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Antiscorpion Venom Activity of an Aromatic Compound having Carbohydrate Moiety Isolated from *Hemidesmus indicus* (Anantamul) Root Extract in Experimental Animal Models

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Abstract

The present study established the anti-scorpion venom activity of an aromatic compound purified from the Indian medicinal plant *Hemidesmus indicus* (anantamul) root extract in experimental animals. The compound (H.I-1) was isolated by silica gel column chromatography and thin layer chromatography and spectral analysis was done by IR and H1 NMR. Animal ethical clearance was obtained before experiment. Anti-scorpion venom activity was evaluated using various *in vivo* tests, urine qualitative analysis, renal toxicity markers (urea and creatinine), hepatotoxicity markers (AST and ALT), myotoxic markers (LDH and CPK) and oxidative stress markers (LPO, GPX and GSH) in animal models. Statistical analysis was done by one way ANOVA, values expressed as mean ± SEM, $P<0.05$ was considered as statistically significant. Silica gel column chromatography I & II produced an active compound (H.I-1) which gave a single spot on TLC. IR and H1 NMR studies revealed that H.I-1 was aromatic compound having sugar (rhamnose) moiety. It could neutralize scorpion (*Heterometrus bengalensis*) venom induced hepatotoxicity, renal toxicity, myotoxicity and oxidative stress in experimental animals. These observations confirmed that the aromatic compound H.I-1, isolated from *Hemidesmus indicus* root extract might be useful in scorpion envenomation and warrants further detailed studies.

Keywords: Scorpion; *Heterometrus bengalensis*; Scorpion venom; Venom neutralization; *Hemidesmus indicus*; Venom toxicity

Introduction

There are 1500 species of venomous scorpions available worldwide, and approximately 25 species are dangerous to humans [1-3]. In India, 90 species of scorpions are available, among which 19 species belongs to the genus *Heterometrus*. *Heterometrus bengalensis* is commonly found in West Bengal, India [4,5]. Envenomation by scorpion remains a serious health problem, causing child mortality and elderly morbidity [6]. Their venom are composed of a complex mixture of polypeptides, enzymes and toxic agents, exhibiting different pharmacological activities [5,7-9]. Hundreds die annually from scorpion envenomation, not less than one million people around the world are predicted to suffer from systematic symptoms [10,11]. No specific antiscorpion antiserum is available in India. In some developed countries, the antiscorpion antivenoms are available for clinical applications. Prophylactic immunization against scorpion envenomation has also been advocated, but acceptable experimental evidences are lacking [12]. Symptomatic folk-traditional treatments are available in many parts of the world, among which the most common is the usage of herbal products like, *Mangifera indica. L*, *Aristolochia indica*, *Solanum indicum L*, *Andrographis paniculata Nees*, *Barringtonia acutangula L*, *Hemidesmus indicus*, *Pluchea indica* and *Aristolochia indica* etc. [13-15]. These plant products are cheap and easily available in rural village areas; some of these plants have been worked out to for their scientific validation against venoms-toxins. Plants like *Hemidesmus indicus*, *Pluchea indica* and *Aristolochia indica* have been found to neutralize scorpion (*Heterometrus bengalensis*) venom induced lethality, edema, urinary changes, plasma recalcification, cardiotoxicity, neurotoxicity, in experimental animal models [16,17]. Based on the above information, the present investigation was explored to identify the active constituent from the root extract of the plant *Hemidesmus indicus*, and to establish its anti scorpion (*Heterometrus bengalensis*) venom activity in animal models.

Methods

Scorpion venom

Adult black scorpions (*Heterometrous bengalensis*) of both sexes were collected from Burdwan district of West Bengal, India, during the rainy seasons (June–September) and were kept in a wire mesh cage. They were provided with food (live cockroach, ant egg), water *ad libitum*. The scorpion venom (SV) was collected once in a month by applying square wave electrical stimulation (12 V, 5 ms) to the telson. The venom was pooled, lyophilized and stored at 4ºC in amber color bottle, until further use. Before use, SV was weighed, dissolve in 0.9% saline and was expressed in terms of dry weight.

Animals

Male Swiss albino mice (20 ± 2 gm) and Wister Albino rats (100 ± 10 gm) were obtained from enlisted supplier of Calcutta University and were maintained in standard laboratory conditions, diet and water *ad libitum*. All animal experiments were approved by the University Animal Ethics Committee, Department of Physiology, University of Calcutta, Kolkata, India and were in accordance with the guidelines of the committee for the purpose of Control and Supervision of

Experiments on Animal (CPCSEA), Government of India (Ref. No.: 820/04/ac/CPCSEA dated 06.08.2004). Animals (n=6) were grouped into: Gr.1- Sham Control, Gr.2- Scorpion Venom Control (0.2 mg/20 gm/i.v), Gr.3- H.I-1 treated (0.1 mg/20 gm/iv).

Hemidesmus indicus root extract, purification and characterization

The plant *Hemidesmus indicus* root was collected commercially from M/s United Chemicals and Allied Products, Kolkata, India and was identified by Prof N Paria, Department of Botany, University of Calcutta. A voucher specimen has been deposited at the Department of Botany, University of Calcutta, Kolkata. The powdered root was extracted in Soxlet apparatus using petroleum ether, chloroform and methanol. The methanol phase was dried and kept at room temperature until further use.

The dried methanolic root extract of *Hemidesmus indicus* (15g) was dissolved in methanol, and centrifuged at 2000 rpm for 30 min. The supernatant was added to silica gel and evaporated to dryness. The sample was resolved through column chromatography I, using silica gel (60-120 mesh) column (85cm x 6cm) and column chromatography II, using silica gel (60-120 mesh). Both the columns was eluted with petroleum ether: chloroform (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 and 0:1), chloroform: methanol (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 and 0:1). The fractions collected were evaporated to dryness and tested for homogeneity by TLC using Silica gel (type G), solvent (chloroform: methanol, 1:1, v/v) and developed by iodine vapour. Spots were marked and R_f was calculated.

The active fraction (H.I-1) was characterized by (1) IR (in KBr) spectra was registered on a infrared spectrometer (Perkin Elmer Model No.732) at the Instrumentation Centre, University of Calcutta (2) H1-NMR (in D_2O) was recorded on a spectrophotometer (Jeol JMS Model No.600) at the Indian Institute of Chemical Biology, Calcutta, India. The signals of the spectrum were analyzed for determination of the functional groups. The active compound (H.I-1) was used for the anti scorpion venom activity in experimental animals.

Urine parameters

Urine analysis was done by injecting SV (0.2 mg/20 gm/i.v) into male albino mice (20 ± 2 g) and after 24 h urine was collected to check the presence of blood and protein using qualitative urine strips (Multistrix SG, Bayer, Germany). Neutralization was done using SV (0.2 mg/20 gm/i.v) and H.I-1 (0.1 mg/20 gm/iv), incubated at 37°C for 30 mins and centrifuged at 2000 rpm × 10 min. The supernatant was injected (iv) for the urine analysis after 24 h. Urine was collected to check the presence of blood and protein using qualitative urine strips (Multistrix SG, Bayer, Germany).

Serum parameters

SV (0.2 mg/20 gm/iv) was injected into male albino mice (20 ± 2 g), blood was collected after 24 h and serum was prepared to assess hepatotoxic markers (SGOT & SGPT), nephrotoxic markers (urea and creatinine) and myotoxic markers (LDH and CPK). Neutralization was done using SV (0.2 mg/20 gm/iv) and H.I-1 (0.1 mg/20 gm/iv) incubated at 37°C for 30 min and centrifuged at 2000 rpm × 10 min. The supernatant was injected (iv) and after 24 h, the serum hepatotoxic, nephrotoxic, myotoxic markers were analyzed using biochemical kits (Merck, India and Labkit, Spain) and UV-Vis spectrophotometer (Analab, India).

Antioxidant parameters

Antioxidant markers (LPO, GPX, and GSH) were measured by injecting SV (0.2/20 gm/iv) into male albino mice (20 ± 2g). After 24 h, liver tissue was collected, homogenized and assayed for the antioxidant markers after Buege and Aust [18], Takahashi et al. [19] and Ellman [20]. Neutralization was done using SV (0.2 mg/20 gm/iv) and H.I-1 (0.1 mg/20 gm/iv), incubated at 37°C for 30 min and centrifuged at 2000 rpm × 10 min. The supernatant was injected (iv) and after 24 h, the liver antioxidant markers were assayed. Serum protein was measured after Lowry et al. [21].

Statistical analysis

All results were expressed as Mean ± SE (n=4). The level of significance of difference between means were determined by Student's t test (two groups) and One way ANOVA (> two groups), $p<0.01$ were considered significant.

Result

Purification and characterization of H.I-1

The *Hemidesmus indicus* extract was fractionated over silica gel (60-120 mesh) column chromatography I, which gave an active fraction eluted with chloroform: methanol (8:2 v/v). This active fraction produced multiple spots on TLC using chloroform: methanol (1:1, v/v).

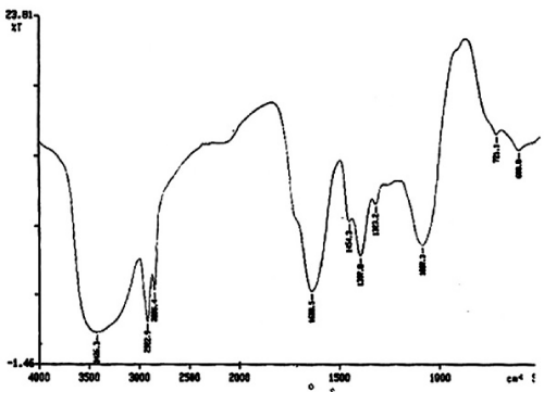

Figure 1: IR-Spectral studies with H.I-1 isolated and purified from the root extract of *Hemidesmus indicus*. The IR spectra of the H.I-1 displayed peaks at δ 3426.3 cm^{-1}, 1638.5 cm^{-1}, 1323.2 cm^{-1} and 1087.3 cm^{-1}, representing =N-H, Nitrates or diketones, alcohols or sulfonamides and C-F or C=S groups, respectively.

This fraction was further rechromatographed on silica gel (100-200 mesh) column chromatography II, which resolved into an active compound eluted by chloroform: methanol (65:35, v/v). On TLC, it gave a single spot (R_f 0.66), using chloroform: methanol (1:1 v/v). The active fraction neutralized scorpion venom induced actions and provisionally designated "H.I-1". The solubility of H.I-1 was found to be 100% in water and 90% in methanol. Yield of H.I-1 was found to be 0.15 ± 0.05 gm%.

IR and H1 NMR spectroscopy of H.I-1

The IR spectra of the H.I-1 displayed peaks at δ 3426.3 cm^{-1}, 1638.5 cm^{-1}, 1323.2 cm^{-1} and 1087.3 cm^{-1}, representing =N-H, Nitrates or diketones, alcohols or sulfonamides and C-F or C=S groups, respectively (Figure 1). H1 NMR spectrum displayed one sugar unit at 1.314 MHz, which was likely to be rhamnose. There was one anomeric signal at 440 MHz and one O-CH3 group, which suggested that H.I-1 was likely to be a sugar containing aromatic compound (Figure 2).

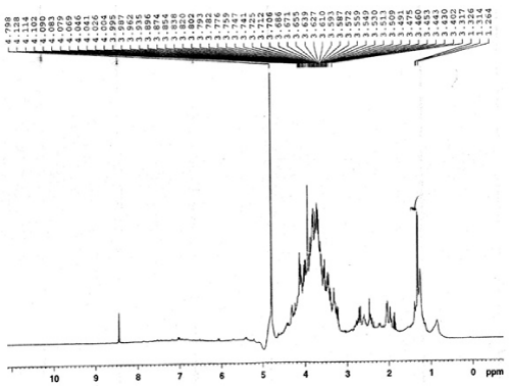

Figure 2: H1-NMR Spectral studies with H.I-1 isolated and purified from the root extract of *Hemidesmus indicus*. H1 NMR spectrum displayed one sugar unit at 1.314 MHz, which was likely to be rhamnose.

Urine parameters

SV (0.2 mg/20 gm/iv) injected in male albino mice showed presence of blood (+++) and protein (+) in urine after 24 h. HI-1 (0.1 mg/20 gm/iv) gave partial protection against SV (0.2 mg/20 gm/iv) induced qualitative urinary changes in male albino mice after 24 h (Figure 3).

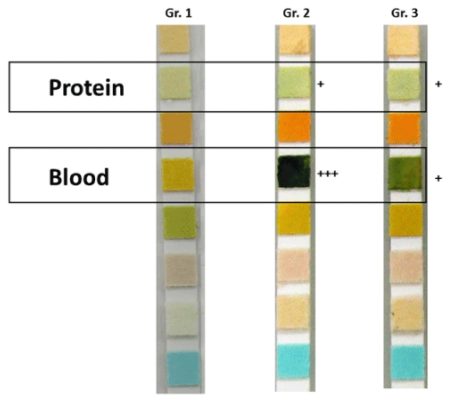

Figure 3: Neutralization of SV-induced urinary changes by H.I-1 in male albino swiss mice. +Trace amount; ++Medium amount; +++High amount.

Serum hepatic markers: SV (0.2 mg/20 gm/iv) injected in male albino mice significantly increased the SGOT & SGPT level after 24 h as compared with control. H.I-1 (0.1 mg/20 gm/iv)) significantly decreased SV induced changes in SGOT and SGPT level in male albino mice after 24 h (Control: SGPT 35.57 ± 3.07 U/L and SGOT 82.77 ± 3.14 U/L; SV-treated: SGPT 98.02 ± 5.87 U/L and SGOT 200.98 ± 3.28 U/L; SV+H.I-1 treated: SGPT 64.87 ± 3.40 U/L and SGOT 122.97 ± 7.02 U/L; P<0.01). % protection against SV-induced hepatotoxic markers offered by H.I-1 was 53.13% (SGPT) and 65.96% (SGOT) in *in vivo* experiments (Table 1).

Serum kidney markers: SV (0.2 mg/20 gm/iv) injected in male albino mice significantly increased the urea and creatinine level after 24 h of treatment as compared with control. H.I-1 (0.1 mg/20 gm/iv)) significantly decreased SV induced changes in urea and creatinine level in male albino mice after 24 h (Control: urea 30.28 ± 1.8 mg/dL and creatinine 0.69 ± 0.06 mg/dL; SV-treated: urea 77.50 ± 4.29 mg/dL and creatinine 1.64 ± 0.16 mg/dL; SV+H.I-1 treated: urea 45.56 ± 2.30 mg/dL and creatinine 0.90 ± 0.02 mg/dL; P<0.01). % protection against SV-induced nephrotoxic markers offered by H.I-1 was 67.3% (urea) and 77.6% (creatinine) in *in vivo* experiments (Table 1).

Serum muscle markers: SV (0.2 mg/20 gm/iv) injected in male albino mice significantly increased the LDH and CPK level after 24 h of treatment as compared with control. H.I-1 (0.1 mg/20 gm/iv)) significantly decreased SV induced changes in LDH and CPK level in male albino mice after 24 h (Control: LDH 136.55 ± 14.30 U/L and CPK 147.05 ± 4.78 U/L; SV-control: LDH 342.72 ± 15.40 U/L and CPK 243.22 ± 8.72 U/L; SV+H.I-1 treated: LDH 190.30 ± 13.02 U/L and CPK 174.73 ± 5.83 U/L; P<0.01). % protection against SV-induced myotoxic markers offered by H.I-1 was 73.8% (LDH) and 69.8% (CPK) in *in vivo* experiments (Table 1).

Antioxidant markers: SV (0.2 mg/20 gm/iv)) injected in male albino mice significantly increased the LPO and GPx level, and decreased GSH level after 24 h as compared with control. H.I-1(0.1 mg/20 m/iv)) significantly decreased SV induced changes in LPO and GPx level and significantly increased GSH level in male albino mice after 24 h (Control: LPO 0.79 ± 0.02 MDA/mg protein; GPx 59.0 ± 3.2 U/mg of protein; GSH 12.99 ± 0.36 µM/mg protein; SV-treated: LPO 2.91 ± 0.11 MDA/mg protein; GPx 139.65 ± 3.5 U/mg of protein; GSH 2.95 ± 0.18 µM/mg protein; SV+H.I-1 treated: LPO 1.50 ± 0.17 MDA/mg protein; GPx 83.72 ± 3.0 U/mg of protein; GSH 8.77 ± 0.42 µM/mg protein; P<0.01). % protection against SV-induced antioxidant markers offered by H.I-1 was 94.6% (LPO), 69.3% (GPx) and 58.4% (GSH) in *in vivo* experiments (Table 1).

Discussion

Scorpion sting is the most under recorded envenomation and is a major public health problem in many underdeveloped tropical countries. The clinical manifestations and severity of the symptoms vary from patients to patients thus making the management individual specific. Maximum symptomatic treatments available against scorpion envenomation, in countries especially like India, where specific anti scorpion antivenoms, are not available. They rely on folk usage of herbal products either orally or as paste applied on the stung parts. There have been many reports, both folk as well as scientific about the Indian medicinal plants and their use against scorpion/snake envenomation [13-15,22-24]. Earlier from this laboratory, it has been established that *Hemidesmus indicus* root extract contained active constituents (2-hydroxy-4-methoxy benzoic acid, lupeol acetate) which antagonized snake venom actions in experimental animals [25,26]. *Hemidesmus indicus* and its active constituent 2-hydroxy-4-methoxy benzoic acid protected against hepatotoxicity in animal model [27,28]. In the present study, an aromatic compound having an active sugar

moiety rhamnose (H.I-1) was isolated from the root extract of *Hemidesmus indicus* which effectively neutralized scorpion (*Heterometrous bengalensis*) venom induced toxicities in animal models. Rhamnose has been found to be effective in hyperglycemia induced cytotoxic effects in type II-diabetes. It is likely that SV induced proinflammatory responses/ organ damages are influenced by rhamnose present in H.I-1.

Parameters	Animal Groups		
	Sham Control	SV Control (0.2 mg/20 gm/i.v)	H.I-1 Treated (0.1 mg/20 gm/i.v)
SGOT (U/L)	82.77 ± 3.14	200.98 ± 3.28$^{\#}$	122.97 ± 7.02*
SGPT (U/L)	35.57 ± 3.07	98.02 ± 5.87$^{\#}$	64.87 ± 3.40*
Urea (mg/dL)	30.28 ± 1.8	77.50 ± 4.29$^{\#}$	45.56 ± 2.30*
Creatinine (mg/dL)	0.69 ± 0.06	1.64 ± 0.16$^{\#}$	0.90 ± 0.02*
LDH (U/L)	136.55 ± 14.30	342.72 ± 15.40$^{\#}$	190.30 ± 13.02*
CPK (U/L)	147.05 ± 4.78	243.22 ± 8.72$^{\#}$	174.73 ± 5.83*
LPO (MDA/mg protein)	0.79 ± 0.02	2.91 ± 0.11$^{\#}$	1.50 ± 0.17*
GPx (U/mg of protein)	59.0 ± 3.2	139.65 ± 3.5$^{\#}$	83.72 ± 3.0*
GSH (μM/mg protein)	12.99 ± 0.36	2.95 ± 0.18$^{\#}$	8.77 ± 0.42*

Table 1: Effect of H.I-1 treatment in SV induced changes in serum markers and antioxidant parameters in animal model. Significant change was observed in serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), urea, creatinine, lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) after treatment with H.I-1 in scorpion venom-induced animals. $^{\#}P<0.05$, when compared to sham control and scorpion venom control animals; $^{*}P<0.05$, when compared to scorpion venom control and H.I-1 treated animals. Values were expressed as mean ± SEM.

Scorpion envenomation leads to organ toxicity and multiple organ failure in young patients [29]. The major organs affected is the liver, kidney and muscle and clinically it is evident that the level of liver, kidney, muscle markers were elevated significantly that leads to multiple organ failure in experimental and clinical conditions [29]. Here, we have observed that SV injected in animals significantly increased the liver, kidney, muscle markers, which was significantly decreased by the herbal compound H.I-1. H.I-1 is likely to be responsible for neutralizing the venom molecule induced damage of the major organs at the molecular level. It is not clear how H.I-1 is involved in the neutralizing process. It may be acting (1) at the enzymatic level (2) targeting the cellular signaling molecules. Envenoming by different scorpions had shown an increase level of circulating enzyme like succinate dehydrogenase, creatine phosphokinase, lactate dehydrogenase ALT, and AST [10,30-36]. These studies also confirmed that scorpion venom target the major organs (liver, kidney, muscle) and produce multi organ failure before death. The herbal compound H.I-1 neutralized the SV-induced toxicities in liver, kidney and muscle, thereby antagonized multi-organ failure induced by SV in animal models.

Scorpion envenomation induces lipid peroxidation and decreases antioxidants in animal model [37-39]. Several biomarkers (LPO, GPx, GSH, etc.) have been found to be involved in stress physiology [40]. In the present study scorpion venom induced oxidative stress was measured by lipid peroxidation, glutathione peroxidase and reduced glutathione content. Treatment with H.I-1 decreased venom induced stress in animal models. It is likely that rhamnose, present in H.I-1 interfere with the pro-oxidant action of SV in animal model.

Conclusion

To conclude, in this present scenario of unavailability of anti-scorpion antiserum in India, the findings from this study will definitely enlightened the value of herbs/herbal compounds against scorpion envenomation as an alternative/supportive treatment subject to more detail studies.

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