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Research Paper

Investigation of skin permeation, *ex vivo* inhibition of venom-induced tissue destruction, and wound healing of African plants used against snakebites

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ABSTRACT

Ethnopharmacological relevance: Snakebite envenomation causes 5000–10,000 mortalities and results in more than 5–15,000 amputations in sub-Saharan Africa alone every year. The inaccessibility of antiserum therapy is a vast problem, and only about 2.5% of the actual need for antiserum in Africa is covered. Numerous plants have shown *in vitro* inhibitory activity against one or more of the hydrolytic enzymes involved in snakebite-induced necrosis. However, a more thorough examination of the plant species in *ex vivo* and *in vitro* cell assay models is needed to test their ability to inhibit necrosis.
Materials and methods: Extracts which had previously shown *in vitro* inhibitory activity against necrosis enzymes, were tested in an *ex vivo* air–liquid-interface model, and a wound healing scratch assay as well as for their ability to permeate the skin barrier and inhibit venom induced cell death.
Results: Of the 14 water extracts and 16 ethanol extracts tested at a concentration of 10 μg/mL, only the ethanol extracts of *Tamarindus indica* and *Paullinia pinnata* resulted in a small but significant increase in cell migration of around 10% compared to treatment with buffer after 24 h treatment. The remaining extracts showed no effect, or they even delayed the cell migration compared to the treatment with buffer. After 48 h treatment, 10 of the tested extracts showed a decreased cell migration compared to no treatment. At a 100 μg/mL concentration all the extracts inhibited cell migration and five extracts killed some of the cells, while four extracts killed all the cells. Ten of the thirty extracts were tested in a Franz cell set-up but none of the extracts tested did permeate the skin barrier over a 48 h period, and will therefore be of very limited use topically in the initial treatment of snakebites in its present form. None of the extracts were able to directly interact with the enzyme to lower the cell toxicity of the venom. Two extracts, *Dichrostachys cinerea* and *Grewia mollis*, were tested in the *ex vivo* model, but none of them inhibited the tissue destruction caused by venom.
Conclusion: On the basis of this study, topical treatment with plant extracts for snakebite-induced tissue necrosis cannot be recommended.

1. Introduction

Many snakebites occur in parts of the world where antiserum therapy is not easily accessible, and only about 2.5% of the actual need for antiserum in Africa is covered (Chippaux, 1998; Brown, 2012). This inaccessibility is a serious problem

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DPBS, Dulbecco's phosphate-buffered saline; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PDGF, Platelet-derived growth factor

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especially in sub-Saharan Africa where approximately 314,000 people are bitten every year—causing 5000–10,000 deaths and 5000–15,000 amputations (Chippaux, 2011). The established and only effective treatment for snakebite envenomations is mono- or polyvalent antiserum. However, antiserum provide limited protection against local tissue damage because the antiserum might lack the antibodies of the toxins involved in local tissue damage or the *in vivo* distribution of neutralizing antibodies is too slow compared to the fast action of the toxins (Rucavado and Lomonte, 1996). The diffusion of toxins from snake venom from the site of the bite depends on the extent of local tissue destruction (Kemparaju and Girish, 2006). This makes the initial inhibition of toxins and enzymes in the bite site highly important. Hence the local acting enzymes are the rate limiting step in the treatment of snakebites, and inhibition of these is believed to increase the survival time of the victims (Girish and Kemparaju, 2005). Older studies shows that in developing countries about 80% of snakebite victims prefer to be treated by a traditional healer, and they are therefore not treated with antivenom therapy (Reid and Theakston, 1983; Chippaux, 1998). Due to a decreasing number of antivenom in Africa from 250.000 to less than 20.000 doses over a 25 years period (Brown, 2012), traditional healers are still of immense importance today. The use of plants is a major part of the traditional practitioners' treatment, and they have extensive knowledge of plant species used as treatment against snakebites. Many articles have listed the ethnopharmacological use of a variety of plants both topically and systemically (Houghton and Osibogun, 1993; Coe and Anderson, 2005; Soares et al., 2005; Molander et al., 2012). Numerous *in vitro* and *in vivo* studies have been performed for assessing the beneficial effect of plants against the enzymes and toxins in snake venom (Otero et al., 2000; Borges et al., 2005; Girish and Kemparaju, 2005; Nunez et al., 2005; Ticli et al., 2005; Cintra-Francischinelli et al., 2008; Leanpolchareanchai et al., 2009; Camargo et al., 2010; Gomes et al., 2010; Pithayanukul et al., 2010). In our previous study of plants used in Mali, South Africa, and DR Congo, 41 plants showed inhibitory activity against one or more of the hydrolytic enzymes hyaluronidase, phospholipase A_2, and protease—which are all believed to cause local necrosis in the skin after snakebite (Molander et al., 2014). The objective of this study was to test the above-mentioned plant species for their ability to inhibit necrosis in more complex *ex vivo* and cell assay models.

2. Materials and methods

2.1. Plant materials and preparation

The plant material used and the preparation of extracts were as previously described in Molander et al. (2014). Plant species and vouchers can be seen in Table 1.

2.2. Neutralization of venom—Ex vivo air–liquid interface model

The model was modified from a porcine wound healing model (granted patent DE 10317400; (Pollok et al., 2011) but venom was injected into dermis of the biopsy instead of creating a wound). Pig ears were delivered from a local abattoir in Hamburg. All the pigs were six months old and of the race crossbred Yorkshire/Deutsches Edelschwein. After washing and disinfection of the pig ears, punch biopsies (⌀6 mm) were taken from the plicae of the ears. Some of the fat and subcurtis was removed to ensure equal heights of the biopsies. Five microliters of 20 mg/mL *Bitis arietans* venom was injected into dermis of the biopsies. The biopsies were placed with dermis down on gauze in culture dishes filled with Dulbecco's modified Eagle's medium (DMEM) supplemented with hydrocortisone, fetal calf serum, penicillin, and streptomycin. The medium was only in contact with the dermis, thus leaving epidermis exposed to the air to create an "air–liquid-interface". The plant extract was redissolved in concentrations of 0.5 mg/mL or 5 mg/mL in Dulbecco's phosphate-buffered saline (DPBS) and 10 μL was injected into the dermis 10 min after the venom injection. The biopsies were incubated with 5% CO_2 at 37 °C for 24 h. The samples were snap-frozen in isopentane pre-cooled with liquid nitrogen, and stored at −80 °C.

Table 1
List of plants tested in this study. Inclusion of plants is based on results found in Molander et al. (2014).

Family	Plant species	Plant part	Voucher	Country	Extract
Amaranthaceae	*Pupalia lappacea* Juss	Herba	1218 (DMT)	Mali	Water
Anacardiaceae	*Lannea acida* A. Rich.	Cortex	1145 (DMT)	Mali	Water and ethanol
	Sclerocarya birrea Hochst.	Cortex	0077 (DMT)	Mali	Water
	Spondias mombin L.	Radix	1148 (DMT)	Mali	Ethanol
	Spondias mombin L.	Cortex	1148 (DMT)	Mali	Ethanol
Clusiaceae	*Psorospermum corymbiferum* Hochr.	Cortex	2651 (DMT)	Mali	Water
Colchicaceae	*Gloriosa superba* L.	Radix	(DMT)	Mali	Water
Combretaceae	*Combretum molle* R. Br. ex G.Don	Folium	1060 (DMT)	Mali	Water and ethanol
Combretaceae	*Guiera senegalensis* J.F. Gmel.	Radix	0991 (DMT)	Mali	Water
Fabaceae	*Bauhinia thonningii* Schumach.	Cortex	B904 (LWI)	DR Congo	Ethanol
	Bauhinia thonningii Schumach.	Radix	0885 (DMT)	Mali	Water and ethanol
	Burkea africana Hook.	Cortex	1168 (DMT)	Mali	Ethanol
	Dichrostachys cinerea (L.) Wight & Arn.	Folium	1798 (DMT)	Mali	Ethanol
	Parkia biglobosa Benth	Cortex	1801 (DMT)	Mali	Ethanol
	Swartzia madagascariensis Desv.	Folium	0527 (DMT)	Mali	Ethanol
	Tamarindus indica L.	Cortex	1545 (DMT)	Mali	Ethanol
Hypoxidaceae	*Curculigo recurvata* W.T.Aiton	Folium	B001 (LWI)	DR Congo	Water
Lamiaceae	*Haumaniastrum galeopsifolium* (Baker) Duv. & Plancke	Herba	B390 (LWI)	DR Congo	Water
	Teucrium kraussii Codd	Herba	AY 1481 (NU)	South Africa	Water
Loganiaceae	*Strychnos innocua* Delile	Folium	B442 (LWI)	DR Congo	Water
Malvaceae	*Dombeya quinqueseta* (Delile) Excell	Cortex	1332 (DMT)	Mali	Ethanol
	Grewia mollis Juss.	Cortex	0573 (DMT)	Mali	Water and ethanol
	Waltheria indica L.	Radix	1339 (DMT)	Mali	Water and ethanol
Rhamnaceae	*Ziziphus mucronata* willd.	Radix	2222 (DMT)	Mali	Ethanol
Sapindaceae	*Paullinia pinnata* L.	Radix	0197 (DMT)	Mali	Ethanol