebetina	GF-1
6	Serine protease VLSP-1	gi|381141431	95.67	19	Macrovipera lebetina	GF-1
7	Thrombin-like enzyme gyroxin B1.7	gi|380875412	75.22	10	Crotalus durissus terri cus	

enzyme, and for plasma kallikrein; RVV displayed comparatively lower activity towards the plasmin substrate, indicating that brinolytic serine proteases with plasmin-like activity are less abundant in RVV (Table 2). Alternatively, this may also be due to enzyme promiscuity toward various pNA substrates. Collectively, these data (Tables 1 and 2) indicate that multiple higher molecular mass serine proteases and metalloproteases account for the observed high proteolytic activity of RVV from Pakistan [2,9,31]. These results are also consistent with the observed coagulopathic toxicity of RVV following human envenomations [2].

3.2. LC-MS/MS analysis of RVV GF peaks

Snake venoms contain many enzyme and non-enzymatic proteins and peptides which exert toxic or lethal effects in prey or snakebite victims [34]. The shotgun proteomic approach, coupled with protein database searches and protein assembly algorithms, has been shown to surpass other MS-based proteomic systems in terms of number and diversity of proteins identi ed and in dynamic range for detection [35]. The ESI-LC-MS/MS analysis has provided an overview of presence of different proteins and peptides in GF fractions of Pakistan RVV (Table 3 and Supplementary Table S1). The relative abundance of proteins and peptides of Pakistan RVV identi ed by LC-MS/MS analysis is shown in Fig. 2. The alignment of MS/MS-derived peptide sequences with the homologous protein/peptide from the Viperidae snake venom database is shown in Supplementary Fig. S2. By LC-MS/MS analysis of tryptic

[38,39]. By peptide mass ngerprinting analysis we have identi ed 2 LAAO isoenzymes in GF-1 fraction of Pakistan RVV (Table 3, Supplementary Table S1, Supplementary Fig. S2). According to LC-MS/MS analysis, these LAAOs corresponding to molecular mass of ~66 kD (Fig. 1B, Supplementary Fig. S1) [11] comprise about 0.6% of the Pakistan RVV proteome (Fig. 2). One of the LAAOs (Accession no. gi|395406796) found in Pakistan RVV was also reported in RVV from Southern India (7.9%) and Sri Lanka (5.2%) [7,8]. However, the relative abundance of LAAOs in RVV from Southern India RVV (7.9%) and Sri Lanka (5.2%) [7,8] is signi cantly higher as compared to Pakistan RVV (Fig. 2).

RVV is a rich source of proteolytic enzymes [40]. Proteolytic enzyme pro ling, in addition to comparison of levels of proteolytic activity in venom from different subspecies of RV, has shed light on the toxicological contribution of proteases to RV envenomations [2,40]. Snake venom proteases have been broadly classi ed into two catalytic types …SVSPs and SVMPs[40,41]. RVV is rich in both SVSPs and SVMPs, and they affect the hemostatic system in victims by proteolytic cleavage of several different blood coagulation factors [31,42,43]; thrombin-like activity is prominent [9,44,45]. In in vitro conditions, crude RVV displays procoagulant activity owing to the predominance of pro-coagulant proteases[2]. In addition, snake venom proteases account for hemorrhage, necrosis and muscular degeneration in victims [40].

Fig. S1) and therefore, they represent the most abundant non-enzymatic proteins of this venom. Interestingly, KSPIs are detected in almost all GF peaks of Pakistan RVV (Table 3) suggesting their interaction with other components, including venom PLA_2s and proteases [77]

Supplementary Table S1, Supplementary Fig. S2). Interestingly, the presence of this particular isoform (bearing the same accession no) was also reported in RVV from Sri Lanka [8] but not in RVV from Southern India [7].

Snake venom vascular endothelial growth factors are one of the 7 members of the vascular endothelial growth factor (VEGF-F) family of proteins and their molecular weights range from 23 ..33 kDa [15]. Evidence has indicated that VEGF-F binds to receptors like KDR and Flt-1 and exhibit potent hypotensive effects and enhances vascular permeability [88]. Two VEGFs from venom of southern India D. russelii russelii [7] and 2 VEGF isoforms from D. russelii siamensis venom [6] have been identi ed. The VEGFs constitute 1.5% of Pakistan RVV (Fig. 2) and 3 isoforms of this protein family have been identi ed in peaks GF-1, 4, and 9 (Table 3, Supplementary Table S1, Supplementary Fig. S1). However, the identi ed VEGFs are exclusively reported in Pakistan RVV and the occurrence of identical isoforms of VEGFs was not reported in RVV from other geographical locations [6..8].

In addition to the enzymatic and non-enzymatic proteins mentioned above, an MS-MS derived peptide showing sequence similarity to a hypothetical protein (accession no. gi|19574645) from Agkistrodon piscivorus piscivorus venom was also identi ed in Pakistan RVV (Table 3, Supplementary Table S1, Supplementary Fig. S2). Due to a lack of information, the biological activity of this protein could not be assigned.

3.5. Pharmacological properties of crude RVV and chromatographic peaks

Some pharmacological properties of crude RVV and gel- ltration peaks are shown in Table 4. However, due to lack of published clinical data, the pharmacological properties of Pakistan RVV could not be correlated with the observed clinical manifestations of RV bite in Pakistan.

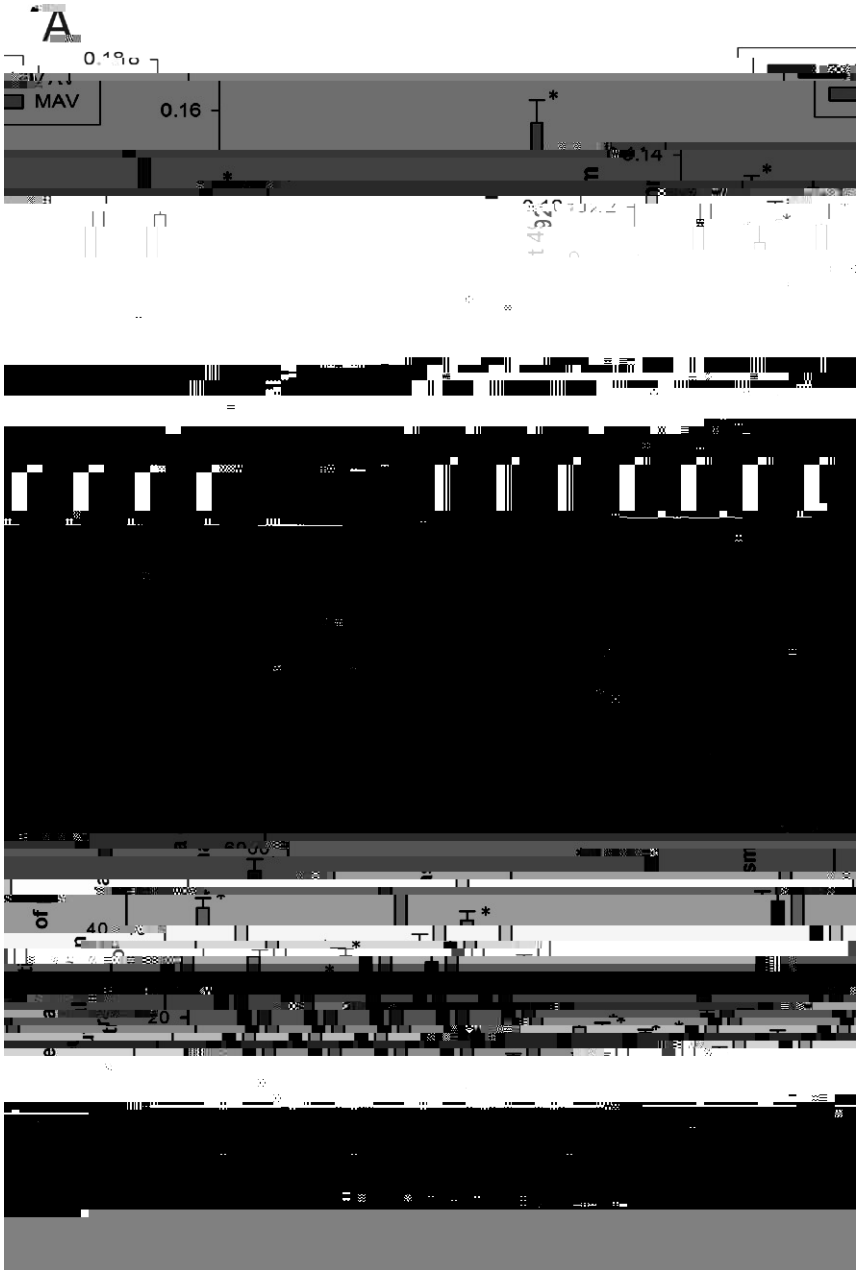

Fig. 3. A. Immunological cross-reactivity of Pakistan RVV and its GF fractions with commercial polyvalent (PAV) and monovalent (MAV) antivenom. Values are mean ± SD of triplicate determinations. B. Neutralization of pro-coagulant/anticoagulant activity of CRVV and its fractions by PAV and MAV. The experiment was conducted as described in the text. Values are mean ± SD of triplicate determinations. CRVV, crude Russell's Viper venom. Signi cance of difference with respect to cross-reactivity of PAV, * $p < 0.05$.

The crude RVV or GF peaks did not exhibit hemolytic activity (Table 4), in accordance with our previous reports showing RVV PLA $_2$ enzymes do not induce direct hemolysis of washed erythrocytes [30] ; however, PLA$_2$s may induce indirect hemolysis in the presence of phospholipids [2,29] . The cytotoxicity of peak GF-1 against Colo-205 and MCF-7 cells was found to be higher than the cytotoxicity exhibited by crude RVV under identical experimental conditions (Table 4), which may be attributed to the presence of the cytotoxic protein Rusvinoxidase (a LAAO enzyme) in this peak [13] . Only peak GF-2 showed brinogen clotting activity (Table 4), resulting from the elution of Russelobin (a thrombin-like serine protease) in this peak [9] .

Crude RVV in in vitro demonstrated pro-coagulant activity (Table 4) due to predominance of procoagulant components in the venom; nev-

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