Effects of Aqueous Stem Extract of Achyranthes aspera on Bitis arietans Venom Protease and Phospholipase A\textsubscript{2} Activity

Hope Chinyere Nwune\textsuperscript{*,} Mohammed Adamu Milala, Hassan Zanna

Department of Biochemistry, Faculty of Science, University of Maiduguri, Maiduguri, Nigeria

Email address: hopecnwune@yahoo.com (H. C. Nwune)

\textsuperscript{*}Corresponding author

To cite this article: Hope Chinyere Nwune, Mohammed Adamu Milala, Hassan Zanna. Effects of Aqueous Stem Extract of Achyranthes aspera on Bitis arietans Venom Protease and Phospholipase A\textsubscript{2} Activity. American Journal of BioScience. Vol. 5, No. 3, 2017, pp. 54-58.
doi: 10.11648/j.ajbio.20170503.13

Received: February 16, 2017; Accepted: March 27, 2017; Published: May 27, 2017

Abstract: Aqueous stem extract of *Achyranthes aspera* was investigated for inhibitory activity against *Bitis arietans* venom protease and phospholipase A\textsubscript{2} activity. The elemental analysis and phytochemical screening of the plant extract were carried out. The activities of protease and phospholipase A\textsubscript{2} (V\textsubscript{o}) of the crude *Bitis arietans* venom was determined and the data obtained was used to estimate K_M, V_max, and K_cat. Inhibition studies were carried out using the same procedure except that different concentrations of the extracts (5\%, 10\%, 15\% for protease assay and 0.5\%, 0.75\%, 1\%, 1.25\% and 1.5\% for phospholipase A\textsubscript{2} assay) were added to the reaction mixture. The result showed that the *Bitis arietans* venom protease had a V\textsubscript{max} of 0.062 ± 0.013 µmol/min, K_M of 0.496 ± 0.095mg/ml and a K_cat of 0.125 ± 0.001min\textsuperscript{-1}. The result also indicates that the *Bitis arietans* phospholipase A\textsubscript{2} had a V\textsubscript{max} of 3.27 ± 0.030min\textsuperscript{-1}, K_M of 8.358 ± 0.050 mg/ml and K_cat of 0.391 ± 0.002min\textsuperscript{-1}. The aqueous stem extract produced a statistically significant (P<0.05) decrease in the V\textsubscript{max}, K_M and K_cat of the *Bitis arietans* venom phospholipase A\textsubscript{2} in a dose dependent manner and a statistically significant (P<0.05) increase in the V\textsubscript{max}, K_M and K_cat of *Bitis arietans* protease in a dose dependent manner. The phytochemical screening revealed the presence of flavonoids, tannins, steroids, saponins and terpenoids in the extract while the elemental analysis revealed the presence of Zn, Cr, Ni, Cd, Mn, Fe and Na. The result suggests that aqueous stem extract of *Achyranthes aspera* inhibited the *Bitis arietans* venom phospholipase A\textsubscript{2} in an uncompetitive manner while the protease activity was stimulated by the extracts. It was observed that the use of the stem of *Achyranthes aspera* may be important in the treatment of snake bites.

Keywords: Achyranthes aspera, Bitis arietans, Antivenom, Protease, Phospholipase A\textsubscript{2}

1. Introduction

*Bitis arietans* (puff adder) belongs to the family viperidae and is one of the most dangerous snakes found in Africa. [10]. Viper venom contains protease and phospholipase A\textsubscript{2}. Snake venom phospholipase A\textsubscript{2} have injurious effects such as haemolysis of red blood cells, anticoagulation and cardiotoxicity. Snake venom proteases on the other hand is responsible for the severe bleeding observed in snake bite victims, interference with blood coagulation and haemostatic plug formation and degradation of the extracellular matrix components of the victims of snakebite [21]. These two enzymes therefore having implicated in such a variety of pathological mechanisms can be said to play a central role in the pathology of *Bitis arietans* envenomation. Therefore possible blockage or inhibiting of their action could unveil a way of ameliorating or totally rendering ineffective the toxicity posed by the venom. *Achyranthes aspera* belonging to the family Amaranthacea is claimed to be used in the North Eastern parts of Nigeria in the treatment of snakebite. Hence this study investigated the invitro effect of the aqueous stem extract on *Bitis arietans* venom protease and phospholipase A\textsubscript{2} activity.
2. Materials and Method

2.1. Chemicals

All the chemicals used in this study were of analytical grade and purchased from various sources.

2.2. Snake Venom

Freeze dried *Bitis arietans* Snake Venom was obtained from the Department of Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria, Nigeria.

2.3. Plant Material

Fresh stems of *Achyranthes aspera* were collected from Biu, Borno State Nigeria. The voucher number was obtained and stored in the herbarium. It was washed and shade dried for two weeks to a constant weight. The dried stems were pounded to fine powder with mortar and pestle.

2.3.1. Extract Preparation

One hundred grams of the plant material was transferred to two liter of round bottom flask containing one liter of water. The condenser was fitted to the flask. The flask and the material were heated for 45 minutes. The solution was decanted to remove debris. This was repeated three times. The filtrate was poured onto an evaporating dish concentrated on a water bath. The extract was transferred to airtight containers for further analysis.

2.3.2. Phytochemical Screening

The presence of anthraquinone, combined anthraquinone, steroidal nucleus, terpenoids, saponin, glycosides, flavonoids, alkaloids, tannins were tested as described by Sofowora, [23], Harborne [11], Trease and Evans [24].

2.3.3. Elemental Analysis

The mineral composition of the extract was determined using UV-spectrometer with computer readout after acid digestion [4].

2.4. Protease Assay

The protease activity was assayed as described by Fahmey et al, [8]. Briefly, 50µl of the crude venom solution (10 mg/ml) was incubated with 500µl of 100mM sodium acetate buffer, pH 4.5, and 100µl of 3% Casein at 37°C. The mixture was made up to 1ml with distilled water. Assays were carried out after 1hr, the reaction was stopped by the addition of 200µl of 20% trichloroacetic acid. This was followed by the removal of the precipitated proteins by centrifugation at 10,000g. The absorbance of the supernatant was measured at 366nm. The activity of the protease is defined as the amount of enzyme required to hydrolyze 1µg of FFA from casein per minute under the standard assay conditions.

2.5. Phospholipase A2 Assay

This was carried out by modification of the method of Haberman and Neumann as described by Okonogi et al, [17]. Here 0.5ml of egg yolk suspension (2 mg mL⁻¹) was introduced into a clean test tube containing 50µl of 1mM CaCl₂. To this, 100µl of 20 mg ml⁻¹ venom solution was added and incubated at 37°C for 1hr. Thereafter, the enzymes was stopped by heating at 100°C for 2 minutes, a drop of phenolphthalein was added and then titrated against 2mM NaOH solution to an end point. The same procedure was carried out in the absence of the enzyme in order to obtain titre value for the blank for adequate comparison to deduce effect of the enzyme on the yolk (deduction of any FFA released). The activity of phospholipase A₂ was defined a the amount of enzyme required to hydrolyze 1mg of FFA from the lecithin present in the egg yolk under the standard conditions.

2.6. Determination of Kₘ, Vₘₐₓ and, Kₖₑₐₜ

The activities of protease and phospholipase A₂ (V₀) was determined in the presence and absence of various concentrations 5%, 10%, 15% for protease and 0.5%, 0.75%,1.0%, 1.25% and 1.5% for phospholipase A₂ assay of the plant extracts. Data obtained was used in estimating the Kₘ, Vₘₐₓ and, Kₖₑₐₜ.

2.7. Statistical Analysis

The Data obtained was presented as mean± standard deviation and analysis of variance was used to compare paired means and a difference was considered statistically significant p<0.05.

3. Results

| Phytochemical                  | Presence/Absence |
|--------------------------------|------------------|
| Alkaloid                       | -                |
| Flavonoid                      | +                |
| Tannins                        | +                |
| Steroid                        | +                |
| Saponins                       | +                |
| Phenolic group                 | -                |
| Terpenoids                     | +                |
| Anthraquinone                  | -                |

**Table 1. Phytochemical screening of aqueous stem extract of Achyranthes aspera.**

| Element | Concentration   |
|---------|-----------------|
| Zn      | 0.07± 0.02      |
| Cr      | 0.05± 0.02      |
| Ni      | 0.71 ± 0.30     |
| Cd      | 0.07± 0.50      |
| Pb      | -               |
| Mn      | 0.20± 0.10      |
| Fe      | 0.45 ± 0.10     |
| K       | 8.50 ± 0.20     |
| Na      | 114.68±10.10    |

**Table 2. Elemental analysis (ppm) of aqueous stem extract of Achyranthes aspera.**

Key: + Present.
- Absent.

Values are mean ± standard deviation for triplicate determinations.
Table 1 shows the results of phytochemical screening of the aqueous stem extract of *Achyranthes aspera*. The result shows the presence of flavonoids, tannins, steroids, phenolic group and terpenoids, while alkaloids and anthraquinones were absent.

Table 2 shows the results of elemental analysis of aqueous stem extract of *Achyranthes aspera*. The result shows that Zn, Cr, Ni, Cd, Pb, Mn, Fe, K and Na are present in the aqueous stem extract and *Achyranthes aspera* at varying concentrations with Na accumulating at highest level while Pb was not detected.

The result of the effect of aqueous stem extract of *Achyranthes aspera* on *Bitis arietans* venom protease activity as shown in Table 3 shows that the aqueous stem extract of *Achyranthes aspera* produced a dose dependent increase in the computed physiological index of efficiency of *Bitis arietans* venom protease. The Michaelis Mentens (Km) and maximum velocity (V_max) of *Bitis arietans* venom protease were all significantly increased in the presence of the extract.

The result of the effect of the aqueous stem extract of *Achyranthes aspera* on *Bitis arietans* venom phospholipase A2 activity as presented in Table 4 shows that the aqueous stem extract of *Achyranthes aspera* produced a dose dependent increase in maintaining electrolyte balance in the body. Richards et al [19] reported that high salt concentration increases the activity of the HIV-1 protease. The addition of salt primarily affects the K_M value and the increase in proteolytic activity is usually attributed to the “salting out” of the hydrophobic substrate in the enzyme binding cleft. The increase in protease activity K_cat/K_M might be due largely to the concentrated presence of K+ and also to the presence of Na+.

4. Discussion

The presence of flavonoids, tannins, steroids, saponins and terpenoids in the aqueous stem extract of *Achyranthes aspera* revealed in this study corroborates with the findings of Dey [6] who reported that *Achyranthes aspera* plant contains sterols, alkaloids, saponins, flavonoids and terpenoids.

Again, the presence of Zn, Cr, Ni, Cd, Mn, Fe, Kand Na in the aqueous stem extract of *Achyranthes aspera* as revealed in this study corroborates with the findings of Jabeen [13] who reported that *Achyranthes aspera* plant contains among others elements Zn, Cr, Ni, Cd,Pb Mn, Fe, K, Na, and Mg.

Shendkar et al [22] reported that the aqueous stem extract of *Achyranthes aspera* contained Na, Cr, Ni, Zn, Pb, Mn, and Fe. Although Pb was found to be absent. Their analysis indicated a higher concentration of K. The presence of Na and K in the extracts implies that the plant may be of help in the entrance of the reaction cavity. The entrance of binding substrate in the enzyme binding cleft. The increase in proteolytic activity is usually attributed to the “salting out” of the hydrophobic substrate. The electrostatic potential of the protease surface at the active site entrance and therefore influence substrate /inhibitor recognition [25].
been associated with chemically active compounds of Achyranthes aspera incorporated in the experimental diet of Labeo rohita significantly enhanced the serum antiproteases level than the fishes fed with control diet which did not contain the extract [18].

The result of the effect of the aqueous stem extract of Achyranthes aspera on Bitis arietans phospholipase A₂ activity shows an uncompetitive pattern of inhibition. The aqueous stem extract of Achyranthes aspera produced a dose dependent decrease in the computed physiological index of efficiency of Bitis arietans phospholipase A₂ activity which indicates a reduction in the number of free fatty acid hydrolyzed from the lecithin present in the egg yolk suspension. This is consistent with the report by Samy et al., [20] that the plant extract of Achyranthes aspera (glycosides) have shown potent snake venom neutralizing activity. The plant extract and partially purified fractions were administered orally to rats envenomed with rattle snake venom. Significant protection against venom induced changes in serum superoxide dismutase and Lipoprotein X levels were seen after administration of purified fractions.

Enzyme inhibiting and protein binding properties have been associated with chemically active compounds of flavonoids, polyphenols, terpenoids, xanthenes etc. These phytochemicals also inhibits phospholipase A₂ activities of both viper and cobra venom. Phenolics especially polyphenols like some tannins bind proteins acting upon both viper and cobra venom. Significant protection against venom induced changes in serum superoxide dismutase and Lipoprotein X levels were seen after administration of purified fractions.

Enzyme inhibiting and protein binding properties have been associated with chemically active compounds of flavonoids, polyphenols, terpenoids, xanthenes etc. These phytochemicals also inhibits phospholipase A₂ activities of both viper and cobra venom. Phenolics especially polyphenols like some tannins bind proteins acting upon component of venom directly and disabling them to act upon the receptors and according to Lans et al., [15]. They could also act by competitive blocking of the receptors.

Herbal constituents active against snake envenomation include among others alkaloids, steroids, tannins and terpenoids [9]. Several plant constituents including flavonoids and terpenoids possess protein binding and enzyme inhibiting properties and also inhibits snake venom phospholipase A₂ of both viper and cobra venom [3]. Polyphenols and tannins are attributable to reduction in enzyme activities [1]. Okonogi et al., [17] suggested that tannins in addition to other plant constituents which are known to unspecifically inactivate proteins to be the likely mechanism involve in detoxifying snake venom. Tannins precipitate proteins and form dark coloured complexes with metals such as iron [7]. Tannins are problems as they unspecifically bind to proteins and thereby may show nonspecific (false positive) activity in enzyme assays [14].

Therefore the aqueous stem extract of Achyranthes aspera could serve as a good source of Bitis arietans antidote and could as well help in designing a novel drug to be used as an antivenin.

5. Conclusion

The stem extract of Achyranthes aspera possess potent snake venom neutralizing capacity and may provide protection against the toxicity posed by Bitis arietans venom hence it may be used for therapeutic purposes in case of snakebite.

References

[1] Abubakar, M. S., Abdulrahman, E. M., Haruna, A. K. and Jahun, B. M. (2000). Invitro snake venom detoxifying action of leaf extract of Guierra senegalensis. Journal of Ethnopharmacology, 69: 253-257.
[2] Aguiyi, J. C. (2011). The Molecular Basis of Natural Products Development, Unijos Inaugural Lecture Series 49, Journal of Natural, Knowledge and Health, Pp. 1-4.
[3] Alam, M. I., Auddy, B. and Gomes, A. (1996). Viper venom neutralization by Indian medicinal plants (Hemidesmus indicus and Pluchea indica) root extract, Phytotherapy Resources: 10: 58-61.
[4] AOAC. Officia methods of analysis. Association of analytical chemist 15th ed. Washington DC: American Chemical Society. 1990; pp12-13.
[5] Bradford, M. M. A. (1976). Rapid and Sensitive Method for the Qualification of Microgram Quantities of Protein Utilizing the Principle of Protein dye Binding. Journal of Analytical Biochemistry, 72: 248-254.
[6] Dey, A. (2011). Achyranthes aspera L: Phytochemical and Pharmacological Aspects, international Journal of pharmaceutical sciences Review and research, 9 (2): 72-76.
[7] Evans, W. C. and Sandners W. B. (2002). Plants in African Traditional Medicine – an Overview in Trease and Evans Pharmacognosy, 15th Edition, Pp. 488-495.
[8] Fahmey, A. S., Ali, A. A. and Mohammed, S. C. (2004). Characterisation of Cysteine Protease from Wheat TriticumAestivum, Biore Technology, 91: 297.
[9] Gomes, A., Das, R., Sarkhel, S., Mishara, R., Mukherjee, S., Bhattacharya, S. and Gomes, A. (2010). Herbs and herbal constituents active against snake bite. Indian Journal of experimental biology, 48: 865-878.
[10] Gray, J. E. (1842). Monographic synopsis of the vipers, or the family viperidae. Zoological Miscellany London, 2 (69): 68-71.
[11] Harbone, J. B. (1973). Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis. Chapman A and Hall London Pp. 279-280.
[12] Ibrahim, M. A., Aliyu, A. B., Abusuufiyana, A., Bashir, M. and Sallau, A. B. (2011). Inhibition of Najanigricolis (Reinhard) Venom Protease Biology: 49: 552-554.
[13] Jabeen, S., Muhammad, T. S., Khan. S. and Hayat, M. Q. (2010). Determination of major and trace elements in ten important folk and therapeutic plants of haripur basin, pakistan. Journal of medicinal plant research, 4 (7): 559-566.
[14] Jager A. K (2015) Plant–based treatment of snakebites indian journal of traditional knowledge 14 (4): 571-573.
[15] Lans, C., Harper, T., Georges, K., Bridgewater, E. (2001). Medicinal and ethnoveterinary remedies of hunters in trinidad BMC compliment. Alternative Medicine, 1: 1-10.
[16] Meendatchisundaram, S., Parameswari, G. and Michael, A. (2009). Studies on the Antivenom Activity of AndrographisPaniculata and AristolichiaIndica Plant Extracts against Daboiaresesseli Venom by Invivo and Invitro Methods, Indian Journal of Therapeutics Africa, 2 (4): 76-79.
[17] Okonogi, Hatton T. Z., Ogiso, A. and Mitsui, S. (1979). Detoxification by PermisionTanin of Snake Venom and Bacterial Toxins. Toxic Journal, 17: 524-527.

[18] Rao, V. Y., Chakrabarti, R. (2004). Enhanced anti-proteases in Labeo Rohita fed with diet containing herbal ingredients. Indian journal of clinical biochemistry, 19 (2): 132-134.

[19] Richards, A. D., Phylip, L. H., Farmeric, W. G., Scarborough, P. E., Alvarez, A., Dunn, B. M., Hirel, P. H., Strop, P., Pavilickova, L., Kostka, V., and Kay, J. (1990). Journal of Biochemistry, 265 (14). 7733.

[20] Samy, R. P., Thwin, M. M., Gopalakrishnakone P. and Ignacimuthu S. (2008). Ethnobotanical survey of folk plants for the treatment of snake bites in southern part of Tamilnadu, Indian journal of ethnopharmacology, 2: 39-45.

[21] Sallau, A. B., Njoku, G. C., Olabisi, A. R., Wuruchekke, A., Abdulkadir, A. A., Isahi, S., Abubakar, M. S. and Ibrahim, S. (2005). Effect of Guerra senegalensis leaf extract on some Echis carinatus venom enzymes. Journal of medical sciences, 5: 280-283.

[22] Shendkar, C. D., Chandrachood, P. S., Pawar, A. B., Lavate, S. M. and Nirmala, R. and Deshpande, N. R. (2011). Quantitative estimation of macro, micronutrients and trace elements by X-ray fluorescence spectroscopy (XRF) from Achyranthes aspera International Journal of chemtech research, 3 (2): 610-613.

[23] Sofowora, A. (1993). Medicinal Plants and Traditional Medicinal in Africa 2nd Edition. Sunshine House, Ibadan, Nigeria. Spectrum Books limited: Screening Plants for Bioactive Agents, Pp. 134-156.

[24] Trease, G. E. and Evans, W. C. (2002). Pharmacognosy 15th edition, Saunder publishers Pp. 42-393.

[25] Vrbka, L., Vondraskı, J., Jagoda-Cwiklik, B., Vacha, R., Jung, B. and Wirth P. (2006). Proceedings of the National academy of Sciences of the United States of America, 103 (42): 15440.