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Protective effect of *Euphorbia hirta* and its components against snake venom induced lethality

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ABSTRACT

Ethnopharmacological relevance: Despite the use of snake anti-venom therapy, herbal medicine is still in practice to treat snakebites. *Euphorbia hirta* is traditionally used as antidote for snakebites and also for numerous other ailments. However, the scientific evidence for its anti-snake venom property is still lacking.

Materials and methods: Methanolic extract of *E. hirta* was evaluated for anti-venom activity under *in vitro* and *ex vivo* conditions. Histopathological changes in the vital organs of the mice were also monitored. UHPLC-SRM/MS was used to estimate the phenolic constituents whereas GC–MS analysis was performed to analyze the volatile metabolites present. The major compound was further evaluated for its contribution to the overall inhibitory potential of the extract.

Results: Methanolic extract of *E. hirta* completely inhibited the venom enzymes under *in vitro* and reduced the edema ratio. The extract increased the survival time (> 24 h) of mice which was further evidenced by histopathological analysis of vital organs. Phytochemical analysis revealed higher content of phenolic (144 mg/g extract) compounds in the extract. UHPLC-SRM/MS demonstrated that ellagic acid, gallic acid and quinic acid are the major phenolics whereas GC–MS analysis revealed pyrogallol as the major constituent (60.07%) among the volatile components of the extract. It was also shown that pyrogallol has the ability to differentially inhibit venom protease but not phospholipase A2.

Conclusion: The present study confirmed that *E. hirta* methanolic extract was able to completely inhibit *Naja naja* venom induced toxicity under *in vitro* as well as *ex vivo* conditions, thus providing scientific evidence to its traditional use.

1. Introduction

Snakebite is still a common medical concern in many of the tropical and sub-tropical Asian countries. World Health Organization has recently categorized snakebite as a neglected tropical disease which requires more attention from national and international health authorities (WHO, 2007). Agriculturists and their families living in rural areas of the country are the most affected community, thus snakebite is represented as a 'disease of poverty' (Harrison et al., 2009). Approximately 2.5 million snakebites occur throughout the world with 85,000 deaths every year. In India alone it is estimated that nearly 35,000–50,000 deaths per year due to snakebite (Gutierrez et al., 2010; Mohapatra et al., 2011). Of the 216 species in India, 53 are poisonous (Warrell, 2004). Indian spectacled cobra *Naja naja* (Elapidae family) is one among the big four snakes (*N. naja*, *Daboia russelii*, *Echis carinatus* and *Bungarus caeruleus*) responsible for such high morbidity and mortality.

Snake venom is composed of more than 90% proteins, of which most of them are enzymes (Gomes et al., 2010). A large number of victims are inflicted with permanent physical injury due to local tissue necrosis and psychological sequelae (Hansdak et al., 1998). The composition of venom depends on many factors including species of the snake, geographical and seasonal variations and others, thus reflecting the complications involved in snakebite treatment.

Though anti-snake venom (ASV) is the only specific and authorized treatment available to treat snakebites, failure in its supply to the rural places and affordability are the major factors contributing to the severity and complexity of this public health problem (Harrison et al., 2009; Warrell, 2008). Massive doses of the administered anti-venom (Sharma et al., 2005) often lead to adverse reactions (Ariaratnam et al., 2001; Gupta and Peshin, 2012) and post-bite complications (Abubakar et al., 2010; Warrell, 2010; Tsagalis, 2011; Vishal et al., 2012).Therefore, investigation on

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complementary/alternative remedy for snakebite treatment is mandatory.

Plant extracts are being traditionally used in the treatment of snakebite envenomations, especially in remote areas where hospital facilities are limited (Borges et al., 2001). Though reports indicate that many of these herbal plant formulations exhibit anti-venom activity (Gomes et al., 2010), the molecular basis of the anti-venom activity of several of these herbal extracts is still elusive. *Euphorbia hirta* L., belonging to the family of Euphorbiaceae, is a small annual herb common to tropical countries. *E. hirta* is used in traditional medicine for various diseases including asthma, amebic dysentery, bronchitis, coughs, colds, diarrhea, emphysema, fever, heartburn, intestinal parasites, kidney stones, laryngeal spasms, menstrual problems, peptic ulcers, sterility, vomiting and venereal diseases are well documented (Sandeep et al., 2009; Rajesh et al., 2010; Shih and Cherng, 2012). The pharmaceutical application of this plant includes antibacterial, antifungal, anti-allergic, anti-inflammatory, antidiarrheal, antioxidant, anti-tumor, anti-diabetic, anti-hypertensive, anti-malarial and also immunomodulatory activities (Huang et al., 2012). The tribal people living in and around Vellore District of Tamil Nadu, India, have long been using aerial parts of this plant in the treatment of snake bites. The decoction of the whole plant extract is administered orally and applied topically to treat poisonous snakebites (Samy et al., 2008). However, the scientific evidence for its anti-venom activity is yet to be investigated. Therefore, the present investigation, the first of its kind on *E. hirta*, unravels its anti-ophidian activity against Indian cobra *N. naja* venom toxicity.

2. Materials and methods

2.1. Plant collection

The present investigation was carried out by conducting surveys and questionnaire with tribal people and irulars living in Irularpatti, Vallimalai, Alamalrengapuram and Ammorupalli in Vellore district of Tamil Nadu, India. Traditional healers, called 'Vaidyars' from different indigenous groups were also approached for the documentation of the medicinal plants used in the treatment of snakebite. The plant was collected from Irrularpatti village, Gudiyatham District, Tamil Nadu, India and it was authenticated by Dr.K. Madhava Chetty (Assistant Professor, Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India).A voucher specimen was deposited in the Herbarium of the same department under the number SVUTY-EU/1067.

2.2. Plant extraction

Five hundred grams of shade dried and powdered whole plant material was immersed in methanol at the ratio of 1:2 (w/v) for 24 h with continuous stirring. The extraction was repeated for 3 days by changing the solvent in every 24 h and the filtrate was concentrated using rotary evaporator under reduced pressure. The dried extract was further dissolved in 10 mM phosphate buffered saline (PBS pH 7.4) and the supernatant was used for all enzyme inhibition studies. The activity of the venom in absence of plant extract served as the negative control.

2.3. Venom and chemicals

Lyophilized snake venom (*N. naja*) was purchased from Irula snake catcher's Industrial co-operative society limited, Chennai, India. The venom was dissolved in 0.9% saline and centrifuged at 2500 rpm for 10 min. The supernatant was used for the study. Hyaluronic acid was purchased from Sigma-Aldrich (US). The lyophilized polyvalent anti-snake venom (AV) was purchased from Vins Bioproducts Limited, Andhra Pradesh, India (Batch no. 01AS13016). Until unless mentioned, all enzyme inhibition studies with AV were carried out at a ratio of 1:60 w/w. Pyrogallol was purchased from SRL Pvt. Ltd. and dissolved in 10 mM PBS, pH 7.4 for enzyme inhibition studies. All other reagents and solvents were of high quality analytical grade and were purchased from SD Fine chemicals/Sisco Research Laboratory Pvt. Ltd./ HiMedia.

2.4. Protease inhibition

2.4.1. Caseinolytic inhibition by plate assay

50 μg of *N. naja* venom was pre-incubated with various concentrations of *E. hirta* extract or AV for 1 h at 37 °C. Protease activity was then determined using 1% casein as substrate in 1% agarose plates according to the method of Okoroma et al. (2012). Briefly, the pre-incubated samples were loaded into 3 mm diameter wells of casein–agarose plates (0.1 M Tris–HCl buffer, pH 8.0) and incubated overnight at 37 °C. Plates were then stained with Coomassie Brilliant Blue-R 250 for 1 h and destained with methanol:water:acetic acid (50:40:10). The percentage of protease inhibition was calculated by measuring the zone of clearance in the presence of plant extract or AV. Zone of clearance values obtained for the venom in the absence of the plant extract served as negative control while venom treated with AV served as positive control in all the experiments.

2.4.2. Caseinolytic inhibition by SDS-PAGE

10 μg of *N. naja* venom was pre-incubated with various concentrations of *E. hirta* extract or AV for 1 h at 37 °C. These samples were further incubated with 15 μl of 1% casein (dissolved (w/v) in 0.1 M Tris–HCl buffer, pH 8.0, 37 °C). After 30min the reaction was stopped by adding SDS loading buffer (containing 50 mM Tris–HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 100 mM β-mercaptoethanol and 0.1% (w/v) bromophenol blue). SDS-PAGE of the casein treated with venom in presence and absence of plant extract or AV was then performed according to the method previously described by Laemmli (1970) using 15% acrylamide gel.

2.5. Phospholipase (PLA_2) inhibition

50 μg of *N. naja* venom was pre-incubated with various concentrations of *E. hirta* extract or AV for 1 h at 37 °C. PLA_2 activity was then evaluated using egg yolk as substrate in 1% agarose plates according to the method of Gutierrez et al. (1998). The pre-incubated samples were then loaded into 3 mm diameter wells of 1% agarose plates containing 0.6% egg yolk and 5 mM $CaCl_2$ followed by overnight incubation at 37 °C. The PLA_2 inhibition was calculated by measuring the zone of clearance in the presence and absence of plant extract or AV. PLA_2 activity of venom in the absence of plant extract served as negative control.

2.6. Hemolytic inhibition

100 μg of *N. naja* venom was pre-incubated with various concentrations of *E. hirta* extract or AV in a volume of 200 μl PBS for 1 h at 37 °C. The assay protocol was followed by the method of Boman and Kaletta (1957) with slight modifications. Briefly, human erythrocytes and 10 mM PBS (pH 7.4) were mixed (1:8 v/v) and 100 μl of this suspension was incubated with pre-incubated venom samples for 2 h at 37 °C. The reaction was stopped by adding 1 ml of ice cold PBS and centrifuged at 2500 rpm for 10 min at 4 °C. The amount of hemoglobin released in the supernatant was estimated at 540 nm.