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ORIGINAL ARTICLE

Antiprotease Activity of Indigenous Medicinal Plants against Pakistani *Echis carinatus* Venom

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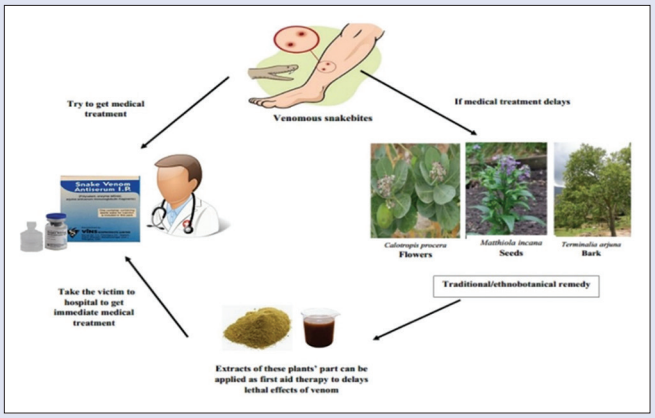
ABSTRACT

Background: Snakebite is a commonly neglected tropical disease globally. According to the World Health Organization, tens of thousands of deaths have been reported due to the venomous snakes previously. Among such snakes, vipers of the genus *Echis* are extremely important clinically. In folk medicine, plants are commonly used for the treatment of snakebites even though snake venom antiserum is the only efficacious treatment currently known. **Objectives:** This study was conducted to investigate the inhibitory potential of extracts from Pakistani medicinal plants against protease enzyme abundant in *Echis carinatus* venom. **Materials and Methods:** Organic extracts from different indigenous plant species and parts were used for *in vitro* determination of their inhibition against protease activity in snake venom. Active methanolic extracts were further fractioned using different solvents, and these fractions were also tested for antiprotease activity. **Results:** Results of this study show that *Calotropis procera* (Wild.) R. Br., *Matthiola incana* (L.) R. Br., and *Terminalia arjuna* Wight and Arn were able to neutralize the protease enzyme by 63%, 71%, and 66%, respectively. *Trichodesma indicum* (L.) R. Br. showed 51% inhibition of protease activity. **Conclusion:** The present study indicated about *C. procera* (Wild.) R. Br., *M. incana* (L.) R. Br., and *T. arjuna* Wight and Arn possessed inhibitor (s) against protease enzyme present in *E. carinatus* venom and would be worthwhile for development as treatment against envenomation in future.

Key words: *Calotropis procera*, *Echis carinatus*, *Matthiola incana*, natural remedy, Pakistani, *Terminalia arjuna*

SUMMARY

- Snake envenomation is a common yet neglected problem worldwide including Pakistan. Medicinal plants possess compounds that act differently (either individually or synergistically) to overcome snake venom potency by inhibiting its distribution and reducing physiological reactions, thus leading to complete neutralization of venom toxicity. The purpose of this study was to evaluate the inhibitory potential of selected medicinal plants against the protease enzyme of *Echis carinatus* venom. Results indicate that *Calotropis procera* (Wild.) R. Br., *Matthiola incana* (L.) R. Br., and *Terminalia arjuna* Wight and Arn have the ability to neutralize protease enzyme.



Abbreviations used: *E. carinatus*: *Echis carinatus*; EDTA: Ethylenediaminetetraacetic acid; SVMPs: Snake venom metalloproteases; NIH: National Institutes of Health.

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INTRODUCTION

Snakebite is a common yet neglected medical problem throughout some parts of the world. It has a close association with rural communities and is often regarded as a "disease of poverty."[1] Epidemiological data concerning snakebites show that about 1.8 million venomous snakebites occur annually, which leads to nearly 125,000 deaths.[2] Effects of snake venom range from minor consequences, including edema and pain,[3] to more severe conditions such as permanent tissue damage, renal failure, hemorrhage, hematuria, hemoptysis, anemia, melena, hypotension, and hematemesis.[4] It is a public health issue, particularly in Asian and African countries such as Pakistan, Indonesia, Mali, and Nigeria predominantly facing 20,000 deaths/year; Bangladesh about 6000 deaths/year; and Cameroon, India, and Ghana about 50,000 deaths/year.[5,6]

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Among numerous snakes, vipers of the genus *Echis* are extremely important clinically and have been added to the WHO Category 1 list of neglected tropical diseases.[7] In South Asia, bites caused by *Echis carinatus* is a severe threat as it leads to a significant number of deaths. In Pakistan, *E. carinatus* is generally found throughout the country. However, it has high population densities in Astola Island (Makran Coast, Balochistan) as well as in the deserts of Cholistan and Thar.[8]

E. carinatus is a concoction of bioactive molecules attributed for degradation of tissue structure as well as hemorrhage.[9] The majority of proteins that have been studied extensively in *E. carinatus* venom are metalloproteinase, disintegrins, and phospholipases A_2. Metalloproteinases present in venom are involved in different hemostatic activities including inhibition of platelet aggregation through binding with glycoprotein IIb–IIIa receptors, enzymatic degradation of basement membrane (i.e., laminin, collagen IV, and fibronectin), hydrolysis of endothelial cell proteins (i.e., integrins and cadherins) as well as activation of prothrombin.[10] Metalloproteases from *E. carinatus* venom have been defined as prothrombin activators. These activators inhibit clotting factors that induce coagulation, thus ultimately leading to microvascular thrombosis.[11]

Indeed, antiserum is the only known efficacious therapy for the management of snakebites. However, venom antiserum has some limitations associated with it, including cost, significant preparation time, and special storage conditions. It has the potential to neutralize systematic but not local effects,[12] and sometimes, it causes adverse reactions in victims after administration.[13] Most snakebite cases happen in a rural area where snake venom antiserum is not available. Due to all these limitations, the management of snakebites through antiserum is quite a challenge for medical professionals in affected areas. Because of this, plant-based management of snakebite could be quite advantageous. Pakistan has an incredible diversity of plants. Plants are rich sources of active compounds that are currently used by people to treat numerous diseases, including snakebites. Numerous studies have reported plants neutralizing the effects of *E. carinatus* venom components.[14-19] The present research was conducted to evaluate the antiprotease activity of various medicinal plants that are traditionally used as an antivenom against snakebite ubiquitously present in Pakistan.

MATERIALS AND METHODS

Snake venom and chemicals

Pakistani *E. carinatus* venom was gifted from the National Institutes of Health (NIH), Islamabad, Pakistan. Lyophilized venom was stored in light-resistant bottles at 2°C–8°C. The rest of all chemicals were purchased from Merck unless and otherwise described.

Medicinal plants

For this study, plants were selected based on previous ethnobotanical evidence of anti-snake venom activity. Medicinal plants were collected from various regions of Pakistan; however, few were procured from the local market named Naswari Bazar in Rawalpindi. All plants were identified by expert botanist Dr. Zafar Ullah Zafar, and voucher specimens were submitted to the herbarium of the Department of Botany, B. Z. University, Multan, Pakistan. Complete detail about medicinal plants is summarized in Table 1.

Extraction process

Shade-dried parts of the plants were crushed using an electric grinder. Dried powder was soaked in methanol at ambient temperature for a period of 4 weeks. Filtration was done initially with ordinary filter paper and subsequently with Whatman filter paper 41. After filtration, methanol was allowed to evaporate at room temperature and prepared plant extracts were stored for further use.[25]

Enzymatic assay for proteases

Antiprotease activity of medicinal plants was analyzed through casein as a substrate. Briefly, reaction mixture containing 0.5% casein (in Tris-HCl, pH 7.5) and venom (0.1–1.6 mg; phosphate buffer, pH 8) was incubated at 37°C for 10 min. After incubation, the reaction was stopped by the addition of trichloroacetic acid and mixture was filtered. Filtrate was used to estimate enzyme activity using L-tyrosine as standard. In the above analysis, one unit of enzyme activity was expressed as 0.02 µmole of tyrosine released per hour. For inhibition studies, venom was preincubated with plant extracts at 37°C for 15 min.[28,29]

Fractionation of active plant extracts

Active methanolic plant extracts were further fractioned using different solvents including n-hexane, chloroform, dichloromethane, and ethyl acetate.[30] After drying, all fractions were again tested for antiprotease activity using the previously described assay.

Phytochemical analysis

Phytochemical screening was performed for both active methanolic plant extracts and their active fractions using standard analytical

Table 1: List of Pakistani medicinal plants to neutralize snakebite problem

Medicinal plants (voucher number)	Family	Part used	References
Adiantum capillus-veneris L. (R.R. Stewart F.W.Pak. 4[2])	Pteridaceae	Whole plant	[20]
Albizia lebbeck (L.) Benth. (R.R. Stewart F.W.Pak. 381[9])	Fabaceae	Seeds	[20]
Althaea officinalis L. (R.R. Stewart F.W.Pak. 477[6])	Malvaceae	Roots	[21]
Calotropis procera (Wild.) R. Br. (R.R. Stewart F.W.Pak. 566[6])	Apocynaceae	Flower	[20]
Citrullus colocynthis (L.) Schrad. (R.R. Stewart F.W.Pak. 702[10])	Cucurbitaceae	Fruit	[20]
Curcuma longa L. (R.R. Stewart F.W.Pak. 66[3])	Zingiberaceae	Rhizome	[22]
Eclipta prostrata (L.) L. Mint (R.R. Stewart F.W.Pak. 743[5])	Asteraceae	Whole plant	[20]
Eugenia jambolana Willd. ex O. Berg (R.R. Stewart F.W.Pak. 504[2])	Myrtaceae	Fruit	[20]
Fagonia arabica L. (R.R. Stewart F.W. Pak. 433[2])	Zygophyllaceae	Leaves and twigs	[23]
Lepidium sativum L. (R.R. Stewart F.W.Pak. 319[4])	Brassicaceae	Whole plant	[24]
Matthiola incana (L.) R. Br. (R.R. Stewart F.W.Pak. 322[2])	Brassicaceae	Seeds	[25]
Momordica charantia L. (R.R. Stewart F.W. Pak. 706[1])	Cucurbitaceae	Fruits	[22]
Trichodesma indicum (L.) R. Br. (R.R. Stewart F.W.Pak. 604[3])	Boraginaceae	Leaves	[26]
Psoralea corylifolia L. (R.R. Stewart F.W. Pak. 418[1])	Fabaceae	Seeds	[25]
Rubia cordifolia L. (R.R. Stewart F.W. Pak. 689[4])	Rubiaceae	Roots	[20]
Sapindus mukorossi Gaertn. (R.R. Stewart F.W. Pak. 463[3])	Sapindaceae	Fruits	[20]
Swertia chirayita (Roxb. ex Flem.) Karst. (R.R. Stewart F.W.Pak. 561[4])	Gentianaceae	Stems	[27]
Terminalia arjuna Wight and Arn. (R.R. Stewart F.W.Pak. 502[4])	Combretaceae	Bark	[20]

procedures. A complete picture of phytochemical screening is given in Table 2.

RESULTS AND DISCUSSION

The protease enzyme works by causing the breakdown of proteins into their structural amino acid units. Standard curves (absorbance vs. concentration) were constructed using different concentrations of L-tyrosine [Figure 1]. Various concentrations of *E. carinatus* venom were tested using casein as a standard substrate to check protease activity. Activities at venom concentration of 0.1, 0.2, 0.4, 0.8, and 1.6 mg were found to be 68, 75, 120, 178, and 259 units/ml, respectively [Table 3]. A standard concentration (0.8 mg) was then used to evaluate the antiprotease activity of Pakistani medicinal plants. In the current study, snake venom antiserum and ethylenediaminetetraacetic acid (EDTA) were used as reference standards. The results of antiprotease activity from medicinal plant extracts, EDTA, and snake venom are shown in Table 4. Different fractions of active plants' extract were further tested for their antiprotease activity against snake venom. The antiprotease activity of different fractions of *C. procera* (Wild.) R. Br., *Matthiola incana* (L.) R. Br., and *T. arjuna* Wight and Arn is presented in Tables 5-7 respectively. In case of *M. incana* (L.) R. Br., maximum inhibition was achieved for n-hexane and ethyl acetate fractions. However, maximum inhibition was achieved with *C. procera* (Wild.) R. Br. and *T. arjuna* Wight and Arn with n-hexane and ethyl acetate fractions, respectively. The phytochemical analysis of *C. procera* (Wild.) R. Br., *M. incana* (L.) R. Br., and *T. arjuna* Wight and Arn is shown in Table 8. Active fractions of plant extracts were also analyzed for their phytochemical content, and the results of this analysis are summarized in Table 9.

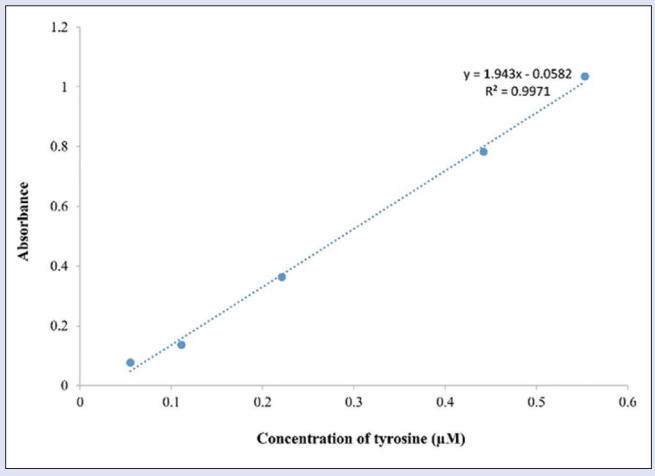

Figure 1: Standard curve for proteolytic activity. L-tyrosine was used as an indicator of protein degradation

Bites caused by snakes of family Viperidae are attributed to the high morbidity and mortality rates globally. In Pakistan, an increase in snake bites is believed to be caused by deforestation and migration of poisonous snakes toward human settlements.[38] Among numerous viper snakes, *E. carinatus* is the most dangerous one. *E. carinatus* venom is a rich source of serine and metalloproteases. Majority of the *E. carinatus* activities are due to the presence of metalloproteases (90%). Snake venom metalloproteases have a significant relation with extracellular membrane and coagulation cascades which results in a variety of hemostatic dysfunctions and tissue damage. Snake venom interaction with coagulation cascades leads to intravascular coagulation. Procoagulant effect of *E. carinatus* venom is due to the presence of ecarin and carinactivase, both of which are metalloproteases, which activates prothrombin activator.[39] Mortality after snake venom envenomation is typically caused by systemic hemostatic disruption, which usually results in systemic hemorrhage. Hemorrhage is the most significant effect caused by snake bites of family Viperidae. The main event behind this lethal effect is microvasculature damage caused by snake venom.[12,40,41]

Medicinal plants are the most accessible resource for the treatment of numerous diseases including snake bites. People of Pakistan usually rely on herbal medicines for the treatment of snake bites. These medicines are easily available by traditional practitioners in those areas.[20] This extensive use of plant-based medicine for snakebite management is ascribed to a range of bioactive compounds in plants that have the ability to neutralize snake venom effects. Phytochemicals neutralize the venom effect either by reducing its diffusion or causing disintegration of venom components.[42] In this regard, the present research was designed to evaluate the antiprotease activity of 18 medicinal plant extracts against *E. carinatus* venom.

The results of this study show that percentage inhibition for snake venom antiserum and EDTA was found to be 78.5% and 64%, respectively [Table 4]. Among 18 selected medicinal plants, three plants, i.e., *C. procera* (Wild.) R. Br., *M. incana* (L.) W. T. Aiton, and *T. arjuna* Wight and Arn, were able to significantly neutralize the protease activity of *E. carinatus* venom, which was comparable to standard inhibitors. *C. procera* (Wild.) R. Br. inhibited the activity by 63%, *M. incana* (L.) W. T. Aiton 71%, and *T. arjuna* Wight and Arn by 66%. Parentage inhibition for *Trichodesma indicum* (L.) R. Br. was found to be 51%. All other plants show inhibition activity < 50% [Table 4]. *C. procera* (Wild.) R. Br. n-hexane fraction was able to neutralize the protease enzyme close to the crude extract [Table 5]. Percentage inhibition for n-hexane fraction was 64.6%. For *M. incana* (L.) W. T. Aiton, two fractions, i.e., n-hexane and ethyl acetate, were effective. N-hexane fraction inhibits protease activity by 71.7% and ethyl acetate fraction by 74.8% [Table 6], whereas for *T. arjuna* Wight and Arn, ethyl acetate fraction exhibited the inhibitory effect (65.8%) close to crude extract [Table 7]. Various studies have reported such neutralizing potential of plants against protease activity of *E. carinatus* venom. *Albizia lebbeck* L. seeds' extract was able to inhibit hyaluronidase (IC_{50} = 91.95 µg; $P < 0.0001$) and protease (IC_{50} = 36.32 µg;

Table 2: Brief summary of phytochemical analysis

Phytochemicals	Tests	Results	References
Alkaloids	Plant extract + 1% HCl+steam. Add Wagner's reagent (6 drops)	Reddish-brown precipitate	[31]
Carbohydrates	2 ml extract + 5 drops of iodine solution	Blue color	[32]
Fatty acids	0.5 ml crude extract + 5 ml ether+ evaporate on filter paper	Transparency on filter paper	[33]
Flavonoids	Extract + dilute NaOH + dilute HCl	Intense yellow color which turns colorless with dilute HCl	[34]
Glycosides	Extract + glacial acetic acid + 1 drop of $FeCl_3$ + H_2SO_4	Formation of brown ring	[35]
Phenols/tannins	Plant extract + 2% ferric chloride solution	Black or blue-green color	[36]
Proteins	2 ml extract + 1 ml 40% NaOH + few drops of 1% $CuSO_4$	Violet color	[37]
Saponin	Filtrate + 5 ml distilled water. Shaken well	Frothing	[34]
Steroids/terpenoids	5 ml extract + 2 ml of chloroform + 3 ml of H_2SO_4	Reddish brown color of solvent interface	[35]

Table 3: Protease activity posed by Pakistani snake *Echis carinatus* venom

Concentrations of venom (1 mg/1 ml)	Absorbance at 660 nm (mean±SD)	Enzyme activity (units/ml)
0.1 mg	0.046±0.0029	68
0.2 mg	0.057±0.0032	75
0.4 mg	0.125±0.0036	120
0.8 mg	0.214±0.0054	178
1.6 mg	0.336±0.0032	259

SD: Standard deviation

Table 4: Antiprotease activity of selected medicinal plants (0.8 mg/0.8 ml) from Pakistan to neutralize *Echis carinatus* (0.8 mg/0.8 ml) venom

Evaluated antidote	Protease activity (units/ml)	Inhibition (%)
Adiantum capillus-veneris L.	105	41
Albizia lebbeck (L.) Benth.	133	25
Althaea officinalis L.	113	36
Calotropis procera (Wild.) R. Br.	65	63
Citrullus colocynthis (L.) Schrad.	127.6	28
Curcuma longa L.	148.6	16.5
Eclipta prostrata (L.) L. Mint	121.5	31.7
Eugenia jambolana Willd. ex O. Berg	100.5	43.5
Fagonia arabica L.	127.6	28
Lepidium sativum L.	51.5	71
Matthiola incana (L.) R. Br.	136.8	23
Momordica charantia L.	87	51
Trichodesma indicum (L.) R. Br.	142.5	20
Psoralea corylifolia L.	102	42.5
Rubia cordifolia L.	119	33
Sapindus mukorossi Gaertn.	127	28
Swertia chirayita (Roxb. ex Flem.) Karst.	101	43
Terminalia arjuna Wight and Arn	60	66
Standard inhibitors		
EDTA (1.2 mM)	63.7	64
Antisera	38	78.5

EDTA: Ethylenediaminetetraacetic acid

Table 5: Antiprotease activity of different fractions of *Calotropis procera* (Wild.) R. Br. extract

Botanical name	Fractions	Protease activity (units/ml)	Inhibition (%)
Calotropis procera (Wild.) R. Br.	n-Hexane	63	64.6
	Chloroform	122	31
	Dichloromethane	155.7	12.6
	Ethyl acetate	92	48

Table 6: Antiprotease activity of different fractions of *Matthiola incana* (L.) R. Br.

Botanical name	Fractions	Protease activity (units/ml)	Inhibition (%)
Matthiola incana (L.) R. Br.	n-Hexane	50	71.7
	Chloroform	97.5	45
	Dichloromethane	101.8	42.8
	Ethyl acetate	44.8	74.8

Table 7: Antiprotease activity of different fractions of *Terminalia arjuna* Wight and Arn

Botanical name	Fractions	Protease activity (units/ml)	Inhibition (%)
Terminalia arjuna Wight and Arn	n-Hexane	92	48
	Chloroform	156.7	12
	Dichloromethane	86	51
	Ethyl acetate	61	65.8

Table 8: Phytochemicals analysis of *Calotropis procera* (Wild.) R. Br, *Matthiola incana* (L.) R. Br., and *Terminalia arjuna* Wight and Arn crude extracts

Phytochemicals	*Calotropis procera* (Wild.) R. Br.	*Matthiola incana (L.)* R. Br.	*Terminalia arjuna* Wight and Arn
Carbohydrates	+	+	+
Alkaloids	+	+	+
Fatty acids	–	+	–
Flavonoids	+	+	+
Glycosides	+	+	+
Phenols/tannins	+	+	+
Proteins	+	–	+
Saponin	+	+	+
Steroids/terpenoids	+	+	+

+ indicates presence while–represents absence of phytochemicals

$P < 0.0001$) activities through *in vitro* evaluation.[14] *Cassia auriculata* L. leave extracts neutralized the protease enzyme activity of *E. carinatus* venom by 96%.[18] This study showed that extract of *Cassia auriculata* L. leaves also inhibited hemorrhagic, edematogenic, myotoxic, and lethal effects of *E. carinatus* venom. Another study revealed that *Tabernaemontana alternifolia* (Roxb) roots' extract showed the neutralization ability against the protease enzyme of *E. carinatus* venom.[19] Girish *et al*.[43] reported the root extract of *Mimosa pudica* L. against protease enzyme of *E. carinatus* venom. This study revealed that aqueous root extract was able to neutralize protease activity in a dose-dependent manner.

Phytochemical analysis of active fractions of plants' extract enunciated about various phytochemicals such as alkaloids, flavonoids, phenols, saponins, and steroids. A complete summary of phytochemical screening is encompassed in Tables 8 and 9. Many *in vitro* and *in vivo* studies were reported to confer potential of alkaloids, terpenoids, polyphenols, flavonoids, saponins, and glycosides as an antidote.[44] The presence of these phytochemicals in active plant extracts could be the reason behind the neutralization of protease enzymes abundant in *E. carinatus* venom. Future investigation about isolation and characterization of active metabolites from potential plant extracts could play a tremendous role in the development of effective antidote against *E. carinatus* snake bite.

CONCLUSION

The present study indicated about *C. procera* (Wild.) R. Br., *M. incana* (L.) R. Br., and *T. arjuna* Wight and Arn possessed inhibitor(s) against protease enzyme present in *E. carinatus* venom and would be worthwhile for development as treatment against envenomation in the future.

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Nil.

Conflicts of interest

There are no conflicts of interest.

Table 9: Phytochemicals analysis of active fractions of *Calotropis procera* (Wild) R. Br., *Matthiola incana* (L.) R. Br., and *Terminalia arjuna* Wight and Arn crude extracts

Phytochemicals	*Calotropis procera* (Wild.) R. Br. n-Hexane	*Matthiola incana* (L.) R. Br.		*Terminalia arjuna* Wight and Arn Ethyl acetate
		n-Hexane	Ethyl-acetate	
Alkaloids	+	+	+	+
Fatty acids	–	–	–	–
Carbohydrates	–	–	–	–
Flavonoids	+	+	+	+
Glycosides	+	+	+	+
Phenols/Tannins	+	+	+	+
Proteins	–	–	–	–
Saponin	+	+	+	+
Steroids/terpenoids	+	+	+	+

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