AN IN-VITRO STUDY OF THE EFFECT OF HYDROETHANOL EXTRACT OF *ANNONA SENEGALENSIS* LEAVES ON *BITIS RHINOCEROS* (VIPERIDAE) VENOM INDUCED COAGULOPATHY.

ABSTRACT

Bites of *Bitis Rhinoceros* (Viperidae) usually cause a hemorrhagic syndrome in the victims. This is a medical emergency. Antivenom immunotherapy remains effective. However, its availability and cost still limiting its use in Africa in general and in Côte d'Ivoire particularly. The roots of a plant of the Annonaceae family (*Annona senegalensis*) are used in Côte d'Ivoire to treat cases of envenomations by Viperidae. The objective of this work is to study the modulation of the effect of *Bitis rhinoceros* venom on coagulation parameters by the hydroethanol extract of *Annona senegalensis* leaves.

The phytochemical study of the plant was carried out by monitoring effect of the venom on three blood coagulation parameters (PT, APTT, Fibrinogen) from healthy individuals in the presence and absence of the hydro-ethanolic extract. Phytochemistry revealed the presence of saponins, tannins, and polyphenolic compounds including flavonoids in this plant.

The study of the effect of the venom showed that at the concentration of 10^{-2} mg/mL the venom of *Bitis rhinoceros* modifies some coagulation parameters by the decrease of half of the prothrombin rate, the increase of the coagulation time. It has no effect on the fibrinogen level.

The effect of this venom is inhibited by 1 mg/mL of the hydroethanol extract. The leaves of *Annona senegalensis* contain molecules that could neutralize the action of the venom in the coagulation disorder.

Keywords :*Bitis rhinoceros*, *Anonna senegalensis* hemostasis; phytochemistry

1. Introduction

Among tropical diseases, snakebites are recognized as a major problem and considered one of the neglected diseases by the World Health Organization [1, 2]. The annual number of snakebites exceeds 5 million and the number of deaths following snakebites is 125,000 [3]. To these figures, we must add 400,000 patients who suffer serious functional sequelae:

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amputations, renal failure, neurological sequelae [4]**.** Ophidian envenomations in Africa represent a public health problem, due to their frequency and severity, but also because of the difficulties of their management. It is estimated that there are more than one million snake bites per year in Africa, followed by 500,000 envenomations, of which 20 to 30% are serious, with nearly 25,000 deaths [5]. In some Africa countries, snakebites account for more than a quarter of accidental deaths in adults [6]. However, in Black Africa, only 15% of victims are hospitalized and at most 10% of those who need it receive anti-venom immunotherapy. Viperidae are responsible for 90% of ophidian envenomations in tropical Africa, particularly in the savannah and the Sahel [7]. Among the species found there is a predominance of Bitis and Echis. Bitis generally cause severe bites [8] which lead to the rapid onset of the hemorrhagic syndrome caused by the envenomation. This hemorrhagic syndrome is due to the degradation of the parameters of hemostasis which leads to the loss of the blood clotting capacity of the bitten individual. This envenomation requires rapid treatment because it results in amputation due to necrosis of the bitten limb, and even death of the individual. Unfortunately, the relatively high cost of anti-venomous serotherapy makes this treatment inaccessible to most if not all of the African population.

Plant wealth is one of the best ways to alleviate this problem because in traditional medicine, plants have been widely used in the formulation of medicinal recipes for centuries [9]**.** The use of these plants in developing countries is of increasing health and economic importance [10]. A good number of plants have been reported to have good potential for the treatment of snakebite [11]. In Africa in general and in Côte d'Ivoire in particular, Annona senegalensis is one of the plants used against snake bites in traditional settings and in pharmacological studies [12 ; 13 ; 14]. To participate in the scientific justification of its therapeutic use in traditional settings against VIPERIDAE bites, this study was conducted. The objective of this work was to study the modulation of the effect of the venom of *Bitis rhinoceros* on the parameters of coagulation by the hydroethanol extract of the leaves of *Annona senegalensis*.

2. Materials and methods

2.1. Materials

2.1.1. Vegetal material

The plant material used was leaves of *Annona senegalensis* (Annonaceae). They were collected in the region of Yamoussoukro (Central Côte d'Ivoire) and identified at the Centre

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National de Floristique of the Université Felix Houphouët-Boigny in Cocody under herbarium number UCI001073.

2.1.2. Blood samples

- Inclusion criteria

To be included in the evaluation, the subjects' blood must have normal coagulation parameters proven by coagulation tests. That is, the prothrombin rate (PT) must be between 80 and 100%, the activated partial thromboplastin time (APTT) between 21 and 28 seconds and the fibrinogen level between 2 and 4 g/l. Sampling must be done on healthy volunteers. The blood must come from people of both sexes, regardless of age.

Non-inclusion criteria

Sick people are not included in this study. People with bleeding diseases are not included, including those who have received a transfusion in the previous 2 months or have taken anticoagulant drugs.

- Human Blood

Blood collection was done after an interview for informed consent and the identity of individuals is kept anonymous. The blood samples were taken from healthy people (men and women).

2.1.3. Snake venom

The venom used for this study was that of *Bitis rhinoceros*. This venom was delivered in freeze-dried form by the herpetology laboratory of Adiopodoumé of the Institut Pasteur of Côte d'Ivoire. It was then stored at 4°C.

2.2. METHODS

2.2.1. Harvest and conditioning of plant material

The leaves of *Annona senegalensis* were harvested in the month of April 2019, in the morning before sunrise because the secondary metabolites synthesized by the plant are available and have not yet been used by it, in the absence of sunlight. They were washed, cut and dried in the shade at room temperature (25-30ºC) for three weeks at the Laboratory of Pharmacodynamics-Biochemistry of University Felix Houphouët-Boigny in Cocody.

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Then, using an electric grinder, they were ground into powder. The obtained grind was stored at 4°C for further use.

2.2.2. Preparation of the 70% ethanolic extract

The extraction was carried out according to the method of Zirihi *et al.* [15]. Indeed, 100 grams of plant powder were dissolved in a mixture of 1000 mL of solvent consisting of 700 mL of 96° ethanol and 300 mL of distilled water, then homogenized in a Blender at room temperature (25-30°C). The homogenate obtained was first wrung out in a white cloth square. Then, double filtered on hydrophilic cotton and once on whatman 3 mm paper. The filtrate obtained was evaporated in an oven at 50°C for 48h. This gave the crude hydroethanol extract 70%. The mass of extract obtained was stored in sterile, clean, dry bottles and then kept at 4 °C for later use.

2.2.3. Tri phytochemical analysis of secondary metabolites

The tri phytochemical analysis is a method of characterization of the main phytochemical compounds of the plant such as sterols, polyterpenes, alkaloids, tannins, polyphenols, flavonoids, quinones and saponins. This method was carried out on the hydroethanol extract of *Annona senegalensis* leaves, according to the protocol of Békro *et al.* [16].

2.2.4. In vitro anti-hemorrhagic test

2.2.4.1. Collection and conditioning of blood samples

The blood sample was taken following an interview and the signing of an informed consent by the volunteers, whose age varied between 20 and 45 years.

The sample was taken at the elbow in blue tubes containing sodium citrate (anticoagulant), at the Institut Pasteur of Côte d'Ivoire in Cocody, more precisely at the URAP (Reception and Sample Collection Unit). This blood sample is left to rest for ten (10) minutes then centrifuged at 3200 rpm for five (5) minutes. The plasma is then collected in hemolysis tubes for further testing.

2.2.4.2. Hemostasis or coagulation tests of the sampled subjects

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The hemostasis test was performed on 30 blood samples, 15 for men and 15 for women. This step made it possible to select the persons whose coagulation parameters were normal for further testing.

The performance of the hemostasis test concerned three parameters, namely: the determination of the prothrombin level (PT), the determination of the activated partial thromboplastin time (APTT), and the determination of the fibrinogen level (FIB). The prothrombin level was measured by taking 50µL of plasma from each subject in a hemostasis cuvette. This plasma was incubated at 37°C for 1 minute in a coagulometer chamber (SYSMEX CA-104). After one minute of incubation, 100µL of an activator (Innovin) pre-incubated at 37°C for 10mn is added to the 50µL of plasma to start the prothrombin assay reaction. At the end of the reaction, the device prints the prothrombin level. The activated partial thromboplastin time test was performed as before. Indeed, to 50µL of plasma from each subject collected in a hemostasis cuvette, 50µL of prewarmed kaolin cephalin reagent (Actin FS) is added. The mixture is incubated at 37°C in a coagulometer chamber for 3 minutes. At the end of the incubation, 50µL of 0.025mol/L calcium chloride is added to it and the activated partial thromboplastin time is printed by the coagulometer after the reaction. For fibrinogen level determination, 10µL of plasma from each subject was diluted in 90µL of Buffer in a hemostasis cuvette. The mixture was incubated at 37°C in a coagulometer chamber for 1 min. After incubation, 50µL of Thrombin was added and the fibrinogen level was printed by the coagulometer at the end of the reaction. The PT is normal if it is between 80% and 100%. The APTT is expressed as a second and its normal values are between 21s and 28s. The fibrinogen level shall be between 2 and 4 g/L.

2.2.4.3. Test of the effect of venom on hemostasis parameters

To perform the test, 6 men and 6 women were selected. Each person was sampled with 3 tubes containing sodium citrate (anticoagulant).

The test required the preparation of concentrations of the venom solution in physiological water. The test was done according to the method of Dandjesso et *al*. [17]. It consists of contacting the venom and plasma before assaying the coagulation parameters of this plasma. Indeed, 180µL of plasma is collected in three hemolysis tubes numbered T1, T2, T3. In the first two tubes 20µL of venom of respective concentrations 10^{-1} mg/mL and 10^{-2} g/mL were mixed with 180µL of plasma. This distribution of venom in each tube reduced the venom concentration in tube T1 to 10^{-2} mg/mL and tube T2 to 10^{-3} mg/mL. Tube T3 (Control tube)

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received 20µL of 0.9% physiological water in place of venom. These mixtures were incubated for 30 minutes at 37°C. At the end of the incubation, the coagulation parameters of each reaction medium were determined as before. All the different tests were repeated three times.

2.2.4.4. Test of the effect of the 70% ethanolic extract on the activity of the venom with respect to hemostasis parameters

The same samples from the previous test were used to perform this test.

The test was done using extract solutions prepared with 0.9% physiological water.

Following the same method of Dandjesso et *al*. [17], this test consisted of first contacting the venom and the extract for a 15 min, then mixing them with plasma for 30 min before assaying the coagulation parameters.

-Effect of 70% ethanolic extract of *A. senegalensis* leaves on the three coagulation parameters of plasma from different subjects:

180µL of plasma was introduced into 2 hemolysis tubes. The test tube received 20µL of 10 mg/mL extract solution. This dissolution reduced the concentration of the extract to 1 mg/mL. The control tube received 20µL of physiological water. The mixtures were incubated for 30 minutes. The various coagulation tests were performed on these solutions.

-Effect of 70% ethanolic extract on venom activity:

This study was done by introducing 10µL of 20 mg/mL extract solution into three hemolysis tubes numbered T1, T2, T3. In the first two tubes (Test tubes), 10µL of the venom was added to them so that T1 received the concentration of 2.10^{-1} mg/mL and T2 the concentration of 2.10^{-2} mg/mL. As for tube T3 (Control tube), it received 10µL of physiological water. These mixtures were incubated at 37°C for 15 minutes. After the 15 minutes of incubation, 180µL of plasma was dissolved in these different mixtures. These dissolutions resulted in an extract concentration of 1 mg/mL in each tube and a venom concentration of 10^{-2} mg/mL in tube T1 and 10^{-3} mg/mL in tube T2. These newly obtained reaction media were incubated for 30 minutes at 37°C. At the end of the incubation, the coagulation parameters of each new reaction medium were determined as before. All the different tests were repeated three times.

2.2.5. Statistical analysis

Statistical analyses of the experimental results were performed using Graph PadPrism 8.4.3 software (Microsoft, USA). The values are presented as mean ± standard error. Data were evaluated by the one-way ANOVA analysis method followed by Tukey's multiple comparison test at the 5% threshold to assess the significance of observed differences. If $P<0.05$ the

difference between the values is considered significant and if P>0.05 this difference is not significant. In addition, the graphical representations were made with the same software.

3. Results

3.1. Phytochemical compounds of the hydroethanol extract of *A. senegalensis* leaves

The phytochemical sceening allowed to highlight a variety of secondary metabolites in the hydro-ethanolic extract of leaves. Polyphenols, flavonoids, saponins, sterols , terpenes and tannins were revealed, but quinones and alkaloids were not detected during this investigation (Table I).

3.2. Values of hemostasis parameters (PT, APTT and FIB) of plasma in the different solutions obtained.

The values of the three hemostasis parameters (PT, APTT and FIB) of the different solutions are summarized by the histograms represented in Figures 1; 2 and 3.

Figure 1, which shows the histogram of the prothrombin rate (PT), reveals that the normal values of PT for men and women are 101.2 ± 3.16% and 109.03 ± 3.37% respectively. With physiological water, the PT of men is 95.13 ± 5.95% and that of women is 107.3 ± 9.25%. This parameter is 97.23 ± 8.98% for men and 109.73 ± 5.95% for women in the presence of the 70% ethanolic extract at 1 mg/mL. In the presence of venom at concentrations of 10^{-2} and 10^{-3} mg/mL the PT are 40.37 ± 0.81% and 98.27 ± 2.37% for men and 45.87 ± 3.52% and 111.83 ± 2.62% for women, respectively. In the presence of 1 mg/mL extract in contact with the 10^{-2} and 10^{-3} mg/mL venom solutions, the PT values ranged from 113.57 ± 18.51% and 97.43 ± 0.55% for men and from 110.83 ± 1.15% and 110.43 ± 3.76% for women. The prothrombin levels of both men and women were normal because their values were overall between 80% and 100%. Ethanolic extract 70% at 1 mg/mL does not alter the normal TP value of both sexes. In the presence of 10^{-3} mg/mL venom the TP values are practically within the range of normal TP values, whereas at the concentration of 10^{-2} mg/mL venom, the TP values are reduced to below half of the TP values of the test persons. When exposed to 70% ethanolic extract at 1 mg/mL for 15 min, the venom had no effect on prothrombin. The activated partial thromboplastin time (APTT) is summarized in Figure 2, which shows that the APTT of women and men are 27.20 ± 0.44 s and 23.70 ± 0.70 s, respectively. In the presence of physiological water, ethanolic extract and 10^{-3} mg/mL venom this parameter remains normal as it varies from 24.13 ± 0.75 to 27.93 ± 0.64 s. But in the presence of venom at 10^{-2}

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mg/mL the activated partial thromboplastin time is twice as high as the normal APTT for women (64.97 ± 9.62 s) and men (65.73 ± 1.07 s). When the 1mg/mL extract is contacted with the 10^{-2} mg/mL venom solutions, the APTT of the woman and man is normal with values of 24.20 ± 0.35 s and 27.37 ± 0.70 s respectively. Ethanolic extract 70% at 1 mg/mL has no effect on APTT because it does not alter the normal value of APTT of both sexes. In the presence of venom at 10^{-2} mg/mL the APTT values of the test persons doubled. When exposed to 70% ethanolic extract at 1mg/mL for 15 min, the venom had no effect on this parameter.

Figure 3, which shows the serum fibrinogen level, reveals that this level is 3.03 ± 0.06 g/L in women serum and 1.73 ± 0.29 g/L in men serum. These fibrinogen values remained virtually unchanged in the presence of all products tested. Venom at 10^{-2} and 10^{-3} mg/mL and extract at 1 mg/mL had no effect on fibrinogen levels because it did not alter the normal value of this parameter.

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Table 1: Secondary metabolites from the leaves of *Annona senegalensis*

Substances sought	Results obtained
Alkaloids	-
Polyphenols	+
Flavonoids	+

Quinones	-
Saponins	+
Sterols et Terpenes	+
Tannins (catechic)	+
Tannins (gallic)	+

Legend : + presence ;

- absence

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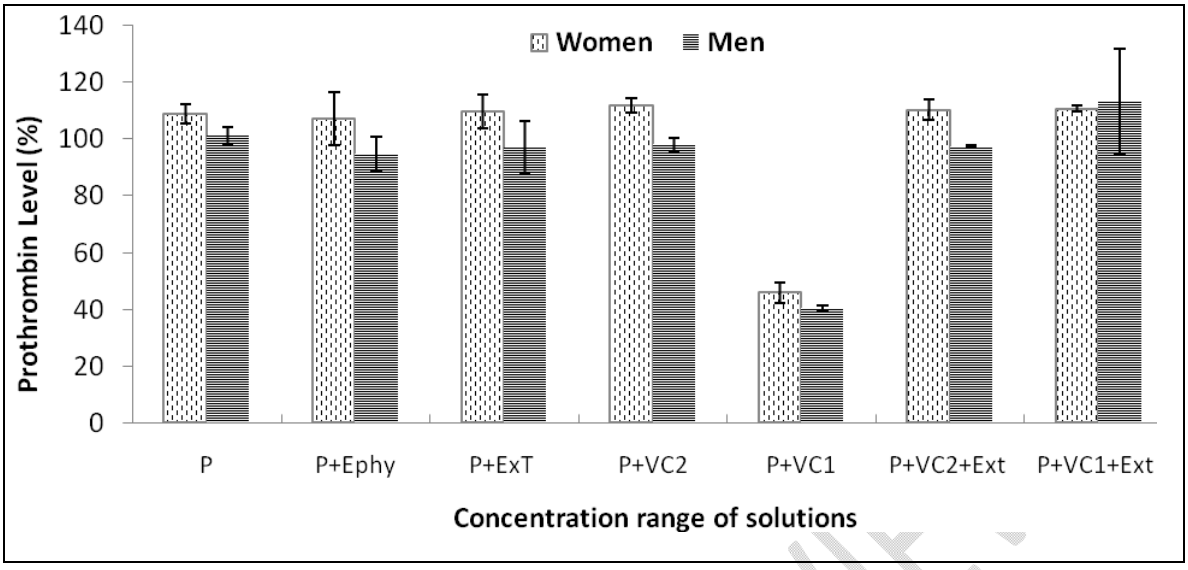
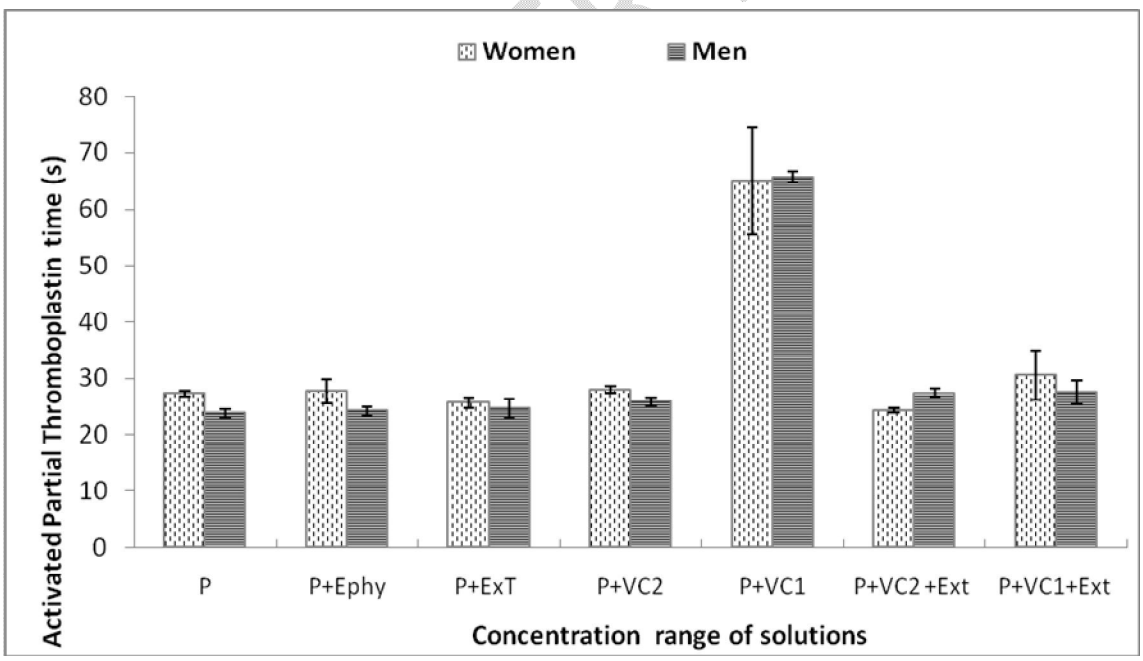


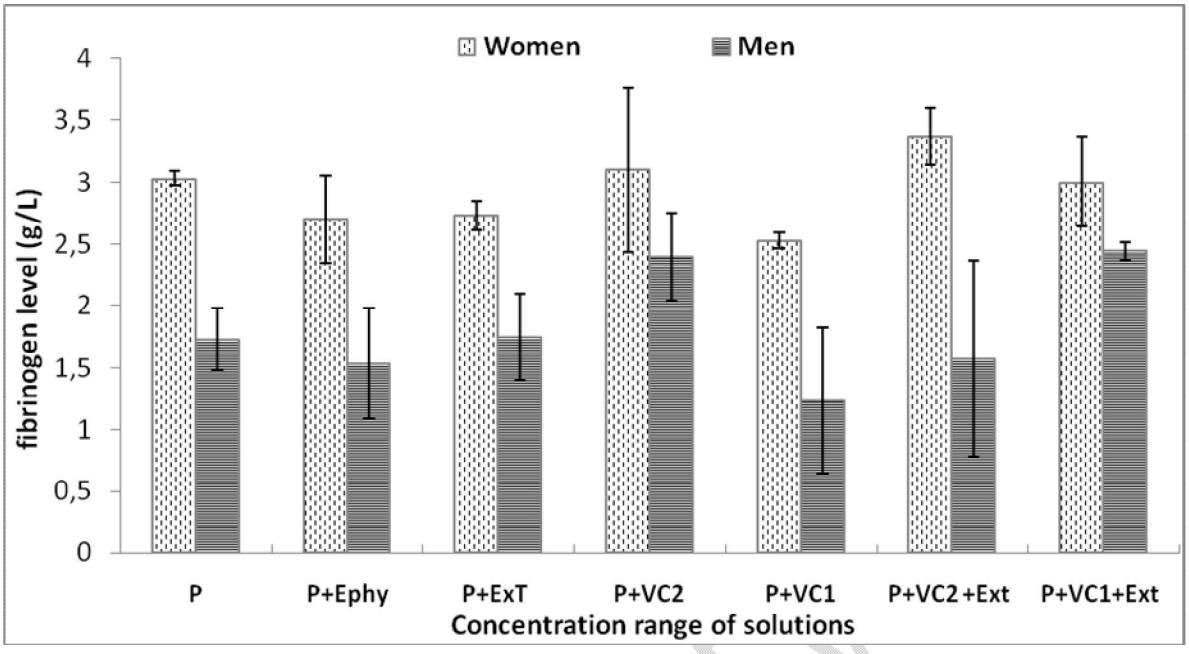
NB: P: Patient Plasma; P+EPhy: Patient Plasma + Physiological Water; P+Ext: Patient Plasma + Extract at 1 mg/mL; P+ Vc_1: Patient Plasma + Venom at 10^{-2} mg/mL; P+Vc_2: Patient plasma + venom at 10^{-3} mg/mL; P+Vc_1+Ext: Patient plasma + Venom at 10^{-2}mg/mL + extract at 1mg/mL; P+Vc_2+Ext: Patient Plasma + venom at 10^{-3}mg/mL + Extract at 1mg/mL.

Figure 1: Prothrombin value of different solutions



NB: P: Patient Plasma; P+EPhy: Patient Plasma + Physiological Water; P+Ext: Patient Plasma + Extract at 1 mg/mL; P+ Vc_1: Patient Plasma + Venom at 10^{-2} mg/mL; P+Vc_2: Patient plasma + venom at 10^{-3} mg/mL; P+Vc_1+Ext: Patient plasma + Venom at 10^{-2}mg/mL + extract at 1mg/mL; P+Vc_2+Ext: Patient Plasma + venom at 10^{-3}mg/mL + Extract at 1mg/mL.

Figure 2: Activated Partial Thromboplastin Time value of the differnt solutions



NB: P: Patient Plasma; P+EPhy: Patient Plasma + Physiological Water; P+Ext: Patient Plasma + Extract at 1 mg/mL; P+ Vc_1: Patient Plasma + Venom at 10^{-2} mg/mL; P+Vc_2: Patient plasma + venom at 10^{-3} mg/mL; P+Vc_1+Ext: Patient plasma + Venom at 10^{-2}mg/mL + extract at 1mg/mL; P+Vc_2+Ext: Patient Plasma + venom at 10^{-3}mg/mL + Extract at 1mg/mL.

Figure 3: Fibrinogen value of the different solutions

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4. Discussion

This work was carried out with the aim of studying the modulation of the effect of the venom of *Bitis rhinoceros* on the parameters of coagulation by the hydro-ethanolic extract of the leaves of *Annona senegalensis*, while giving the chemical composition of these leaves.

The phytochemical analysis of the hydroethanolic extract of *Annona senegalensis* revealed the presence of important secondary metabolites such as: polyphenols, flavonoids, catechic tannins, gall tannins, sterols, terpenes and saponins. Alkaloids and quinones were not detected in this extract. The presence of these major chemical groups would participate in many biological activities of *Annona senegalensis*. These results are in agreement with those of Igwe & Nwobodo and Korotoumou *et al.* [18, 19] who showed the presence of these different chemical groups in aqueous, hydro ethanolic and methanolic extracts of *Annona senegalensis* leaves. These results also confirm the work of Yeo *et al*. [20] who did not find alkaloids, nor quinonic substances in the ethanolic extract of the leaves of this plant harvested in the north of Côte d'Ivoire. However, the work of Dandjesso *et al.* [17] which was conducted on the hydro-ethanolic extract of the leaves of this plant in Cotonou(Benin), did not reveal the presence of flavonoids, nor sterols. Also, the work of Sylvains *et al.* [21] on the roots of this plant with the hydro-methanolic solvent did not reveal the presence of flavonoids and sterols, nor terpenes. This difference could be due to the place of collection, drying conditions, climatic conditions and soil. The study of the effect of hydroethanol extract of *Annona senegalensis* leaves on coagulation parameters showed that the values of prothrombin rate (109.03 ± 3.37% for women and 101.2 ± 3. 16% for men), activated partial thromboplastin time (27.2 ± 0.44 s for women and 23.7±0.7 s for men) and fibrinogen level (3.03 ± 0.06 g/L for women and 1.73±0.25 g/L for men) of healthy individuals were practically unchanged in plasmas containing this extract. Statistical analysis of these results, compared with the control results, showed no significant difference at P = 0.05. Our results are in agreement with those of Djandjesso et *al*. [17] who obtained PT and APTT values of the plasma with the alcoholic extract of the leaves of the same plant identical to the TP and TCA values of the Control plasma. Ethanolic extract 70% at 1 mg/mL has no effect on intrinsic (factor I, II, V, IX, X, XI and XII) and extrinsic (factor I, II, V, VII, X) clotting factors [17].

Regarding the study of the effect of venom on the three coagulation parameters, it was noticed that in the presence of venom 10^{-3} mg/mL the values of PT, APTT and fibrinogen are significantly identical to the values of the parameters of the test persons. But at the concentration of 10^{-2} mg/mL venom, the PT values are reduced to less than half of the PT

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values of the test persons. The venom may contain enzymes that degrade prothrombin present in the plasma of men and women at the concentration of 10^{-2} mg/mL. This venom at 10^{-2} mg/mL doubles the APTT values of those tested. The prolonged APTTs could be due to the presence in the venom, of inhibitors of coagulation factors in the plasma of men and women, at the concentration of 10^{-2} mg/mL [22 ; 23]. The venom contains substances that, at the concentration of 10^{-2} mg/mL, would have a degrading or inhibitory effect on intrinsic (factor I, II, V, IX, X, XI, and XII) and extrinsic (factor I, II, V, VII, X) coagulation factors [17]. The concentrations of fibrinogen in plasma containing 10^{-2} mg/mL of venom were not altered, so venom at this concentration has no effect on this parameter.

When exposed to 70% ethanolic extract at 1mg/mL for 15 minutes, the venom had no effect on the Prothrombin Rate and Activated partial thromboplastin time as it did not alter the values of these parameters. The 70% ethanolic extract could prevent the activity of venom in inhibiting or degrading plasma coagulation factors.

5. Conclusion

This study made it possible to obtain results which reveal that *Bitis rhinoceros* venom at a concentration of 10^{-2} mg/mL modifies some coagulation parameters by halving the prothrombin level, lengthening the Activated Partial Thromboplastin Time. It has no effect on fibrinogen levels. The effect of this venom is inhibited by 1 mg/mL of the hydro-ethanolic extract. The leaves of *Annona senegalensis* contain molecules that could neutralize the action of the venom in the bleeding disorder. Research on the effect of venom requires its extension to other coagulation parameters and the identification of the substances responsible for this effect.

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