1 Succinctus

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Vasoconstriction induced by salivary gland extracts from ixodid ticks

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ABSTRACT

In their quest for blood, most haematophagous parasites secrete vasodilators in their saliva to counter the host haemostatic response of vasoconstriction. Surprisingly, salivary gland extracts from adult female *Dermacentor reticulatus* and *Rhipicephalus appendiculatus* ticks induced constriction in a rat femoral artery model; males induced vasoconstriction or vasodilation depending on the time of feeding. Based on comparative HPLC fractionation, the active compounds inducing vasoconstriction do not appear to be prostaglandins (which ticks normally use as vasodilators). Vasoconstriction may be unique to ixodid ticks, helping them control blood flow during their prolonged blood-feeding of up to 10 days or more.

Keywords: Tick, Salivary glands, Vasoactivity, Vasoconstriction, Vasodilation, Rat femoral artery

41   Haematophagy is a complex task compounded by the presence of haemostatic mechanisms

42 used by the host to prevent blood loss (i.e. platelet aggregation, vasoconstriction and blood

43 clotting). Haematophagous parasites respond to host haemostasis by secreting from their salivary

44 glands a cocktail of anticoagulant, antiplatelet and vasodilatory compounds. The critical role of

45 vasodilation in haematophagy is illustrated by the universality of vasodilatory molecules produced

46 by blood-feeders. For example, ticks use prostaglandins (PGE_2, $PGF2_\alpha$, PGI_2) (Bowman et al.,

47 1996), triatomes produce a nitric oxide binding protein (Champagne et al., 1995), tabanids produce

48 vasotab peptide similar to Kazal-type protease inhibitors (Takáč et al., 2006), sand flies secrete

49 maxadilan peptide that mimics pituitary adenylate cyclase activating peptide (PACAP) activity

50 (Lerner et al., 2007), leeches use histamine (Hildebrandt and Lemke, 2011), and vampire bat

51 submaxillary glands express PACAP and other potential vasodilators (Francischetti et al., 2013).

52   As part of our research to identify compounds responsible for anti-haemostatic activities of

53 tick saliva, we investigated the vasoactive effects of salivary gland extracts (SGE) from two ixodid

54 tick species, *Dermacentor reticulatus* and *Rhipicephalus appendiculatus*. Both species are

55 important disease vectors: *D. reticulatus* transmits pathogenic bacteria (e.g. *Rickettsia slovaca,*

56 *Francisella tularensis*) and protozoa (e.g. *Babesia canis*) (Salman and Tarrés-Call, 2013) and *R.*

57 *appendiculatus* is the vector of *Theileria parva*, the cause of the devastating East Coast fever in

58 cattle (Nuttall and Hindle, 1913). Surprisingly, we found most vasoactivity of the SGE was

59 expressed as constriction rather than relaxation in a bioassay using pre-constricted rat femoral

60 arteries.

61   To prepare SGE), adult ticks from laboratory colonies were fed in groups within retaining

62 chambers glued to the shaved backs of guinea pigs (Trik strain, weight 250 – 300 g). To examine

63 the dynamics of vasoactivity, SGE was prepared from unfed ticks and ticks fed on guinea pigs for

64 2, 6 or 9 days. Adult female *R. appendiculatus* take approximately 9 days to engorge, involving a

65 'slow phase' and a 'rapid phase'. At 2 days, the ticks are in the slow phase, preparing their cuticle

66 for expansion to accommodate the extraordinary bloodmeal ($\geq 200 \times$ their unfed body weight); at

67 day 6 they transition to the rapid phase; and at day 9 the ticks are completing the rapid phase of

68 feeding when 50% of their bloodmeal is consumed. Meanwhile, adult males feed intermittently and

69 mate with feeding females on the host. Salivary glands were dissected under chilled 150 mM PBS,

70 pH 7.2, pooled in Eppendorf tubes and immediately frozen at –70 °C. Prior to the assays, batches of

71 salivary glands were quickly thawed, subjected to 80 °C for 5 min, homogenised, and the

72 supernatants of the SGE preparations were pooled. The soluble protein concentration in the SGE

73 was determined using the Bradford assay adapted to microplates with BSA as a standard.

74 Vasoactivity of the SGE preparations was measured by myography using rat femoral artery

75 preconstricted with phenylephrine (Takáč et al., 2006). Femoral arteries were obtained from

76 laboratory rats (Wistar strain, males, 12 week old, weight 300 – 350 g). All laboratory animals were

77 purchased from the Department of Toxicology and Dobrá Voda breeding station (Institute of

78 Experimental Pharmacology, Slovak Academy of Sciences (SAS), Slovak Republic) and

79 maintained according to guidelines for the care and use of laboratory animals (Act of the

80 Government of the Slovak Republic 2003). All procedures and experimental protocols were

81 approved by the Ethical Committee of the Institute of Normal and Pathological Physiology SAS,

82 and conform to the European Convention on Animal Protection and Guidelines on Research

83 Animal Use.

84   The arteries were cleaned of adherent connective tissue and cut into 1.5 mm ring segments.

85 Two stainless steel wires were passed through the lumen of the artery segments and they were

86 mounted on a myograph capable of measuring the isometric wall tension in a bath of Krebs-Ringer

87 solution at 37 °C, pH 7.4, gassed with 95% O_2 and 5% CO_2. An initial tension of 13.3 kPa was

88 applied and the preparation was allowed to rest for 30 min. After this period, 10 µM phenylephrine

89 and 10 µM acetylcholine were added; if endothelium was present, acetylcholine induced relaxation

90 of phenylephrine-induced constriction. After confirming the presence of endothelium, the segments

91 were washed and then preconstricted by addition of phenylephrine. The plateau of the contractile

92 response induced by 10 µM phenylephrine was taken as a measure of 100% contraction.

93   Vasodilatory and vasoconstrictive activities were expressed as the percentage decrease and

94   increase, respectively, of phenylephrine-induced constriction. Differences in the vasoactivity of tick

95   SGE derived from different feeding phases of individual species and sexes were evaluated by the

96   Kruskal-Wallis test with post hoc paired comparisons; P <0.05 was considered significant. Each

97   experiment was repeated at least three times; a dose response was observed for most preparations.

98   SGE preparations from the two species showed similar vasoactive profiles (Fig. 1). All SGE

99   preparations from adult females induced vasoconstriction whereas SGE from 6 day fed males

100 induced vasodilation, while all other preparations caused constriction. In SGE derived from *R.*

101 *appendiculatus* females, activity significantly increased with feeding, with the highest activity in 9

102 day fed ticks (P <0.05). In contrast, the strongest vasoconstriction observed for female *D.*

103 *reticulatus* was at day 6 of feeding (P <0.05) (Fig. 1).

104   The different dynamics of SGE vasoactive activity between adult females and males observed

105 for *D. reticulatus* and *R. appendiculatus* may well reflect their feeding and mating behaviour. Both

106 species are members of the Rhipicephalinae subfamily, and show similar feeding and mating

107 behaviour although their geographical distribution and hosts differ (Perry et al., 1991; Hillyard,

108 1996). They mate on the host, the male attaching and feeding for a short period before detaching,

109 seeking a feeding female, mating and then reattaching adjacent to the mated female. Males secrete

110 male-specific saliva proteins that help their female mate to feed, a form of molecular "mate

111 guarding" (Wang et al., 1998). Possibly, the vasodilation observed with male SGE obtained at 6

112 days of feeding reflects the time when the male is reattaching to the host after mating and secreting

113 mate-protecting saliva molecules. Vasoconstriction was also induced using SGE from unfed ticks of

114 both species. However, 40 – 60 salivary glands were required to produce the 80 µg dose of unfed

115 SGEs tested for activity whereas, for example, the same dose was achieved with only three salivary

116 gland equivalents from female *R. appendiculatus* fed for 9 days.

117   Typically, ixodid ticks induce vasodilation by secreting prostaglandins in their saliva. Ticks of

118 different genera have been shown to contain prostaglandins PGE_2, $PGF_{2\alpha}$ and PGI_2 (Bowman et al.,

119   1996). All are potent skin vasodilators; $PGF_{2\alpha}$ is the most potent prostaglandin to induce

120   vasodilation in cattle (Kemp et al., 1983). In addition, prostaglandins aid the ectoparasites by

121   modulating the host inflammatory and immune responses (Sá-Nunes et al., 2007). Prostaglandins of

122   the 2-series (i.e. PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2) are synthesized from the arachidonic acid precursor via

123   the cyclooxygenase pathway (Holtzman, 1991). Ticks cannot synthesize fatty acids with more than

124   one double bond and lack the ability to desaturate or elongate dietary fatty acids. Consequently, all

125   fatty acids with more than one double bond, including arachidonic acid, must be sequestered by

126   ticks from their blood meal. Prostaglandins are synthesised from arachidonic acid sequestered at

127   comparatively high levels in the salivary glands (Bowman et al., 1996). Although prostaglandins

128   cause vascular smooth muscle to relax and blood flow to increase, at high concentrations they can

129   induce vasoconstriction (Somova and Bojkov, 1983). Moreover, PGE_2 itself is a weak contractile

130   agent but exhibits pronounced synergism with other contractile agents (Hung et al., 2006). To date,

131   prostaglandins are the only vasoactive compounds identified in ixodid tick saliva.

132   To determine whether the observed vasoactivity might result from prostaglandin-like

133   molecules, molecular sieving Reversed Phase – HPLC (RP – HPLC) was performed on SGE

134   prepared from glands of females and males of both tick species, fed for 6 – 7 days, using 200

135   salivary gland pairs per sample. Following heat treatment and clarification (as described above),

136   SGEs were resuspended in 500 µl of 10% acetonitrile (ACN) with 0.1% trifluoroacetic acid (TFA)

137   and UV was monitored at 210 nm and 220 nm with a Beckman 126/168 DAD HPLC system.

138   Fractions were assayed for vasoactivity. The first purification was performed with a Vydac C4

139   column, 4.6 mm inner diameter (ID) $\times$ 250 mm, 5 µm particle size. The purification was performed

140   at 10 – 100% ACN gradient with 0.1% TFA, flow rate 1 ml/min and 1% ACN/min concentration

141   gain. The second purification was performed with a Beckman Ultrasphere C18 column, 4.6 mm ID

142   $\times$ 250 mm, 5 µm particle size and a gradient of 10 – 40% ACN with 0.1% TFA, flow rate 1 ml/min

143   with 0.5% ACN/min increment. The third purification was performed with a Vydac C18 column,

144   4.6 mm ID $\times$ 250 mm,5 µm particle size, under the same conditions as described in the second

145   purification. Collected fractions were concentrated in a vacuum evaporator (Speed-Vac, Savant

146   Instruments, USA). The fourth purification of the active fraction was performed with a Tosoh

147   Biosep, TSKgel Super ODS column, 2.0 mm ID $\times$10.0 cm, 2 µm particle size and a 20–40% ACN

148   gradient with 0.1% TFA. A prostaglandin HPLC mixture containing 6-keto prostaglandin $PGF_{1\alpha}$,

149    $PGF_{2\alpha}$, PGE_2, and PGE_1 standards (Cayman Chemical Company, MI, USA), and a PGB_1 standard

150   (Sigma-Aldrich Chemie GmbH, Germany) were used for comparative analysis of the elution

151   spectra. These standards were analysed with a Tosoh Biosep, TSKgel Super ODS column, using the

152   same RP-HPLC conditions as for the Step 4 (fourth purification as described above) analysis of the

153   active SGE fraction.

154   HPLC fractions derived from SGE of both female and male *D. reticulatus* and *R.*

155   *appendiculatus* fed for $6 - 7$ days were tested for vasoactivity. Unlike the vasoactivity assays, there

156   was considerable variation in the activity profiles of fractionated HPLC samples, both between

157   species and between conspecific females and males. Furthermore, all the HPLC fractions obtained

158   from male SGE showed vasoconstriction and none showed the vasodilation observed with

159   unfractionated male SGE of both species fed for 6 days. The most potent fractions were obtained

160   from SGE of female *D. reticulatus,* with a single peak at Step 1 of fractionation showing 785%

161   constriction. This fraction was taken through to Step 4 when a peak at retention time 2.3 min still

162   induced a relatively high level of constriction (130%). This peak showed maximum UV absorbance

163   at 206 and 256 nm (Fig. 2). Comparison of the retention time and absorbance profile of the purified

164   SGE fraction with those obtained with prostaglandin standards subjected to the same RP-HPLC

165   conditions, revealed no similarities (Figs. 2, 3 respectively; Supplementary Fig. S1). Thus the

166   relatively high vasoconstrictive activity of the purified fraction from $6 - 7$ day fed female *D.*

167   *reticulatus* SGE does not appear to be due to the presence of either $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, PGE_1, or

168   PGB_1. The apparent absence of prostaglandins in the fractions may have been the result of the

169   method of preparing and processing SGE, and does not exclude the presence of prostaglandins in

170   the salivary glands. Although prostaglandins normally have an antihypertensive action,

171 prostaglandin endoperoxide (PGH_2), thromboxane and isoprostanes can vasoconstrict blood vessels

172 (Welch et al., 2007). However, none of these arachidonic acid derivatives has been reported in tick

173 saliva. Thromboxane-scavenging saliva proteins have been reported from some argasid tick species,

174 although their function is thought to be inhibition rather than induction of vasoconstriction (Mans

175 and Ribeiro, 2008). Heat treatment of PGE_2, using the same conditions as those used in preparing

176 the SGE samples, resulted in a 50% loss of vasoconstriction activity when tested in the rat femoral

177 artery model. This contrasts with the relative heat stability of the purified female *D. reticulatus*

178 vasoconstrictor. Heat treatment at 80 °C for 5 min of crude SGE followed by clarification is

179 commonly used to inhibit protease activity and to remove proteins >20 kD. This procedure was

180 used in the isolation of the 6.1 kD vasotab peptide from horse fly (*Hybomitra bimaculata*) SGE

181 (Takáč et al., 2006). Overall, the results obtained for 6 − 7 day fed female *D. reticulatus* SGE are

182 consistent with a small phenylalanine-rich peptide as the active ingredient in the purified fraction,

183 however, further analysis using mass spectrometry is required to identify this vasoconstrictor.

184 The RP-HPLC results for *D. reticulatus* female and male SGE differed considerably, as was the

185 case for *R. appendiculatus*, suggesting the presence of different vasoactive ingredients in females

186 and males (Fig. 2, Supplementary Figs. S2 − S4). Sexual dimorphism at the level of bioactive

187 salivary gland products has been reported previously for ixodid ticks (Wang et al., 1998). The

188 comparatively low level of vasoactivity observed with fractions of 6 − 7 day fed female *R.*

189 *appendiculatus* SGE indicate that peak vasoconstrictive activity is at around 9 days of feeding

190 compared with 6 − 7 days for *D. reticulatus* (Fig. 1, Supplementary Figs. S3 − S4).

191 It appears counter intuitive that haematophagous parasites should induce vasoconstriction.

192 However, ixodid ticks are exceptional in that a single blood meal takes days if not weeks to

193 complete. The largest blood meal is acquired by adult females, where nutrients are converted into

194 thousands of eggs and then they die, taking 2 weeks of continual attachment and feeding on a host

195 to complete engorgement, and increasing their body weight ≥100-fold (Kaufman, 2007). Females

196 acquire most of the bloodmeal (>50%) in the last 24 h of feeding (the rapid feeding phase),

197 spending much of the attachment period creating a feeding pool and preparing their exoskeleton for

198 enormous expansion. Hence vasoconstriction may be a means of regulating bloodflow and possibly

199 reducing inflammation during the prolonged feeding period of female ixodid ticks.

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203 **References**

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Legends to figures

255

256 **Fig. 1.** Vasoactivity of salivary gland extracts (SGE) from unfed (80 µg only) and partially fed (2

257 day (2D), 6D, 9D) *Rhipicephalus appendiculatus* and *Dermacentor reticulatus* females and

258 males. Rat femoral artery preconstricted with phenylephrine was treated with 20 µg, 40 µg or 80

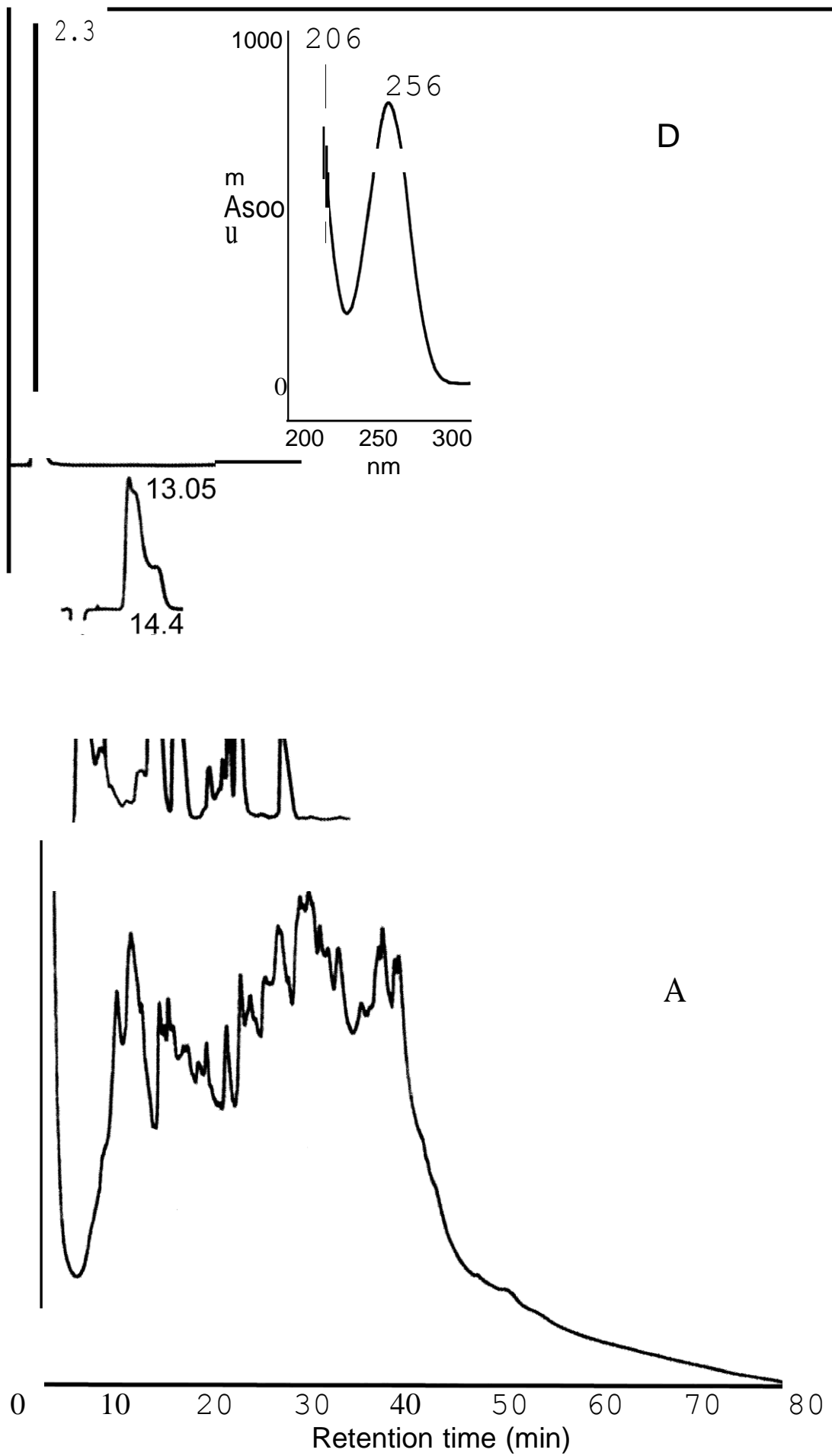259 µg of SGE. Values represent means ± S.E.M.

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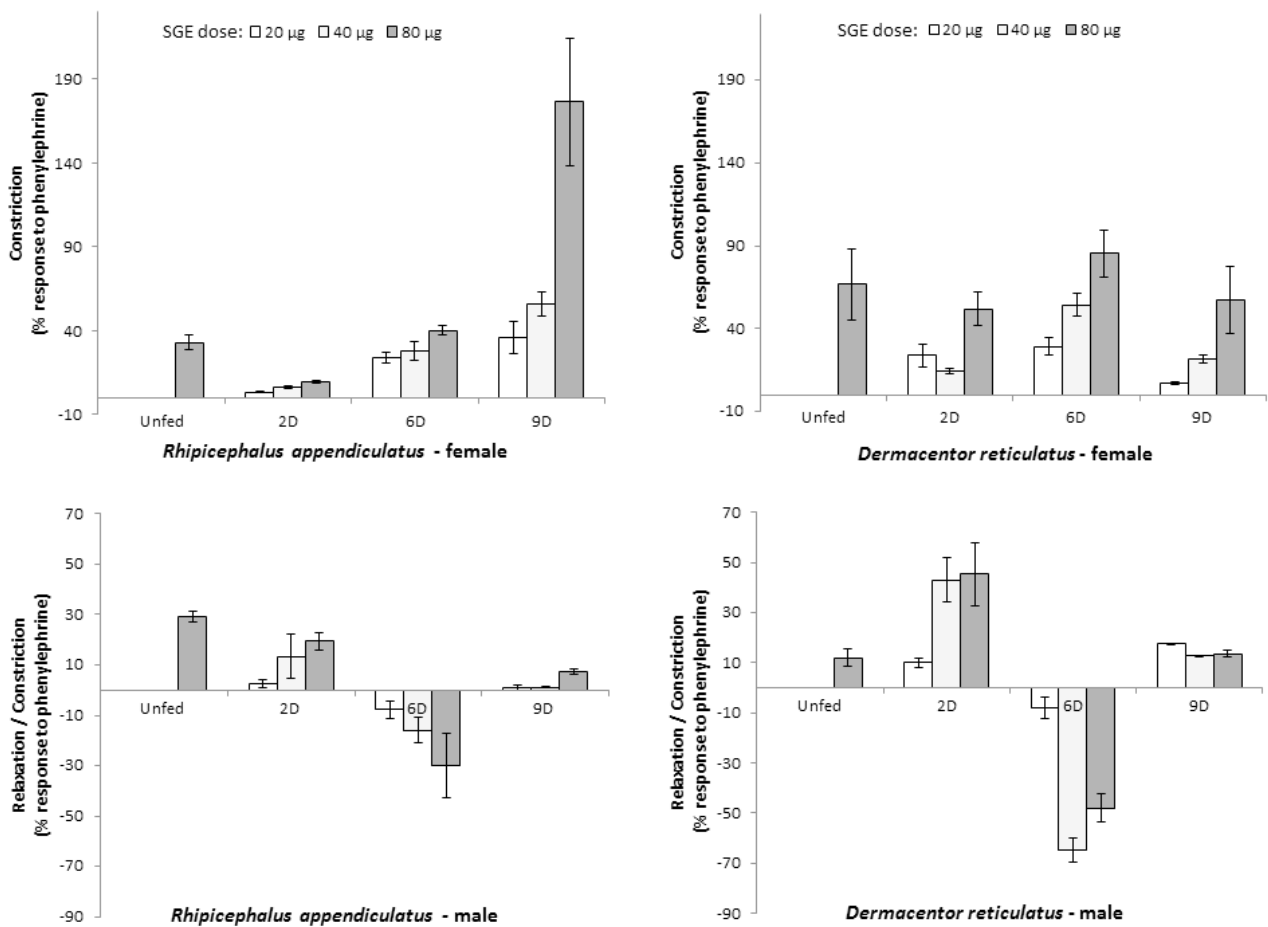
Fig. 2. Reversed Phase (RP)-HPLC chromatogram of salivary gland extracts from 6 − 7 day fed female *Dermacentor reticulatus*. The most potent fractions used for further purification are marked with retention times (min) on the graph. (A) First purification step active fraction at 3.5 min (785% constriction); (B) Second purification step using A-3.5 min gave active fraction at 14.4 min (273% constriction); (C) Third purification using B-14.4 min gave active fraction at 13.05 min (64% constriction); (D) Fourth purification step using C-13.05 min gave single active peak at 2.3 min (130% constriction). Inset: UV spectrum of D-2.3 min showing maximum absorbance (mAU, milli absorbance units) at wavelengths of 206 nm and 256 nm.

2.3

1000

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256

m
Asoo
u

0

200   250   300
nm

D

13.05

14.4

A

0   10   20   30   40   50   60   70   80
Retention time (min)

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Fig. 3. Reversed Phase (RP)-HPLC chromatogram of HPLC prostaglandin mixture (6-keto

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PGF$_{1\alpha}$, PGF$_{2\alpha}$, PGE$_2$, PGE$_1$) showing UV spectra of prostaglandin standards at maximum

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275 absorbance (mAU, milli absorbance units) (insets) and acetonitrile gradient. The prostaglandin

276 mixture was subjected to the same RP-HPLC conditions as used in the fourth purification step
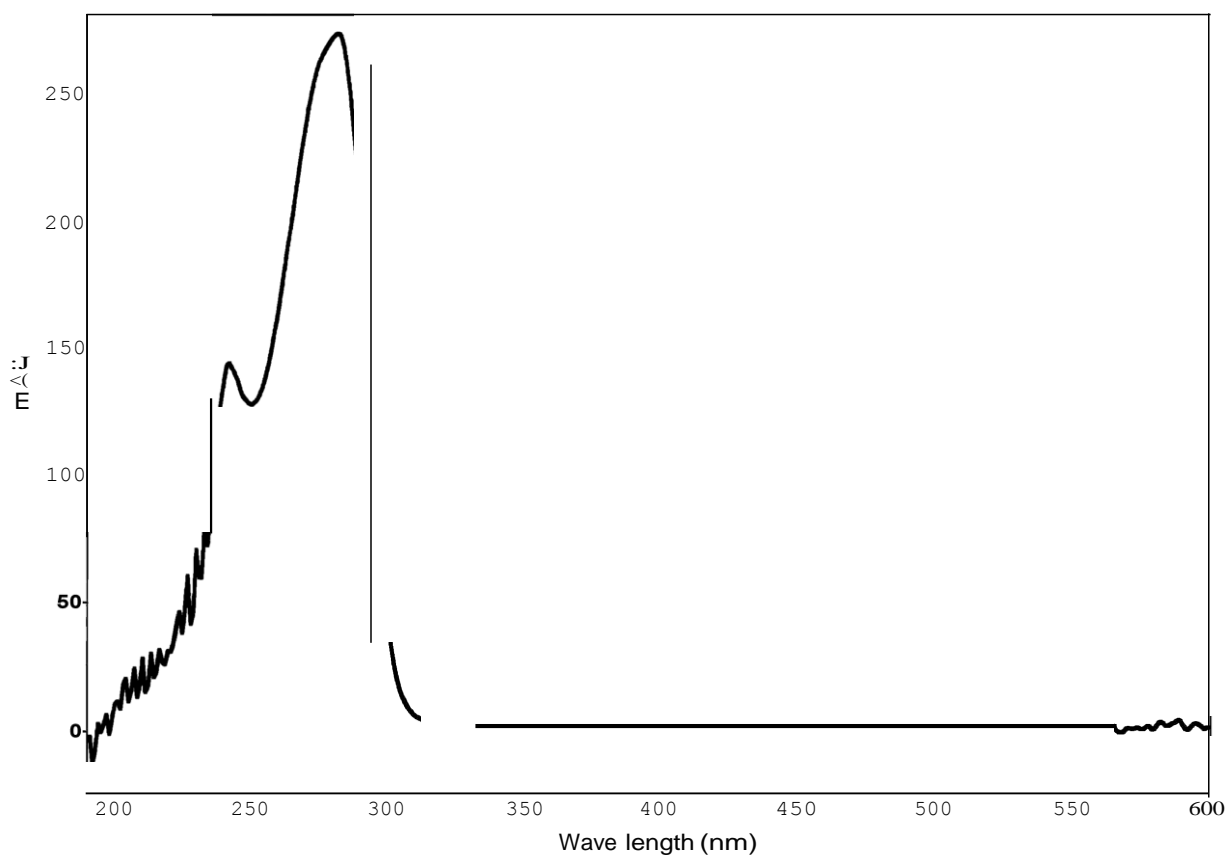
277 of salivary gland extracts shown in Fig. 2D.

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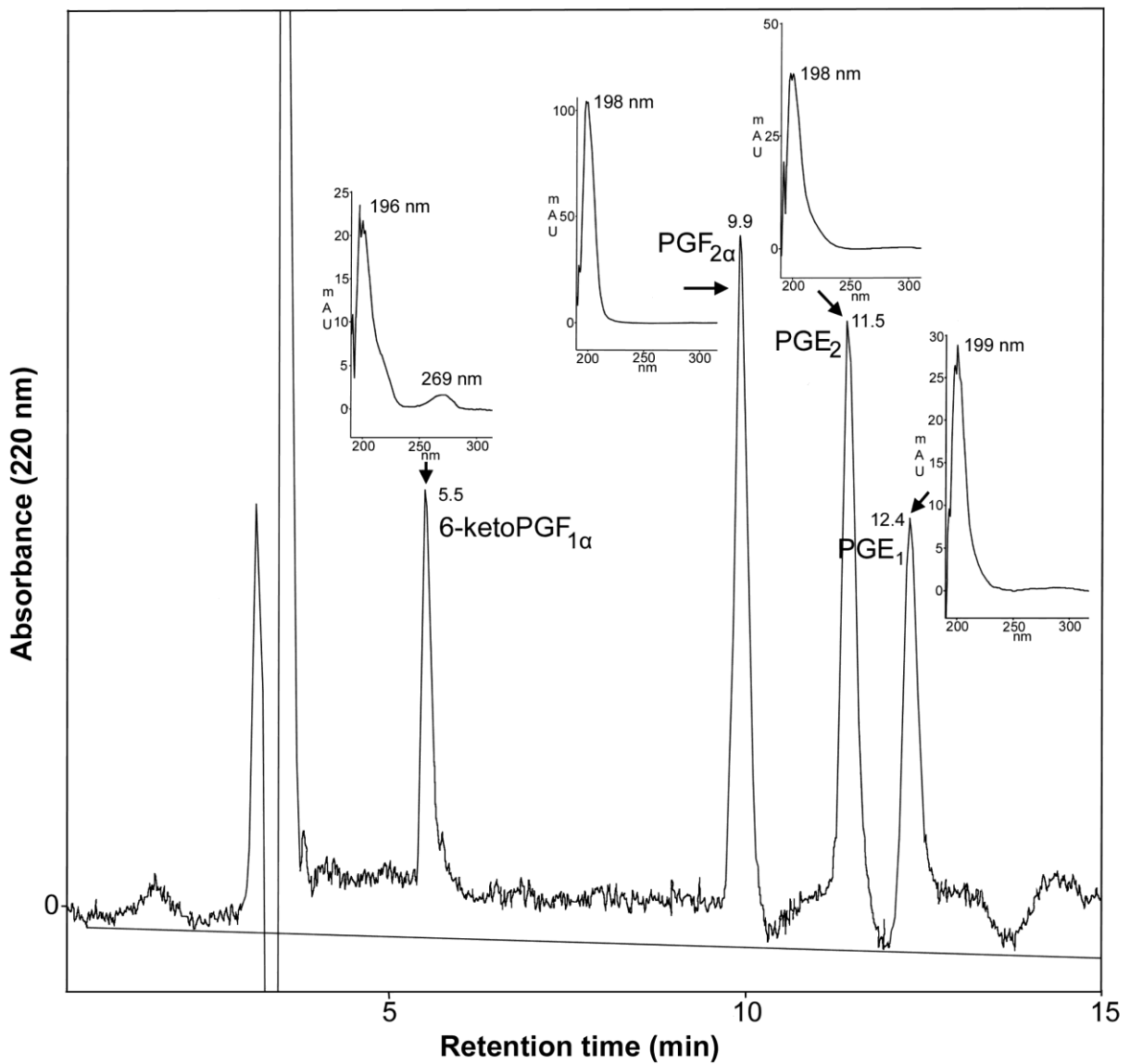
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281 Supplementary data

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283 Supplementary Fig. St. UV spectrogram of prostaglandin PGB $_1$ standard (Sigma-Aldrich Chemie

284 GmbH, Germany) at maximum absorbance (mAU, milli absorbance units).
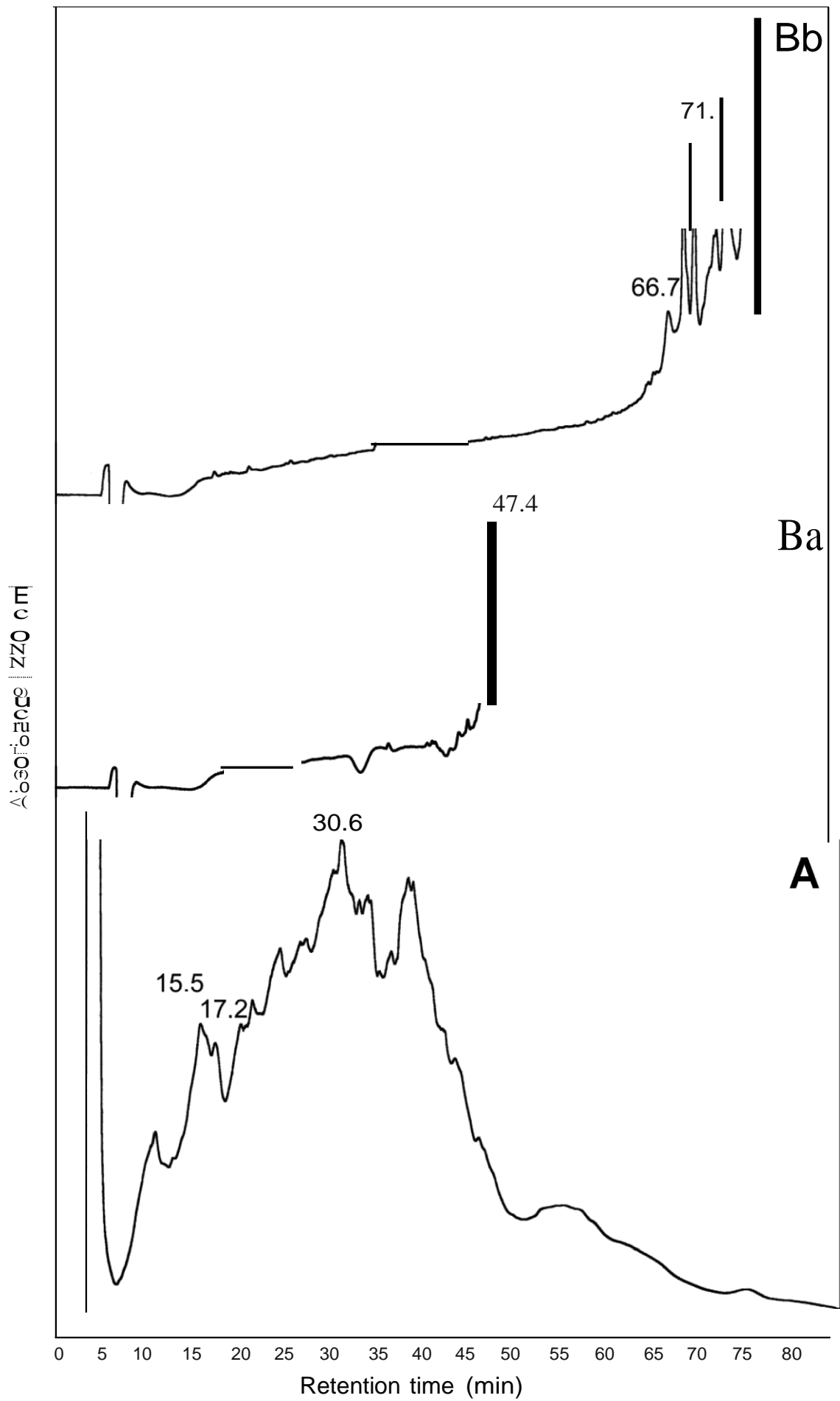

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287   **Supplementary Fig. S2.** Reversed Phase (RP)-HPLC chromatogram of salivary gland extracts

288   from 6 – 7 day fed male *Dermacentor reticulatus*. The most potent fractions used for further

289   purification are marked with retention times (min). (A) First purification step active fractions at

290   13.5 min (152% constriction), 28.2 min (28% constriction) and 31.8 min (47% constriction). (Ba)

291   Second purification step using A-13.5 min gave active fraction at 45.8 min (27% constriction). (Bb)

292   Second purification step using A-28.2 min gave active fractions at 22.2 min (33% constriction) and

293   52.8 min (22% constriction). (Bc) Second purification step using A-31.8 min gave active fractions

294   at 12.6 min (21% constriction) and 43.2 min (21% constriction).

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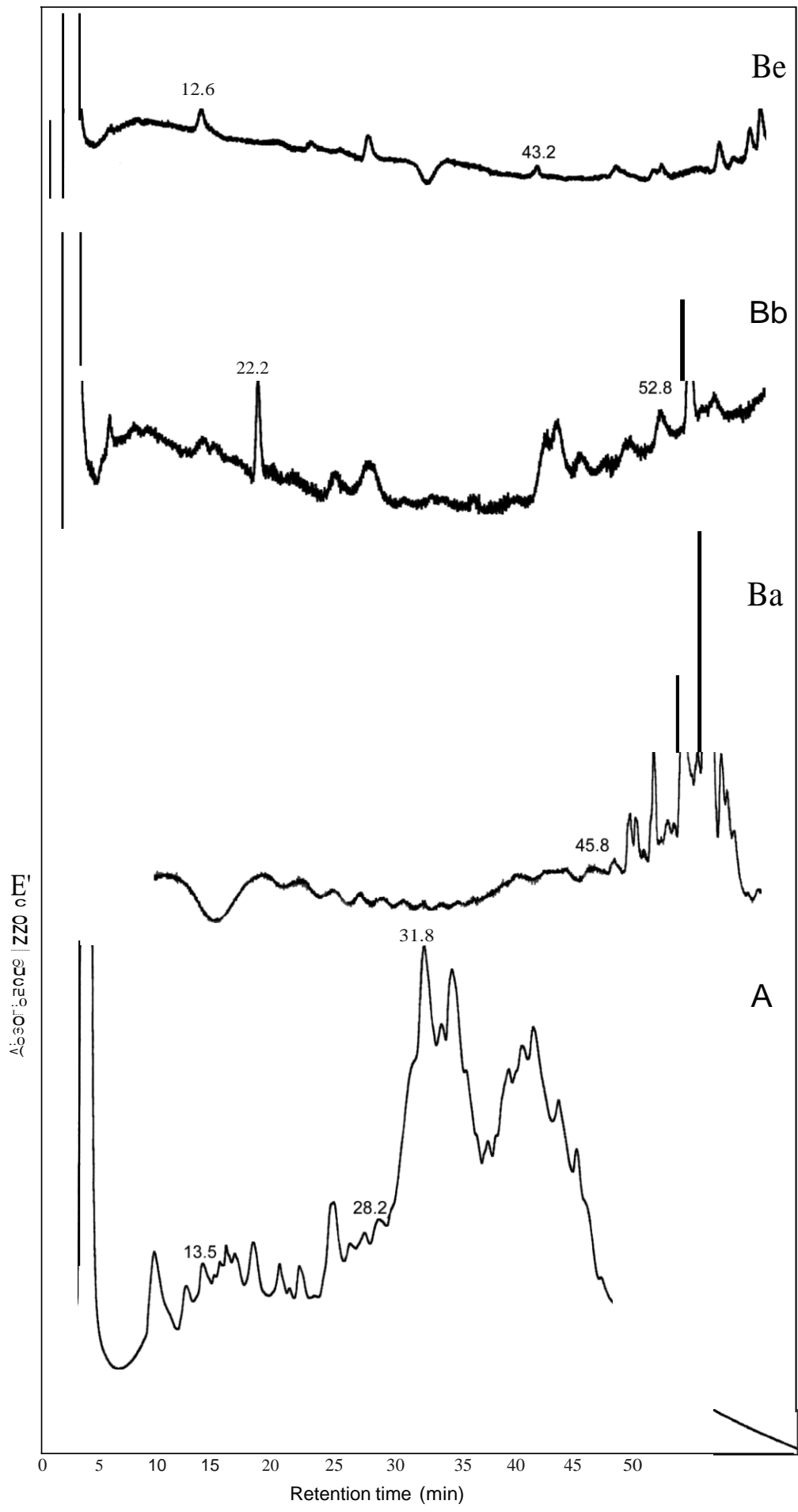
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297 **Supplementary Fig. S3.** Reversed Phase (RP)-HPLC chromatogram of salivary gland extracts

298 from 6 – 7 day fed female *Rhipicephalus appendiculatus*. The most potent fractions used for further

299 purification are marked with retention times (min). (A) First purification step active fractions at

300 15.5 min (29% constriction), 17.2 min (32% constriction) and 30.6 min (15% constriction). (Ba)

301 Second purification step using either A-15.5 min or A-17.2 min gave active fraction at 47.4 min

302 (9% constriction). (Bb) Second purification step using A-30.6 min gave active fractions at 66.7 min

303 (12% constriction) and 71.2 min (24% constriction).

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Supplementary Fig. S4. Reversed Phase (RP)-HPLC chromatogram of salivary gland extracts from 6–7 day-fed male *Rhipicephalus appendiculatus*. The most potent fractions used for further purification are marked with retention times (min). First purification step active fractions at (Aa) 3.4 min (14% constriction) and (Ba) 3.7 min (20% constriction). (Ab) Second purification step using Aa-3.4 min gave active fraction at 10.2 min (9% constriction). (Bb) Second purification step using Ba-3.7 min gave active fraction at 7.2 min (32% constriction). (Ac) Third purification step using Ab-10.2 min gave one active peak at 6.8 min (31% constriction). (Bc) Third purification step using Bb-7.2 min gave one major active peak at 7.1 min (40% constriction).

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A

B