IN VIVO ANTIVENOMOUS EFFECT OF THE MINERALS MUCUNA PRURIENS AND MILLETTIA PINNATA (FABACEAE) ON ORYCTOLAGUS CUNICULUS

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ABSTRACT

The objective of this study was to establish the scientific basis for the traditional antivenomous use of the minerals \textit{Mucuna pruriens} and \textit{Milletta pinnata} (Fabaceae). During this study, some rabbits were previously collected, others were scarified as a preventive or curative measure, and still others were poisoned with the venom of \textit{Naja nigricollis} and then treated with the minerals from both plants, and then they were collected separately according to the level of handling in order to determine their biochemical and hematological parameters respectively. Finally, the rabbits were observed at all these handling stages. The results of the various observations showed that: - all control or scarified rabbits are generally calm, docile and easy to handle;- all venomed rabbits have in most cases difficulty moving around, are agitated, have difficulty lifting and relaxing the leg on which the injection was made and this 20 to 30 minutes after injecting them with the venom ;- some rabbits died a few hours after the injection of the venom despite their treatment, while others survived a few days later. Thus, these results have shown that the minerals from each plant have antivenomous actions on the venom of \textit{Naja nigricollis}. However, minerals from \textit{Milletta pinnata} have shown significant effects both preventively and curatively. Therefore, these minerals may be an obvious resource for the development of phytomedicines against ophidian envenimation.

KEYWORDS: Antivenomous, minerals, \textit{Mucuna pruriens}, \textit{Milletta pinnata}, phytomedicines.

INTRODUCTION

The expansion of cities and the reduction of natural environments have brought humans into contact with wild animals (Piédallu \textit{et al.}, 2016; Akaffou \textit{et al.}, 2019). These contacts often create a problem of biodiversity conservation which is most frequently at the origin of human-wildlife conflicts (Marchand, 2013; Akaffou \textit{et al.}, 2019). This problem is acutely present between humans and ophidians (Nonga and Haruna, 2015; Akaffou \textit{et al.}, 2019). Indeed, these ophidians are at the origin of the envenimations. Throughout the world, according to Chippaux and Goyffon, 1997, ophid envenimation is a serious public health problem, with more than 125,000 deaths per year reported. In contrast, according to Arfaoui \textit{et al.}, 2009; El Koraichi \textit{et al.}, 2011; Coulibaly \textit{et al.}, 2013, the annual incidence of these snake bites exceeds six millions worldwide. Africa comes in first place with 1,100,000 bites; 600,000 envenimations; 25,000 deaths for a population of 800,000,000 (Coulibaly \textit{et al.}, 2013). Nevertheless, african urban populations have access to health centers to benefit from conventional treatment, which is western anti-venom serotherapy (AVS). In addition, this western anti-venom serotherapy is very expensive and requires delicate storage conditions that most health centers in Africa do not have. In any case, it is still not available. On the other hand, those in rural areas, in addition to having low incomes, do not have access to health centers. Therefore, they do not have the same privileges as those in urban areas. So, the snake bite poses a real medical and socio-economic problem to these populations (Bon, 1994; Grema and Koné, 2003).

The species of snakes that cause damage belong to the family of vipersidae (\textit{Bitis}, \textit{Echis}, \textit{Atractaspis}, \textit{Causus}, \textit{Atheris}) and the family of elapidae (\textit{Naja}, \textit{Dendrouspis}) among which is the \textit{Naja nigricollis}. This snake (\textit{Naja}...
nigricollis) or spitting cobra from tropical Africa and some asian najas have venoms rich in cytoxins that can be responsible for necrosis. But also, they can cause the complete destruction of the bitten limb, death from asphyxia due to paralysis of the respiratory muscles and blood circulation disorders (Goyffon and Chippaux, 1990).

For these major reasons, african populations (urban and/or rural) use medicinal plants to treat themselves. The World Health Organization (WHO) estimates that in 2013, approximately 80% of the populations of developing countries will be using traditional medicine, and in particular herbal medicine, for their health care needs. In effect, the african floristic heritage is very rich in medicinal plants whose effectiveness is proven. Some authors have shown that the continent abounds in nearly 5000 medicinal species (Adjanohoun and Aké-Assi., 1979; Okou, 2012 ; Okou et al., 2018).

In west Africa, particularly in Benin, 80 percent of snake bites reported using traditional treatment rather than modern western medicine (Chippaux, 1989; Grena and Koné, 2003). In Côte d'Ivoire, cases of elapid envenimation are treated by the roots of Securidaca longepedunculata (Polygalaceae) (Koné, 1980; Somé et al., 2002), whereas those due to vipers are cared for with the roots of Anonna senegalensis (Annonaceae) (Koné, 1998; Somé et al., 2002). In this country, certain populations in the region of Bouaké (Centre) use two Fabaceae, namely Mucuna pruriens and Millettia pinnata to treat cases of envenomation.

It is with a view torationally exploiting this heritage, to provide a scientific basis for the use of these medicinal plants and to contribute to the discovery of new drug leads that the present study was carried out.

MATERIALS AND METHODS

Materials
Biological material
Plant material
The plant material is composed of Millettia pinnata and Mucuna pruriens. These plants were harvested in December 2019, in the locality of Bouaké (Central Côte d'Ivoire).

Animal material
For this study, 36 rabbits (19 males and 17 females of Hyplus breed) aged two and a half months were purchased from a breeder in the locality of Daloa (Côte d'Ivoire). After the acclimatization period, the weight of the rabbits varied between 1.45 and 2.4 kg. Besides this animal model, viper skulls and the venom of Naja nigricollis (Spitting Cobra) were also used. The viper skulls were provided by a medico-druggist meanwhile the venom of Naja nigricollis was provided by the Pasteur Institute of Adiopodoumé (Côte d'Ivoire).

Methods
Mineral preparation method
For its realization the various plants were harvested in Bouaké, washed, cut then dried in the shelter of the sun, at room temperature during one week. The plant organs were then dried in an oven at a temperature of 70 °C for three days. After this drying time, the organs (plant and animal) obtained were incinerated in a muffle furnace for 13 hours at 550 °C. The resulting ashes were weighed using a precision balance. They are unctuous with the exception of the viper skull which is rough. The colors vary from gray to brown.

The combination of ashes from the various organic products resulted in the following potions:
- P1 consists of the ashes of the two plants and the skull of a viper;
- P2, P3 and P4 are respectively and only made up of ashes of Mucuna pruriens, Millettia pinnata and the skull of viper;
- P5 is composed of the ashes of Mucuna pruriens and Millettia pinnata;
- P6 consists of the ashes of the skull of viper and Mucuna pruriens;
- P7 is constituted by the ashes of the skull of viper and Millettia pinnata.

Calculation of incineration efficiency
The formula below was used to calculate the weight of dry matter of the organs used.

\[
Ac = \frac{\text{Mass of ashes}}{\text{Dry matter}} \times 100
\]

Ac: Ash content

Scarification method for experimental batches
To scarify the experimental batches, the following potions:
- P1 was used for lots 2 (preventive) and 9 (curative);
- P2 has been used for Lot 3;
- P3 has been used for lots 4 (preventive) and 10 (curative);
- P4 served for lot 5;
- P5 is used for lot 6;
- P6 is used for Lot 7;
- P7 has been used for lot 8.

Each experimental batch consisted of two males and one female. However, before scarification, the affected areas (toes of the left paw and tarsus of the right paw) were exposed with a pair of scissors. Then, a separate quantity of 0.45 mg of the potion previously prepared was applied to each affected area of each given batch. Experimental testing began four days after scarification.

In vitro hemolysis test method
To carry out this test, two control batches were set up. Lot 11 consists of three males and lot 12 is composed of three females. Among these control lots, a blood sample was taken from one of them in order to perform the in
**vitro** hemolysis test of *Naja nigricollis* venom. To carry out this test, 10 tubes were used, including a control tube and 9 experimental tubes. The stock solution was prepared in tube 1 by dissolving 1.6 mg of venom crystals in 1 mL of physiological water. In the remaining 9 tubes (tubes 2 to 10) a volume of 0.5 mL of physiological solution was added. The venom concentration ranges were prepared using the double dilution technique of geometric ratio 1/2. It consisted of taking 0.5 mL of the stock solution (tube 1) and transferring it to 0.5 mL of the physiological solution in tube 2 and homogenizing it. This procedure was repeated up to tube 9. From tube 9, a volume of 0.5 mL was collected and subsequently discarded. Thus, concentrations in the tubes ranged from 1.6 mg/mL to 6.26.10^{-3} mg/mL. To these 9 experimental tubes and to the control tube (tube 10), 5 drops of rabbit whole blood was added and homogenized manually. After homogenization, all preparations were incubated at room temperature for 30 to 40 minutes for microscopic observation. This observation was carried out at magnification 40 (X40). For this purpose, the preparations in tubes 1 (SM); 2; 3 and tube 10 (control tube) were diluted distinctly to 1/5th. Then each dilution was spread between slide and slide by putting the dilution of tube 10 (control) and an experimental dilution (e.g. tube 1). This last operation was also performed for tube 2 and tube 3.

**Method of blood collection**

In general, blood samples were taken from the short saphenous vein and/or the femoral vein. The restraint method was performed by three people. The areas where these veins are located were previously exposed with a pair of scissors. The vacutainers into which the needles were inserted allowed the sampling to be carried out using the tubes. The resulting tubes were stored in a cooler containing ice and then transported to the laboratory for analysis.

**Method of carrying out experimental tests**

For the experimental tests, 2 mg of venom crystals were dissolved in 0.5 mL of physiological solution to have a concentration of 4 mg/mL. This concentration was injected into the rabbits. Indeed, according to Fumba, 1983, the median lethal dose for a 2 kg rabbit is 2 mg/kg body weight in intramuscular injection.

**RESULTS AND DISCUSSION**

**Results**

**Results of the hemolysing power of venom in vitro**

Figures 1 (A and B); 2 (A and C) and 3 (A and D) are the results of the effects of different concentrations of the venom tested *in vitro* on red blood cells. These figures show that, in general, the density of red blood cells varies compared to the control depending on the concentrations tested. However, this density is higher when the concentration is low (0.4 mg/mL) and low when the concentration is high (1.6 mg/mL).

![Figure 1: Normal red blood cells (A) and hemolysing effect of 0.4 mg/mL of *Naja nigricollis* venom on normal red blood cells (B).](image1)

![Figure 2: Normal red blood cells (A) and hemolysing effect of 0.8 mg/mL of *Naja nigricollis* venom on normal red blood cells (C).](image2)
Figure 3: Normal red blood cells (A) and hemolysing effect of 1.6 mg/mL of *Naja nigricollis* venom on normal red blood cells (D).

**RABBIT TEST RESULTS**

**Results of the calculation of the ash content of the organs**

Table 1 shows the result of the ash content (Ac) calculation. For 100 g of the viper skull, the ash content is 15.98%. This rate is higher than that of *Mucuna pruriens*, which in turn is higher than that of *Millettia pinnata*.

<table>
<thead>
<tr>
<th>Material</th>
<th>Quantity of dry matter (g)</th>
<th>Quantity of ash (g)</th>
<th>Ash content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mucuna pruriens</em></td>
<td>100</td>
<td>4.89</td>
<td>4.89</td>
</tr>
<tr>
<td><em>Millettia pinnata</em></td>
<td>100</td>
<td>5.21</td>
<td>5.21</td>
</tr>
<tr>
<td>Skull of viper</td>
<td>100</td>
<td>15.98</td>
<td>15.98</td>
</tr>
</tbody>
</table>

**Results of the appearance of control rabbits (reference or scarified)**

Figure 4 (A and B) shows reference control rabbits and controls scarified as a preventive measure. The figure shows that the rabbits are calm, docile and easy to handle.

**Results of the aspect of rabbits treated preventively and envenomized**

Figure 5 (A, B, C and D) is the result of the effects of the injected venom on the rabbits studied. This figure shows that after 20 to 30 minutes of observation, the rabbits have difficulty moving around, are agitated, and have difficulty lifting and relaxing the leg on which the injection was made. They lie down and slip showing that they lose their balance.

Figure 4: Control rabbit (A) and scarified rabbit (B)

Figure 5: Effect of injected venom on rabbits (A, B, C and D)
Figure 5: Aspects of the rabbits 20 minutes (A and B) and 30 minutes (C and D) after their envenomization.

Figure 6 (A and B) is the one obtained a few hours after the injection of the venom. In figure 6A, the death of the rabbit is observed. The rabbit has grown in size and may have oral and nasal hemorrhage; while in figure 6B, paralysis is prolonged.

Figure 6: Aspects of rabbits a few hours (11 hours) after envenomization

Results of the appearance of rabbits that have been envenomized and treated with P1 and P3 as curative treatment

Figure 7 (A and B) is the one obtained after a few days of treatment with potion 1 (P1) as a cure. In this figure 7A, paralysis is prolonged, while the death of the rabbit is seen in figure 7B. The dead rabbit has gained volume, has a runny nose and mouth.

Figure 7: Aspects of rabbits three days after envenomization and treatment as a cure.
Figure 8 (A and B) is the result obtained a few days after treatment with potion 3 (P 3) as a cure. It shows that the paralysis is prolonged in figure 8A, while the rabbit is alive (normal) in figure 8B.

Figure 8: Aspects of rabbits three days after envenomization and curative treatment.

Table 2 summarizes the overall outcome of rabbits, whether or not they survive after their preventive or curative treatment. It shows that:

1. At day 0 (day of venom injection at each batch), there are zero deaths out of thirty (0%).
2. Day 1, there are seventeen deaths out of thirty (56.67%).
3. On day 2, there are two deaths out of thirteen (15.38%).
4. At days 3 and 4, there are zero deaths out of eleven (0%).

The same table reveals that during the preventive or curative treatment of different batches, the number of deaths varies depending on the various potions used. So, it is noticed that for the batches treated as a preventive measure:

1. The untreated poisoned control lot, the lots treated with viper skull ash (P 4) or with ash consisting of *Mucuna pruriens* and *Millettia pinnata* (P 5) or with ash consisting of both plants and viper skull (P 1) all died one day after treatment (100%).
2. Lots treated with *Mucuna pruriens* ash (P 2) or viper skull ash and *Mucuna pruriens* (P 6) each lost one rabbit in three or 33.33% of their numbers one day after treatment and the rest survived throughout the experiment.
3. The batch treated with viper skull ash and *Millettia pinnata* (P 7) lost two out of three rabbits or 66.67% of its numbers one day after treatment and one remaining rabbit survived during the whole experiment.
4. The batch treated with *Millettia pinnata* ash (P 3), all the animals survived throughout the experiment (100% survivors).
5. Whereas for batches treated for curative purposes:
6. The batch treated with the ash consisting of the two plants and the viper skull (P 1) allowed the survival of one rabbit out of three, i.e. 33.33%.
7. The batch treated with the ash of *Millettia pinnata* (P 3) saved two out of three rabbits, i.e. 66.67%.

In sum, out of a total of thirty (30) rabbits, nineteen (19) died regardless of the type of treatment and the potion used (63.33%).

The following table 2 is a summary of the balance sheet of rabbits surviving or dead after their treatment as a preventive or curative measure.

Table 2: Summary of the balance sheet of rabbits surviving or dying after preventive or curative treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Preventive</th>
<th>Curative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T. E</td>
<td>P 1</td>
<td>P 3</td>
</tr>
<tr>
<td>Day 0</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Day 1</td>
<td>3/3</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Day 2</td>
<td>-</td>
<td>-</td>
<td>0/3</td>
</tr>
<tr>
<td>Day 3</td>
<td>-</td>
<td>-</td>
<td>0/3</td>
</tr>
<tr>
<td>Day 4</td>
<td>-</td>
<td>-</td>
<td>0/3</td>
</tr>
<tr>
<td>Total</td>
<td>3/3</td>
<td>3/3</td>
<td>0/3</td>
</tr>
</tbody>
</table>

T.E: Untreated envenomed witnesses ; -: Absence of animals.

DISCUSSION

The results of the *in vitro* hemolysis test showed that, in general, the venom of *Naja nigricollis* has a hemolyzing effect on rabbit whole blood. Nevertheless, these effects are a function of its concentration. In fact, hemolysis is more important when the concentration is high. This explains why the density is low with the concentration of 1.6 mg/mL; it is higher with the lowest concentration...
used (0.4 mg/mL). This gradual venom action would imply that the effect would be a function of the quantity injected. Therefore, the venom has a dose-dependent action on red blood cells in vitro. These results are in agreement with those of Chippaux, 2006. The author indicates that the effect of elapidae venom is proportional to the quantity of toxin molecules introduced into the organism.

The tests carried out on control (reference) rabbits and those scarified as a preventive measure have resulted in rabbits that are calm, docile and easy to handle. These characteristics are consistent with those described by Langlois, 2013.

Envenomation tests performed on rabbits have shown that 20 to 30 minutes after the injection of the venom, the rabbits are agitated. They have difficulty moving, lifting or relaxing the leg on which the injection was made. They lie down and move around with difficulty showing that they lose their balance. These observations would prove that the venom of elapidae spreads rapidly. In effect, according to Chippaux, 2006, the rapid diffusion of elapidae venom is due to the presence of small size toxins.

If after a few hours of envenimation some rabbits are alive but have paralyzed legs and others are dead, it would mean that:

- In the case of paralysis, there would be a fixation of neurotoxins on the cholinergic receptor of the neuromuscular plaque, thus preventing the transmission of nerve impulses (Chippaux, 2006).
- In the event of death, this would be due to paralysis of the diaphragm leading to respiratory failure (Larréché et al., 2008).

If, in the summary table, the number of deaths and survivors generally varies according to the day or mode of treatment (preventive or curative), it would mean that:

- Depending on the day, there would be a time for the venom to be lethal. This action time varies between thirty minutes and ten hours (Larréché et al., 2008).

This assertion could justify the fact that there were zero (0) deaths on day 0, seventeen (17) deaths out of thirty (30) on day 1, or 56.67%, and two (2) deaths out of thirteen (13), or 15.38%.

However, if on days 3 and 4, there are zero (0) deaths out of eleven (11) or (0 %), it would imply that there were elements that would have prevented the death of these animals. These elements would be due to the presence of the minerals used in the experimental tests.

- Depending on the treatment method, it is noted that:
  ✓ as a preventive measure, if the untreated poison control lot, the lots treated with ash from viper skull (P 4) or with ash consisting of Mucuna pruriens and Millettia pinnata (P 5) or with ash composed of both plants and viper skull (P 1) all died one day after treatment, i.e. 100%, this would mean that in the case of the untreated poison control lot, the venom had a lethal action on this lot; whereas in the other batches the minerals used had no action on the expression of the venom. But, the non-effect of P 1, P 3 and P 5 could be explained by the fact that P 4 which is essentially made up of viper skull ash is inactive on the venom; the amount of viper skull ash used in the preparation of P 1 (a mixture of viper skull ash, Millettia pinnata and Mucuna pruriens) is double that of the others. As such, this inactivity of the viper's skull could cause the slightest effect of P 1 on the venom. As for P 5, its lesser activity could be due to a problem in the dosage of the minerals Mucuna pruriens and Millettia pinnata which enter into its composition or to a problem of competition between the two.
  ✓ Always as a preventive measure, if the batch treated with the viper skull ash and Millettia pinnata (P 7) lost two rabbits out of three of its number one day after treatment and one remaining rabbit survived throughout the experiment, i.e. 66.67 %, this would prove that the minerals contained in the composition of P 7 have a relative activity on the venom. However, the quantities of Millettia pinnata ash and viper skull are the same. Thus, the presence of minerals from the viper skull, which does not normally act on the venom, comes as if to diminish the action of those from Millettia pinnata.
  ✓ As a precautionary measure, if the batches treated with Mucuna pruriens (P 2) or viper skull ash and Mucuna pruriens (P 6) each lost one rabbit in three of their numbers one day after treatment, and the rest survived all along the experiment, i.e. 33.33 %, this would mean that the minerals used in the composition of P 2 have a significant effect on the venom. This significant action is noted with P 6. Nevertheless in P 6, the quantities of Mucuna pruriens and viper skull minerals are the same. In the latter case, the sensitive expression of P 6 may be due to the fact that the minerals from viper skull had no noticeable action on those from Mucuna pruriens.
  ✓ Again as a precautionary measure, if the batch treated with Millettia pinnata ash (P 3), all the animals survived throughout the experiment, i.e. 100 % survivors, this could be explained by the fact that the minerals contained in P 3 have a significant action on the venom.

Ultimately in the design of an anti-venomous drug as a preventive measure, it would be advisable to start with P 3, then P 2 and 6 and finally P 7.

✓ on a curative basis, if the batch treated with the ash consisting of the two plants and the viper skull (P 1) allowed the survival of one rabbit out of three, i.e. 33.33%, this would mean that the minerals...
contained in P 1 which did not have a preventive action have a relative curative action and this despite the same quantities used.

✓ Still as a curative measure, if the batch treated with *Millettia pinnata* ash (P 3) saved two out of three rabbits, i.e. 66.67%, this would mean that the minerals contained in P 3 are significantly active on the venom.

In short, as a curative measure, it would be recommended as an antivenomous drug in a first step P 3 and P 1 later on.

Clearly according to the results of this study P 3 consisting essentially of *Millettia pinnata* can be used as an antivenomous drug as a preventive and curative. And this even if, as a curative measure, this potion did not allow 100% survival.

CONCLUSION

The general objective of this study was to determine the antivenomous effects of the minerals *Mucuna pruriens* and *Millettia pinnata* in rabbits. To these two plants are granted at the traditional level of antivenomous therapeutic virtues. Thus, this study corroborates the objective of our course which is to valorize the medicinal plants of our heritage. The results of this study showed that:

- Venom has a dose-dependent action on red blood cells *in vitro*.
- The minerals of these two plants used have antivenomous effects on the venom.
- The action of the different mixtures of minerals differs according to the plant used, the combinations made and the treatment method.

Thus, the minerals of *Millettia pinnata* have a significant action on the venom as a preventive measure; while as a curative measure its minerals have a significant active effect on the venom. As for the minerals of *Mucuna pruriens* as a preventive measure, they have a significant effect on the venom. Therefore for the design of an antivenom medication, it would be especially advisable to use the minerals of *Millettia pinnata*.

REFERENCES


