INTRODUCTION

Many people now (and historically) world-wide depend on plants and plant extracts as the main source of their medications. The World Health Organization itself has endorsed the use of native medicines and initiated a strategy in 2002 and again 2014-2023 [1] for world-wide development and safe use, which includes extracts as well as pure compounds. Some ethnomedicines have led to lead drugs [2-6] for further development.

Snake envenomation has historically been a life-threatening major challenge to indigenous and other rural peoples worldwide, and as a result, cultures often have anti-venom treatments using local botanicals that are of both pharmacological and anthropological interest to study. In 2009, the World Health Organization officially designated snakebite envenoming an important but neglected tropical disorder [7], with a recognized need for an integration of snakebite envenoming within a global strategy to combat the neglected tropical diseases [8]. Estimates indicate thousands of people in the U.S receive hospital treatment [9-11], with an estimated 4000 deaths in the Americas per year and many more worldwide [12,13].

Although snake envenomation remains a worldwide problem, treatment is limited. If the victim has access to high-level medical care, therapies are supportive, palliative, and may rely on antivenoms. Antivenoms, made as antisera, can be life-saving therapeutics but are expensive, not always readily available [14], requiring as well a hospital setting for administration. Antivenoms are aimed at specific species, which often requires knowing what species is involved, which can be unclear. Even with identification, treatment failures occur and successful treatment does not always prevent significant tissue loss. Thus, the biomedical interest in plants inhibiting venoms as available drugs to the general population and as possible sources of lead drugs continues and for which this paper adds useful information on *Eryngium*.

As this is an endemic, life-threatening medical risk, how did Native American peoples treat snake envenomation, and is there a scientific basis for the practice, a pharmacological validation

**An in vitro evaluation of the Native American ethnomedicinal plant Eryngium yuccifolium as a treatment for snakebite envenomation**

Joseph A. Price III

ABSTRACT

**Aim:** At least seven North American tribes specifically mention the use of *Eryngium* (typically roots) as an anti-snake venom therapy. As snake envenomation is an endemic, life-threatening medical risk, is there a scientific basis for the Native American ethnomedicine? Could this be demonstrated in an assay amenable to mechanistic evaluation and high throughput screening for later isolation and possible evaluation as a source for a lead drug? 

**Materials and Methods:** Proteases, mainly metalloproteases, are thought to be the main pathological agents in most American snake venoms. Water extracts of four plant parts of *Eryngium yuccifolium* were tested for enzyme inhibition in three highly sensitive in vitro protease assays, with multiple venoms.

**Results:** Interestingly, activity was found in all plant parts, not just the roots, in the general protease assay, also in the most specific assay for collagenases, but less so for elastases where enzymatic activity was low, and against five species of American snake venoms. Inhibition spared the activity of a mammalian elastase, suggesting it has some specificity. In dose response assays, inhibitory activity in extracts of *Eryngium* was noticeably more effective than randomly chosen plants and comparable to some others.

**Conclusions:** All data shown here are consistent with pharmacological inhibition of proteases in at least selected venoms of common venomous snakes by *Eryngium* extracts. Moreover, as the genus is widely distributed in America, the ethnomedical practice of using this plant as an anti-snake venom treatment is supportable, may have been common, and suggests further bioactivity and phytochemical studies are warranted.

**KEY WORDS:** *Eryngium*, ethnopharmacology, protease inhibition, snakebite, toxins, venoms
that would also support the study of this genus for possible lead drugs? Several plants in North America have been reputed to have anti-venom properties but have been unverified. Unusually, one genus of plants has a world-wide reputation and ethnomedicinal use for both as an antitoxic and anti-inflammatory, *Eryngium*. In the U.S. these are wild flowers [15,16] of distinctive appearance found in a variety of natural habitats while some varieties are available through commercial plant suppliers. Some may be endangered or threatened in many locales including where habitat is being lost for both coastal and native prairie species [17]. American *Eryngium*, usually *Eryngium aquaticum* or *Eryngium yuccifolium* (often var. *synchaetum* or *Michigan*), are referred to as “rattlesnake master” and ethnomedicinally used (usually topically) to treat snake envenomations (Crotalid). As published, *Eryngium* for this purpose was used by at least six tribes, the indigenous Choctaw, Chickasaw, and Creek [18,19], Meskwaki [20], Cherokee [21], and Mikasuki [22]. One might expect even more accounts, but as Taylor [19] pointed out in her study of five tribes, even if the plants are available to neighboring tribes, they typically did not share medicinal information [18]. Further, the removal of eastern “civilized” tribes to Oklahoma Territory caused their herbalists to adapt to a new ecosystem, and reculturization (often forced) of almost all U.S. tribes has caused much of the herbal knowledge to become isolated or lost. Moreover, unlike Arab countries, since North American peoples did not keep written records, ethnomedical accounts as cited are likely an underestimate of use. Ethnological phytomedicinal uses for *Eryngium* have been reported among other indigenous peoples as well, including as a topical antidote to scorpion envenomation [23,24].

To test the likely efficacy of the ethnological practice, one can ascertain if the pharmacology supports the use of the plant for that therapeutic application with a pathologically relevant *in vitro* system. The venom of most North American snakes is degradative not neurotoxic, and it is argued that their main pathophysiological agents are metalloproteases [25], largely collagenases. Recently, research has included seeking low molecular weight natural antagonists from plants to snake venom, e.g. as reviewed recently [26], or to venom metalloproteases proteases specifically [27], or to proteolytic and phospholipase activities in Bothrops venoms [28]. This is aside from naturally occurring factors in venom-resistant animals, anticipated to often be polypeptides, and thus possibly immunogenic and not therapeutically suitable. Libraries of antiprotease compounds can be screened for their inhibition of protease and collagenase activities using a variety of North American venoms [29]. Moreover, as others have stated, the possible antivenom mechanisms of action by this plant genus should be explored [30].

To evaluate *Eryngium* antivenom activity, the approach was to secure unrelated plants, make aqueous extracts, and test extracts for antiprotease activity. Further, was the activity limited to roots as reflected in historical and popular use; to any one species of snake; and was this a particularly potent activity in this plant compared to other unrelated plants? Is there an *in vitro* activity that would support the ethnomedicinal use of the plant for envenomation? If so, this would also provide a basis for later *in vivo* studies and any phytochemical compound isolations.

**MATERIALS AND METHODS**

Laboratory chemicals were from Sigma-Aldrich®. Water was 18 mΩ filtered on site. Venoms were obtained from Miami Serpentarium Laboratories (Punta Gorda FL, USA), dissolved in saline at 2 mg/mL, cleared at 10,000 × g for 5 min, aliquoted then frozen at −80°C until use. Venoms are mixtures of many enzymes and other agonists and may vary substantially in activity and composition between species and individuals, even to some extent between batches from the same animal.

*E. yuccifolium* Michx. plants were obtained from an established commercial supplier (Ion Exchange, 1878 Old Mission Drive - Harpers Ferry, Iowa). The common herbs dill (*Anethum graveolens* L.), fennel (*Foeniculum vulgare* Mill.), and sage (*Salvia officinalis* L.) were obtained from a local established commercial supplier (Southwood Nurseries, Tulsa, OK, USA). The plant names have been checked with www.theplantlist.org on June 5, 2015. Plants were grown to maturity locally and voucher specimens submitted for identification and archiving. Harvested *E. yuccifolium* plants were separated into flowers, rootlets, leaves, and corm, while aerial parts were used from other plants. Samples were washed in water, dried, and stored at −80°C until used. The composition of crude extracts from different plant tissues would be expected to have some components in common, but otherwise vary, as has been widely reported. Algae names were checked with algaebase (www.algaebase.org). Dried commercially prepared *Chlorella* (Beijerinck) and *Spirulina platensis* (Gamont) Geitler (also listed as *Arthrospira platensis* Comont) were each also purchased from commercial sources: (*Chlorella* sp. [Paragon Plus, Paragon, Torrance, CA, USA] and *S. platensis* [Solaray, Park City, Utah; Earthrise, Calipatria, CA, USA]). The dried algae were rehydrated in water at room temperature briefly before extraction. For all samples, 5 g of each ground tissue or algae were extracted with 15 mL 18 mΩ water at 42°C for 2 h, then re-extracted with 10 mL. Extractions were pooled, prefilledtered through a 1.2 um 32 mm filter (Supor Pall), aliquoted, and stored at −80°C for later assay. For each species, one pool of extract was made from multiple plants and assayed in replicates.

Assays were conducted as published [29] using EnzChek® Protease Assay, EnzChek® Elastase Assay, and EnzChek® Gelatinase/Collagenase Kits from InVitrogen according to kit instructions. The gelatinase substrate has been used for venoms previously [31]. Briefly, experiments were done by manual micropipetting, typically within a single black 96 well (Costar) microplate (typically n = 3 unless otherwise noted), always with plate-wide controls per plate. Reactions consisted of 100 uL of reagents (e.g., venom in buffer, plus plant extract) followed by 100 uL of substrate in buffer.

Due to the known variability of the composition of venoms between various species and within species, batches would be expected to show different levels of activity. In preliminary experiments (not shown), various concentrations of venom
from several different species were incrementally increased until all gave usefully active readings at the same concentration of venom, conditions later used to compare inhibition of the venoms by extracts.

Concentrations of venom and extract vary with the experiment and are shown in the Results section. For assays testing the inhibition of the enzyme activity in venoms, a concentration of enzyme was incubated for 15 min with the inhibitor, then substrate added and the reaction conducted for a sufficient length of time to demonstrate effective enzyme activity in the positive controls, typically 1-2 h. The highest practical concentration of extracts, as noted, were used for qualitative inhibition tests while in the later experiment a dose response was used to compare activities. Controls receiving substrate plus buffer served as the blanks and were included on each microplate. Appropriate wells with enzyme but no inhibitor served as activity controls. Data were always blank adjusted. None of the venoms were autofluorescent at the concentrations tested, i.e. never significantly fluorescent above the buffer blank. Reactions were conducted in triplicate, with the exception of blanks, which were done at either $n = 3$ or $n = 6$.

Measurements were done using a Perkin-Elmer HTS 7000 spectrofluorometer. Fluorescence data are in arbitrary units. Gain settings were customized for each assay to keep the substrate control values low and the active samples within measurable limits producing a workable dynamic range. Reactions were monitored with periodic measurements while single time point fluorescence values were used as per instructions in the kits for activity data unless otherwise noted. Preliminary data handling was done with Microsoft® Excel (Microsoft Office 2010) including macros programmed by me, while graphing and statistical analysis was done with GraphPad Prism version 6.04 for Windows (GraphPad Software, La Jolla, California, USA). All figures showing error bars are plotted as the mean plus/minus the standard error of the mean (SEM).

RESULTS

Most proteases will degrade casein while elastin and gelatin are preferentially degraded by elastases and collagenses, respectively. Each of these substrates was evaluated with a selection of venoms at several venom concentrations. Due to the heterogeneity of venoms, if the samples had been adjusted by dilution to have equal apparent activity, it would still be likely that the variety, number and specific activities of the enzymes would vary. Figure 1 shows the progress of an example reaction over time in each well with casein substrate and Crotalus viridis viridis venom. For each assay used, a substrate blank control treatment was always done on the same microplate, although decay of fluorescence was always minimal, as seen here.

A comparison of activity at the same venom concentration as shown in Figure 2 showed all venoms active, including a comparison control, the neurotoxic but the comparatively mildly degradative venom of Naja naja siamensis (Thailand). At an equivalent concentration (4 ug/mL) to other venoms tested, the activity of Naja naja siamensis was not significantly different from the negative controls (substrate blanks, data not shown). It required approximately 25 times the concentration of Naja naja siamensis venom (100 ug/mL) to display comparable protease activity.

It could be supposed that like many ethnomedicinal claims for plants as anti-venom medicines that the venom inhibitory activity of E. yuccifolium is found in the roots. Testing this hypothesis, extracts made from different parts of the plant were first examined to ensure extraction occurred, seen by the dry weight of extracted materials. As seen in Figure 3, except for the corn extract, which was very different from the other plant part extracts (Tukey’s multiple comparisons test, $P < 0.01$), all were similar, thus verifying the extraction and revealing relatively comparative concentrations of material for later activity analysis.

As seen in Figure 4, contrary to common anti-venom expectations from cultural beliefs, under conditions tested the extracts of leaves, flowers, rootlets, and corn all showed strong protease inhibition activity (different than positive controls,
Tukey’s multiple comparisons test, \( P < 0.001 \), but not different from each other. For subsequent experiments, a pool of extract from aerial parts was used.

To ask if the inhibition was limited to the venom of one species of snake, or to only one type of assay, a panel of venoms was used in the casein (general protease) assay, collagenase (collagenase/gelatinase, often metalloproteases) assay, and elastase assay, respectively. In evaluating inhibition at two levels of five venoms in the casein assay, the inhibition of each venom tested was seen to varying extents, shown in Figure 5, always statistically significant (unpaired \( t \)-tests per species, controls and treated, \( P < 0.05 \)). Tests at lower concentrations of venom were unrevealing due to marginal enzymatic activity in the venom. Thus, the inhibitory component(s) were active with much of the protease(s) activity in the variety of venoms.

Similarly, inhibition of gelatinase/collagenase at two concentrations of five venoms was also seen, shown in Figure 6, always statistically significant (unpaired \( t \)-tests per species, controls and treated, \( P < 0.05 \)). Patterns of inhibition, shown here for the first time and by direct within experiment comparisons, were similar to the general protease assay data (casein). These results might mean that most of the activity seen in the casein assay was due to enzymes with collagenase activity, although this need not be exclusively true.

Next, reactions tested if elastase activity, a degradative factor in venoms, is readily measured in this assay, and if it is inhibited by the plant extract. Porcine elastase (phylogenetically unrelated) served as a comparison control for the five venoms tested to see if inhibition showed specificity. As seen in Figure 7, levels of elastase activity were low compared to the other assays. Under these conditions, only inhibition of \textit{Crotalus atrox} and \textit{C. v. viridis} venom activity was seen (\( P < 0.01 \)). There was no significant inhibition of the porcine elastase, and inhibition was not limited to the least active venoms. Furthermore, the observed inhibition is selective, sparing the porcine enzyme and several venoms’ elastase activity as opposed to a non-discriminant general inhibitor of enzymes, as is the case for many small molecular weight compounds.

To ask if the inhibition of venom activities is particularly strong from \textit{E. yuccifolium} compared to other plants, extracts from five

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**Figure 3:** Tissues from four parts of \textit{E. yuccifolium} were extracted identically, and the yield shown as dry weight per mL of extract (means with SEM)

**Figure 4:** Inhibition of the protease (caseinase) activity of 125 ug/mL of \textit{C. adamanteus} venom by 2 mg/mL extracts of four parts of \textit{E. yuccifolium} (means with SEM)

**Figure 5:** Inhibition of the protease activity (caseinase) of five venoms (means with SEM) after treatment with 2 mg/mL of \textit{E. yuccifolium} extract, repeated at 10 ug/mL and 40 ug/mL of venom. \textit{Ag.c.c.: Agkistrodon contortrix contortrix} (copperhead), \textit{C.ad.: Crotalus adamanteus} (Eastern Diamondback rattlesnake), \textit{C. atrox: Crotalus atrox} (Western Diamondback rattlesnake), \textit{C. v. h.: Crotalus viridis helleri} (southern pacific rattler), \textit{C. v. v.: Crotalus viridis viridis} (Prairie rattlesnake)

**Figure 6:** Inhibition of the collagenase activity of five venoms (means with SEM) after treatment with 2 mg/mL of \textit{E. yuccifolium} extract, repeated at 20 ug/mL and 100 ug/mL of venom. \textit{Ag.c.c.: Agkistrodon contortrix contortrix} (copperhead), \textit{C.ad.: Crotalus adamanteus} (Eastern Diamondback rattlesnake), \textit{C. atrox: Crotalus atrox} (Western Diamondback rattlesnake), \textit{C. v. h.: Crotalus viridis helleri} (southern pacific rattler), \textit{C. v. v.: Crotalus viridis viridis} (Prairie rattlesnake)
unrelated sources, including two algae, were made exactly as had been done for *E. yuccifolium*. Figure 8 shows data from dose-response titrations of extracts using *C. atrox* venom (7.5 ug/mL) as a model. Activity was found for these sources, as might be expected, but some were much more active. Using a comparison at a dilution of 2 as an example, the statistical groupings by Tukey’s multiple range test (*P < 0.05*) were: *E. yuccifolium* leaves/sage were the most potent, *E. yuccifolium* roots (different from sage but not E. leaves), fennel dill/Spirulina, and Chlorella. The fennel-dill-Spirulina grouping of extracts required about 5 times the concentration to get the same level of inhibition as the most potent extracts. Differences in activity became less at lower concentrations (higher dilutions). Complete inhibition was not observed with concentrations tested which is not uncommon with crude extracts and could be an effect of the prevalence of the inhibitor(s) affinity, or mechanisms.

**DISCUSSION**

Worldwide, many plants contain extractable compounds that may have *in vitro* activity as enzyme inhibitors, or at times reported to reduce *in vivo* envenomation pathology [23,24,32-35]. While mechanisms are generally unclear, many low molecular weight nonnitrogenous antivenin compounds in plant extracts may mimic natural inhibitors of the venoms [36], explaining this use [37], while others are nonspecific inhibitors of enzymes, either as direct inhibitors of venom components or maybe anti-inflammatory [38,39].

*Eryngium* genus displays anti-inflammatory [40-49], antimicrobial and anti-oxidant effects [30,50]. Anti-venom protection by *Eryngium* water extract significantly prolongs survival time of guinea pigs challenged with scorpion (*Leiurus quinquestritatus*) venom [31-55]. This type extract also inhibited the hemolytic activity of *Cerastes cerastes* (desert horned viper) highly proteolytic venom [54]. The aqueous root extract of *Eryngium creticum* reduces the hyperglycemic response caused by *Cerastes ceras* in rats [55]. Given the variety of activities documented for *Eryngium*, and that there is a documented effect of the *E. creticum* plant inhibiting both snake and on scorpion venoms, there seems good support for the *Eryngium* extracts as an anti-venom treatment, to which this study adds.

The work reported here adds *in vitro* protease inhibition, a specific mechanism for the actions of the *Eryngium* extracts, as shown with a panel of North American venomous snakes, most noticeable in the general protease and the collagenase assays and provides a system to further study the inhibitors. The presence of the enzyme inhibition activity in all the plant parts tested and not only the roots, should be of interest to phytochemists. The specificity of inhibition, sparing the porcine enzyme, argues against the trivial case in which the *Eryngium* extract is simply a source of nonspecific inhibitors of enzymes. This makes the results more interesting to enzymologists. It will be of interest to later pursue this point further both more thoroughly and widely and to further study the phytochemistry of *Eryngium* species.

As *Eryngium* is widely distributed in America, and all data shown here are consistent with pharmacological inhibition of proteases in at least selected venoms of common local venomous snakes, this validates the effectiveness of this plant’s extract as a source of antivenom agent(s) that may have some specificity, the ethnological practice of using this plant among any others as an anti-snake venom treatment is supportable and may have been common, and suggests further bioactivity and phytochemical studies are warranted. As proteases serve as the major virulence factors of many snakes worldwide, the findings in this paper suggest laboratory and later *in vivo* studies would be appropriate for members of this plant genus in various countries and select venoms of the local snakes.

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