



Research Article

Kinetic Study of the Effect of *Vernonia amygdalina* Leaf Extract Against *Echis ocellatus* Venom Hyaluronidase

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ABSTRACT

Hyaluronidase is an enzyme that catalyses the hydrolysis hyaluronic acid. It is found in human fluids and organs as well as in the venoms of snake, spiders, scorpions and lizards. The objective of this study was to determine the effect of *Vernonia amygdalina* on *Echis ocellatus* Venom Hyaluronidase and to determine the kinetic study of the interaction between *Echis ocellatus* Venom Hyaluronidase and *Vernonia amygdalina* methanol leaf extract. The kinetic study of the interaction between *Echis ocellatus* Venom Hyaluronidase and *Vernonia amygdalina* methanol leaf extract was investigated using Ellman colorimetric method with slight modification and the analysis of the K_i was determined using Dixon plot. The kinetic study showed that the extract caused an uncompetitive type of inhibition, the inhibition was found to be dose dependent. The IC_{50} from the resultant dose dependent graph was found to be 18.78 μM . The *Vernonia amygdalina* methanol leaf extract was found to inhibit *Echis ocellatus* Venom Hyaluronidase and therefore it can be regarded as a potent anti-venom agent.

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INTRODUCTION

Snakebite is an important public health issue in rural regions of tropical and subtropical countries located in Asia, Africa and Latin America (Zameer *et al.*, 2011). In Nigeria, it commonly affects rural population of the Savannah region of the country where farming and animal husbandry are the major occupation. Since the development of snake venom antiserum and its standardization are found to be expensive and difficult, the efforts are continuously being made to invent alternative treatment strategy from medicinal plants. Envenomation of snakebite is caused due to the nature of enzymatic and non-enzymatic toxic compounds present in the poison glands. Hyaluronidase, neurotoxins and cardiotoxins are the major classes of *Echis ocellatus* venom polypeptides involved in the toxicity and pharmacology of snake bite. The role of hyaluronidases in venom is to facilitate the diffusion of venom toxins resulting in clinical manifestations such as hemorrhage coagulation and multiple organ failure (Valenta, *et al.*, 2010). *Vernonia amygdalina*, is a member of the daisy family, it is a small shrub that grows in tropical Africa. *Vernonia amygdalina* typically grows to a height of 2–5 m (6.6–16.4 ft). The leaves are elliptical and up to 20 cm (7.9 in) long. Its bark is rough (Ijeh and Ejike, 2011). *Vernonia amygdalina* is commonly called Congo Bololo in D. R. Congo, bitter leaf in English because of its bitter taste. African common names include grawa (Amharic), ewuro (Yoruba), etidot (Efik), onugbu (Igbo), ityuna (Tiv), oriwo (Edo), Awɔnwono (Akan), chusar-doki or shuwaka (Hausa), mululuza (Luganda), labwori (Acholi), olusia (Luo), ndoleh (Cameroon) and olubirizi (Lusoga) (Egedigwe 2010; Kokwaro 2009; Appiah 2018). Alkaloids, coumestins, steroids, glycoproteins, glycosides, phenols, ptercarpenes, tannins, terpenoids, quinonoid xanthine and miscellaneous chemical groups present in the extract possesses effective antidote against snake venom envenomation (Antony *et al.*, 2010)

Though the definite mechanism of herbal based antidote therapy is not revealed, the majority of the herbal compounds tend to neutralize the toxic venom constituents. Neutralization of venom by phyto constituents includes the process of enzyme inactivation, chelation, adjuvant actions, anti-oxidation and protein folding (Shojaee *et al.*, 2014). It is estimated that about 2.5 million people envenomed every year on a global basis and approximately 5,000-15,000 death were reported in Nigeria per annum. An accurate measure of snake bite venoming remains elusive as victims approach traditional healers for aid and treatment (Chatterjee *et al.*, 2006). Antivenom immunotherapy is the specific treatment available against a snake bite. It is associated with various side effects like pyrogen reactions, serum sickness, along with that, supply of antivenom has logistical, marketing, storage and economic difficulties (Gupta and Peshin 2012). Consequently, it is important to derive an alternative treatment that involves the usage of different venom inhibitor, synthetic or natural that could be a substitute for the action of antivenoms. Over the years, attempts have been made to develop snake venom antagonists especially from medicinal plants. These natural sources could serve as complementary treatment to complement the use of commercial antivenom. In this study, the interaction between *Echis ocellatus* venom Hyaluronidase and *Vernonia amygdalina* methanol leaf extract was determined.

MATERIALS AND METHODS

Plant material Collection and Identification

The fresh leaves of *Vernonia amygdalina* were collected in a separate sterile polythene bags aseptically from Gamboru 3 farm in Maiduguri, Borno state. The plant was identified and authenticated at the herbarium of the Faculty of Pharmacy with the voucher specimen number UMM/FPH/HSR/001, University of Maiduguri.

Preparation of Extract

The leaves were reduced to small pieces and shade dried for and ground into powder using pestle and mortar. 150g of the pulverized sample was extracted with 1.5 L of 70% methanol using cold maceration method as described by Banu and Cathrine (2015). The maceration method was repeated three times after every 24hrs. The mixture was filtered using whiteman No 1 filter paper and allowed to dry under room temperature to obtain the crude extract. The dried crude extract was weighed and stored in a desiccator for further analysis.

Venom Sample

Lyophilize venom sample was obtained from the Department of Biochemistry, University of Maiduguri.

Hyaluronidase activity assay:

5mg/ml of the substrate (Hyaluronic acid) was prepared using sodium acetate (buffer) substrate concentration was prepared in a different concentration. In a cuvette, 200µl buffer, 100µl substrate, 120µl distilled water, 30µl of CTAB, and 50µl hyaluronidase. The volume of the cuvette is 500µl which is subjected into the spectrophotometer at 500nm wavelength and take the absorbance.

Hyaluronidase Inhibition Assay

5mg/ml of the substrate (Hyaluronic acid) was prepared using sodium acetate (buffer). 1mg/ml of the Enzyme (Hyaluronidase) was prepared using buffer. 500µl of the inhibitor was prepared by 500µl of the ethanol. The inhibitor was prepared in different concentration. In the cuvette with 200µl buffer, 100µl substrate, 80µl distilled water, 40µl inhibitor, 30µl CTAB, and 50µl enzyme was subjected into spectrophotometer at 400nm wavelength and taken the absorbance (Di Ferrante *et al.*, 1956).

Kinetic study of Hyaluronidase Inhibition

5mg/ml of the substrate (Hyaluronic acid) was prepared using sodium acetate (buffer). 1mg/ml of the enzyme (Hyaluronidase) was prepared using buffer. 500µl of the inhibitor was prepared by 500µl of the ethanol. A

concentration of substrate was prepared in different concentration. The inhibitor was prepared in different concentration. Each of the prepared concentration of substrate is taken and round all the prepared different concentration of the inhibitor. 200µl buffer, 100µl substrate, 80µl distilled water, 40µl inhibitor, 30µl CTAB, and 50µl enzyme, the cuvette which is 500µl by volume is subjected into spectrophotometer at 400nm wavelength and take the absorbance (Di Ferrante *et al.*, 1956).

Qualitative Phytochemical Screening of *Vernonia. amygdalina* Leaf Extract

Phytochemical analysis of the sample was performed according to standard method as described below:

Test for Alkaloids

Exactly 200mg of the extract was added to 10ml methanol before subsequent shaking and filtration. To 2ml of the filtrate, 1ml of 1% HCl was added before the mixture was then boiled for 5 minutes, filtered and 6 drops of Mayer's reagent were added to 1ml of the filtrate. A positive test was indicated by a creamy precipitate/brownish-red precipitate/orange precipitate.

Test for Tannins

The plant extract 200mg) was added to 10ml distilled water. The process was immediately followed by filtration, addition of 2ml filtrate and also 2ml 10% ferric chloride (FeCl₃). A positive was indicated by a blue black precipitate (Evans, 2002).

Test for Saponins

The extract was subjected to the frothing test. The filtrate (0.5 ml) and 5 ml distilled water was taken and shaken in a test tube. If frothing or foaming persists for five minutes, it will indicate the presence of saponins (Evans, 2002).

Test for Flavonoids

The presence of flavonoids was determined by adding 200 mg of the plant extract to 10 ml ethanol and filtered, 2 ml of the filtered and 5 drops of concentrated HCl and magnesium chips (3 pieces) were taken into a test tube.

The presence of flavonoids was observed by yellow precipitate (Harborne, 1973).

Test for Phenols

To 2 ml of the extract, 2 ml of 5% aqueous ferric was added, formation of blue color indicated the presence of Phenols.

RESULTS

Figure 1 shows double reciprocal plot of the steady state kinetics behavior of

Hyaluronidase at different concentration of Hyaluronic acid. The result showed a gradual increase in steady state kinetic behaviour with increasing concentration from 2 mM⁻¹ to 7 mM⁻¹. At the concentration of 2 mM⁻¹, the steady state kinetic behaviour was 0.15 while at 7 it increases to 0.45 mM⁻¹.

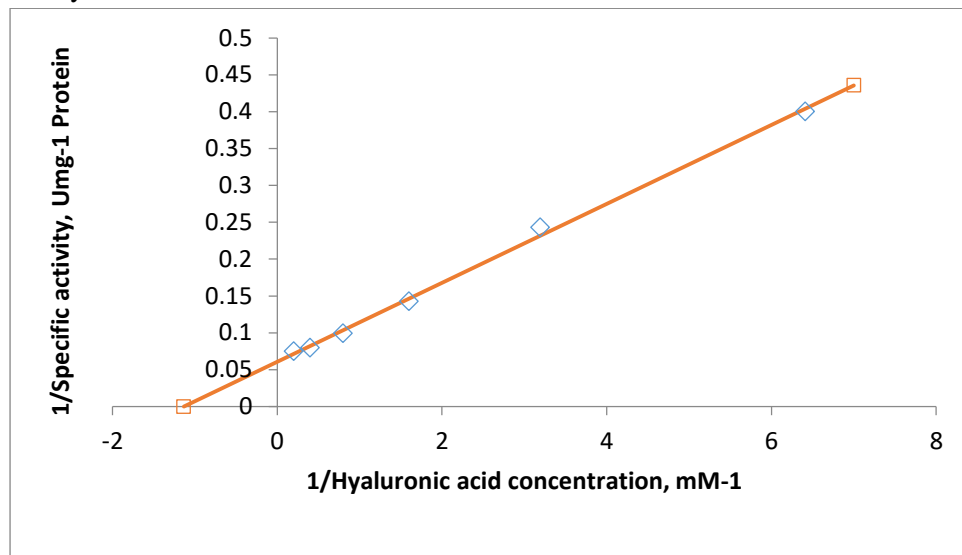


Figure 1: Double reciprocal plot of the steady-state kinetics behavior of Hyaluronidase at different concentration of Hyaluronic acid.

Figure 2 shows dose dependent inhibition of Hyaluronidase activity by different concentration of *V. amygdalina* leaf extract.

An approximate concentration of 8 uM *V. amygdalina* extract reduced the activity by 50 %.

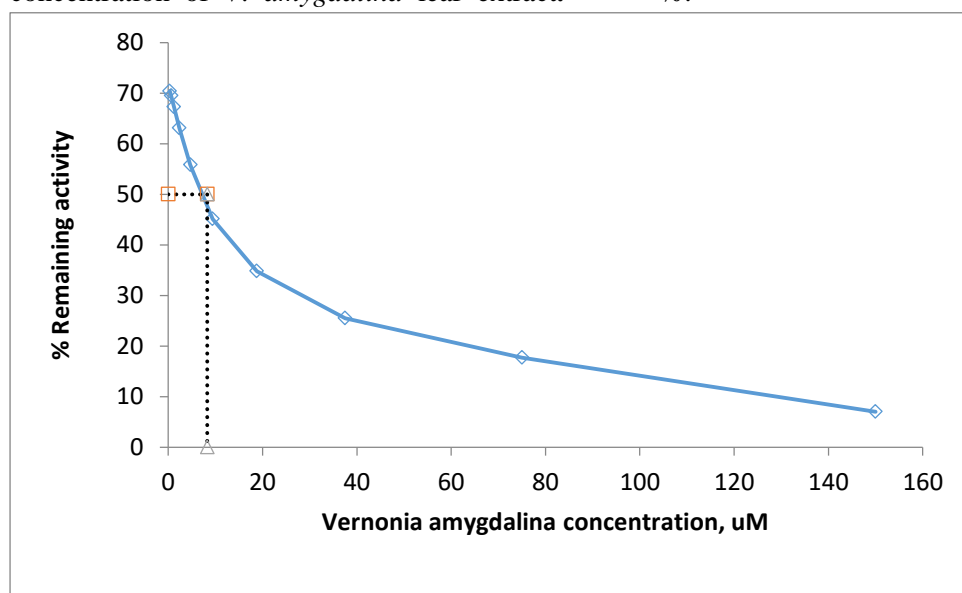


Figure 2: Dose dependent inhibition of Hyaluronidase activity by different concentration of *V. amygdalina* leaf extract.

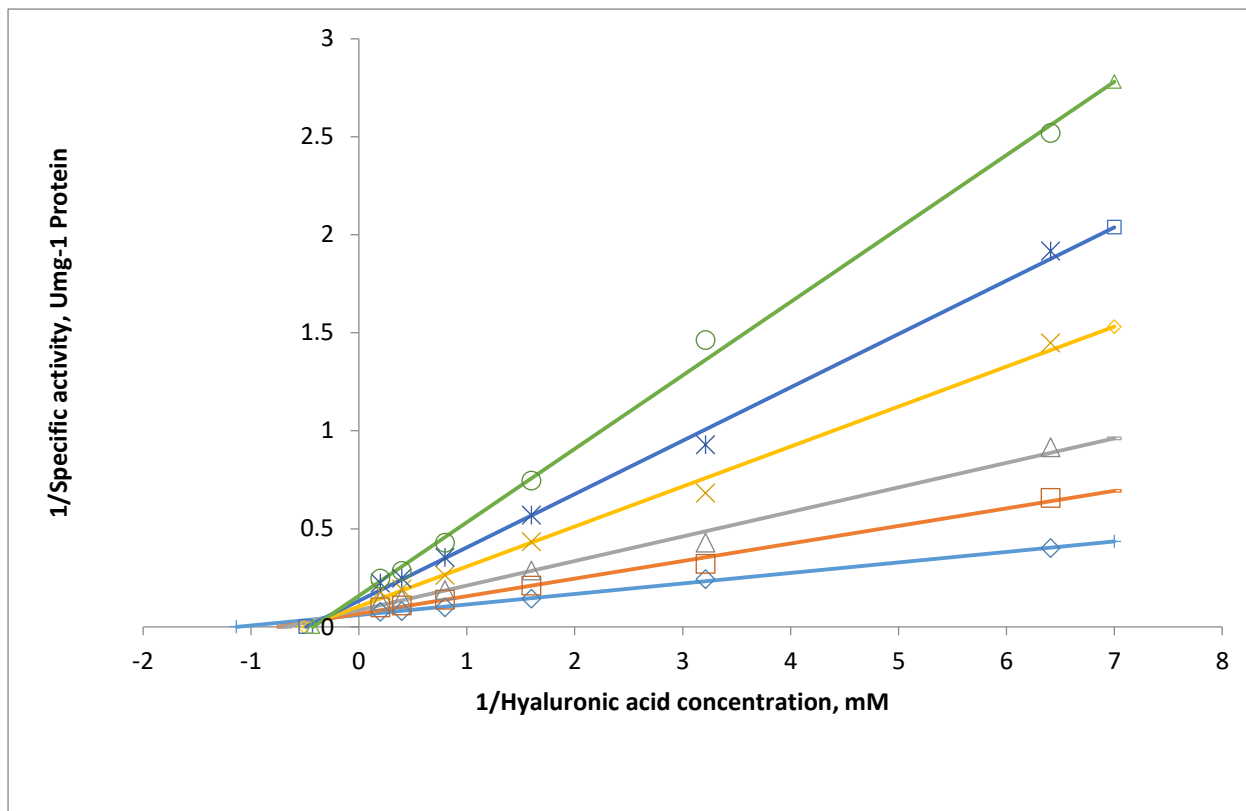


Figure 3 shows inhibition of the Hyaluronidase catalyzed the hydrolysis of Hyaluronic acid by *V. amygdalina* leaf extract. The graph illustrated Lineweaver-Burk plot in the absence of control and in the presence *V. amygdalina*. The x-intercept shift to the left while the y-intercept shift upward suggesting an uncompetitive type of inhibition.

Figure 3: Inhibition of the Hyaluronidase catalyzed the hydrolysis of Hyaluronic acid by *V. amygdalina* leaf extract.

amygdalina leaf extract. The x-intercept shift to the left while the y-intercept shift upward suggesting an uncompetitive type of inhibition. The K_i value was 0.1

Figure 4: Secondary replot of slope and intercept against the concentration of *V.*

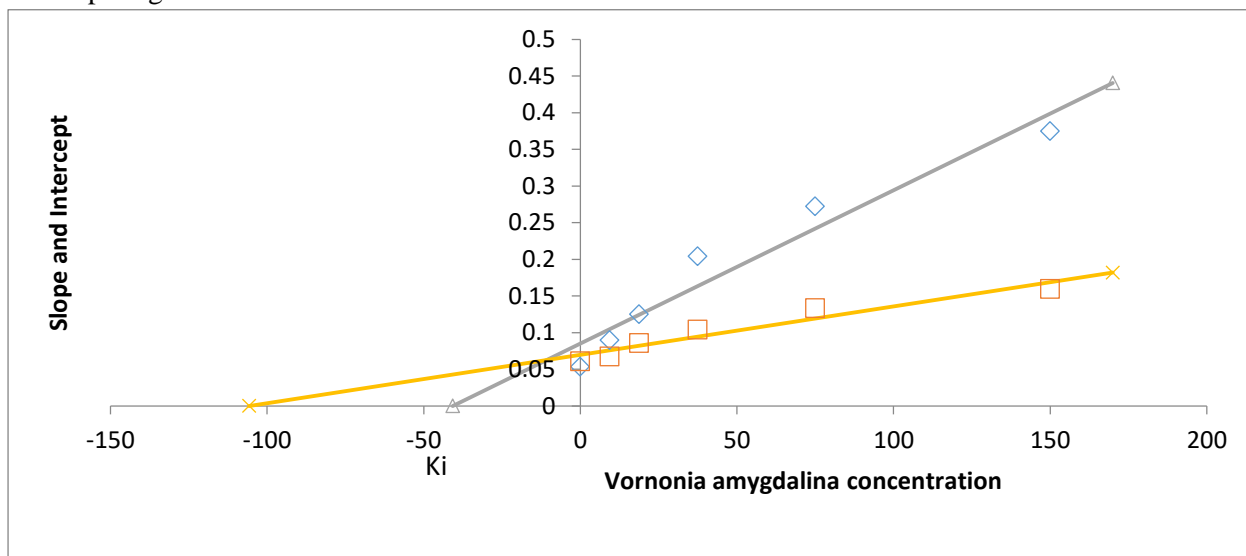


Figure 4: Secondary replot of slope and intercept against the concentration of *V. amygdalina* leaf extract.

DISCUSSION

Vernonia amygdalina is known to have a variety of flavonoids and bitter sesquiterpene lactones which contribute to the bioactivities of this plant (Favi *et al.*, 2008). The presence of these and other secondary plant metabolites (Antony *et al.*) could confer the extract the ability to serve as potent anti-venom. The kinetic study of the interaction between *Echis ocellatus* Venom Hyaluronidase and *Vernonia amygdalina* methanol leaf extract determined how the enzyme Hyaluronidase produced by *Echis ocellatus* interacted with the methanol leaf extract components of *V. amygdalina*. The result produced in figure one showed the specific activity of the enzyme Hyaluronidase at different concentration of the substrate. The activity showed an increase as the concentration of the substrate increases. Similarly, dependence of initial velocity (V_0) on substrate concentration [S], the graph showed the kinetic parameters that define the limits of the curve at high and low [S]. Furthermore, the Lineweaver-Burk plot showed that the extract caused uncompetitive type of inhibition in which the K_m value decreased while V_{max} also decreased with an increase in concentration of *V. amygdalina*. These findings were in line with work done by (Okonogi *et al.*, 1979, Abubakar *et al.*, 2000) which pointed out that the aqueous extract of the plant leaves they used contained tannins which is very much present in *Vernonia amygdalina* leaves (Akindahunsi and Salawu, 2004) which have been reported to unspecifically inactivate proteins and enzymes. Previous studies confirmed that certain naturally occurring substances modify the actions of proteins and enzymes (Haslam, 1996) especially the plant poly phenols which are the precursors of tannin. This can therefore

be suggested to be the mechanism of the detoxifying action of methanol leaf extract of *V. amygdalina* and why it is used in the treatment of snakebite as reported (Bethwell and Daniel, 2006; Abdullahi *et al.*, 2021).

The kinetic studies of the enzyme Hyaluronidase was tested to find out the K_m , V_{max} and K_i . The findings showed that the lesser the K_m value, the more the affinity to the compound or inhibitor to the enzyme. The inhibition of the enzyme Hyaluronidase by the extract was shown to be dose dependent as deduced from the graph in figure 2. This was supported by the findings of (Abdullahi *et al.*, 2021) on the Effect of *Vernonia amygdalina* on phospholipase activity from *Naja mossambica* (Cobra) (Ahmed *et al.*, 2018). Similarly, increased in the concentration of *V. amygdalina* was accompanied by decreased in K_m value. Finally, as the concentration of the inhibitor increased, the V_{max} and K_m values obtained showed that the extract *V. amygdalina* has a high binding affinity to the target enzyme.

CONCLUSION

Since snake venom hyaluronidases contribute to the tissue damage immediately after envenomation, a successful inhibition (uncompetitive) by SG could serve as a potential primary data to establish scientific bases for the use of this plant in some locations in Nigeria. Therefore, with further researches on snake venom hyaluronidases and GS could further establish the use of the plant as antisnake herb or as a potential adjuvant in the treatment of envenomation in Nigeria

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REFERENCES

Abdullahi, A., Omeremime, E. D., Bala, A. M., Yahaya, A. M., Maimuna, B. U.

and Rahinat, G. (2021). Effect of *Vernonia amygdalina* on phospholipase activity from *Naja*

- Mossambica* (Cobra). *GSC Advanced Research and Reviews*, 06(03), 053–060.
- Abubakar, M. S., Sule, M. I., Pateh, U. U., AbdulRahman, A. K., Haruna, B. M. and Jahun, B. M. (2000). *In vitro* snake detoxifying action of leaf extract of *Guiera senegalensis*. *Journal of Ethnopharmacology*, 69, 253–257.
- Ahmed, M., Razaq, A., Mushtaq, N. and Khan, R. A. (2018). *In vitro* kinetics and inhibition of krait snake's venom acetylcholinesterase by *Calligonum polygonoides* extract in relation to the treatment of alzheimer's disease. *Iran Journal of Basic Medical Science*, 21, 869-872.
- Akindahunsi, A. A. and Salawu, S. O. (2004). Phytochemical screening and nutrient-anti-nutrient composition of selected tropical leafy vegetables. *African Journal of Biotechnology*, 14, 497-501.
- Antony, F., Rinko, D., Sumana, S., Roshnara, M., Sanghamitra, M., Shamik, B. and Aparna, G. (2010). *Indian Journal of Experimental Biology*, 48, 865-878.
- Appiah, K. (2018). Medicinal Plants Used in the Ejisu-Juaben Municipality, Southern Ghana: An Ethnobotanical Study. *Medicines*, 6 (1), 1–27.
- Banu, K. S. and Cathrine, L. J. (2015). General techniques involved in phytochemical analysis. *International Journal of Advanced Resesearch in Chemical Science* 2 (4), 25-32.
- Bethwell, O. O. and Daniel, P. K. (2006). Kenyan medicinal plants used as antivenin: a comparison of plant usage. *Journal of Ethn biology and Ethno medicine*, 2, 7.
- Chatterjee, I., Chakravarty, A. K. and Gomes, A. (2006). *Daboia ruselli* and *Naja kaouthia* venom neutralization by lupeol acetate isolated from the root extract of Indian sarsaparilla *Hemidesmus indicus*. *Journal of Ethno Pharmacology*, 106, 38-43.
- Di Ferrante, N. (1956). Turbidimetric measurement of acid mucopolysaccharides and hyaluronidase activity. *Jol. Biology Chemistry*; 220(1), 303-306.
- Egedigwe, C. A. (2010). Effect of dietary incorporation of *Vernonia amygdalina* and *Vernonia colorata* on blood lipid profile and relative organ weights in albino rats (Thesis). Department of Biochemistry, MOUAU, Nigeria.
- Evans, W. C. (2002). Trease and Evans Pharmacognosy, 15th edition. W.B Saunders Company Limited, London. pp 137-139, 230-240.
- Favi, F., Cantrell, C. I., Mebrahtu, T. and Kraemer, M. E. (2008). Leaf peltate glandular trichomes of *Vernonia galamensis* ssp. *Galamensis* var. *ethiopica* gilbert: Development, Ultrastructure, and chemical composition. *International Journal of Plant Science*; 169, 605-614.
- Gupta YK and Peshin SS (2012). Do herbal medicines have potential for managing snakebite envenomation. *Toxicology International*, 19(2); 89-99.
- Harborne, J. B. (1973). Phytochemical methods: A guide to modern techniques of plant analysis. Chapman and Hall Limited, London; Pp. 279.
- Haslam, E. (1996). Natural polyphenols (Vegetable tannins) as drugs: Possible modes of action. *Journal of Natural Product*; 59(2), 205-215.
- Ijeh, I. I. and Ejike, C. E. C. C. (2011). Current perspectives on the medicinal potential of *Vernonia amygdalina* Del. *Journal of Medicinal Plant Research* 5 (7), 1051–1061.
- Kokwaro, J. (2009). Medicinal Plants of East Africa (3rd ed.). Nairobi, Kenya: University of Nairobi Press. ISBN 978-9966-846-84-6.
- Okonogi, T. Z., Hatton, A., Ogiso and Mitsui S, (1979). Detoxification by permission tannin of snake venom and bacterial toxins. *Toxicon*; (17), 524-527.
- Shojaee, G., Parhiz, H., Eshaghi, A., Emami, S. A., Asili, J. and Mosaffa, F. (2014). *Journal of Complement Integrated Medicine*, 11 (2), 121-127.
- Zameer, F., Dhananjaya, B. L., Girish, K. S. and Cletus, J. M. (2011). Anti-venom potential of aqueous extract of stem bark of *Mangifera indica* L. against

Daboia russelli (Russelli's viper)
venom. *Indian journal of
Biochemistry and Biophysics.*
(48), 175-183.

Valenta, J., Stach, Z. and Svitek, M. (2010).
Prague Med. Rep., 111, pp. 69-75