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Phytochemistry and hemostatic properties of some medicinal plants sold as anti-hemorrhagic in Cotonou markets (Benin)

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Abstract

Annona senegalensis (Leaves), *Newbouldia laevis* (Leaves), *Cassytha filiformis* (aerial part), *Cissampelos mucronata* (aerial part) are four species of medicinal plants commonly sold by herbalists in South Benin for treatment of bleeding. Hemostatic tests performed *in vitro* on hydro alcoholic extracts of these plants revealed that all of them have coagulant properties, with a percentage reduction of plasma re-calcification time which is 31% for *C. filiformis* and 39% for *A. senegalensis*. The action of these plant extracts is different from that of the classical pathway of blood coagulation. *A. senegalensis* extract also showed an astringent power. The phytochemical study of these plants revealed that: *C. mucronata* contains alkaloids and tannins; *A. senegalensis* contains tannins, mucilages and reducing compounds; C. *filiformis* contains alkaloids, tannins (gallic tannins and cathetics) and mucilage; *N. leavis* contains tannins, triterpenoids, mucilages and reducing compounds. The traditional use of plants as hemostatic is convicted with these results.

Keywords: Astringent, Hemostatic tests, Herbal, Medicinal plants,

Introduction

In developing countries where 80% of population use traditional medicine for healing (Koumaré, 1989), research on medicinal plants and their development is crucial. In Benin, various herbal recipes are sold by herbalists in the markets for the treatment of a variety of pathological conditions. In the case of bleeding, a major cause of maternal mortality worldwide (Galownia *et al.,* 2006, USAID, 2007), plants are used alone or in combination. Among the plants sold only as antihemorrhagic recipe in southern Benin, the most common are *Annona senegalensis, Newbouldia laevis, Cassytha filiformis, Cissampelos mucronata* (Klotoé, 2011). The present study aims to check *in vitro* haemostatic properties of some extracts of these plants, identify the major chemical groups present in the plants and research the mechanism of action of plant extracts.

Materials and methods

Study setting

Phytochemical analyzes were performed in the laboratory of Pharmacognosy and essential oils of Benin Center for Scientific and Technical Research in Porto-Novo. This laboratory is located opposite to the National Assembly of Benin.

Hemostatic tests were performed in the Research Laboratory in Applied Biology of the Polytechnic School of Abomey-Calavi at University of Abomey-Calavi (UAC).

Material

Plant material

It consists of hydro-alcoholic extract of: a) aerial part of *A. mucronata Cissampelos Rich* (EHCm), b) Leaves of *Annona senegalensis Pers*. (EHAs), c) aerial part of *Cassytha filiformis L.* (EHCf), d) Leaves of *Newbouldia leavis P. Beauv.* (EHNi)

The plants were harvested from a collection site in Cococodji in December 2011. They were authenticated at the National Herbarium of Benin.

Biological sample

Milk: It was about the whole milk brand "President" commercially purchased.

Plasma: It was obtained by centrifugation at 3500 rpm for 10 minutes of blood samples taken from rabbit sodium on sodium's citrate 0.109 M.

Methods

This study was conducted in two phases: The first phase devoted to the evaluation of hemostatic properties and the second devoted to phytochemical analysis.

Preparation of plant extracts

We performed as part of this work, soaking in a water-alcohol solution. For that, 100 g of plant powder was macerated in 01 liter of a water-alcohol solution (distilled water, alcohol 96% V / V) for 48 h under magnetic stirring at room temperature. The macerate obtained was filtered with cotton wool and the filtrate was concentrated using a rotary evaporator (Heidolph) at

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40°C and then lyophilized. The extract was kept in clean, dry bottles.

Plant extracts thus obtained were diluted at 5% or 10% in distilled water for hemostatic tests.

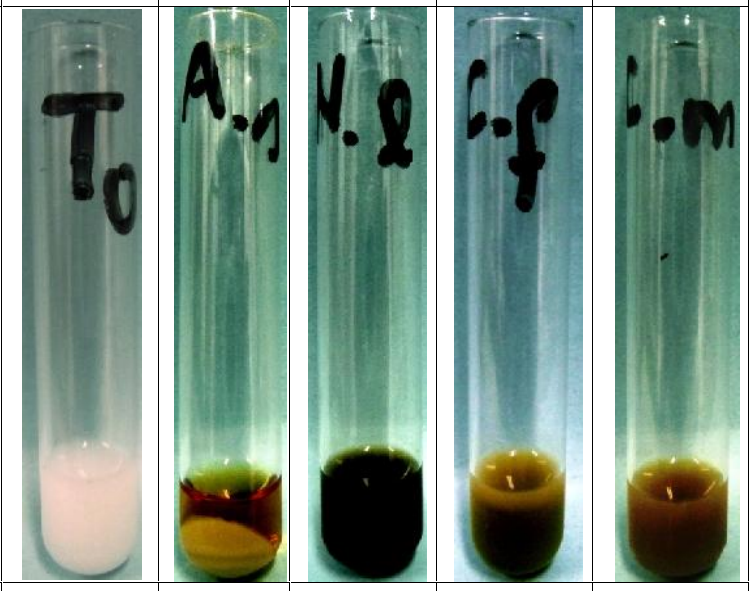
In vitro study of hemostatic properties

Water-alcohol extracts of plants were used, during which the astringency was evaluated by the precipitation of test milk. The pro-coagulant activity of the plant extracts was then assessed through the measurement of plasma re-calcification time (PRT). Finally, the mechanism of action of plant extracts that gave an interesting result for the PRT was sought through the measurement of prothrombin time (PT) and activated partial thromboplastin time (APTT).

Studies of astringent properties

Precipitation of milk: Two tubes, one for the test and the other as control were used. The test tube contained 1ml of plant extract (5%) and the control test contained 1 ml of distilled water. 100 µl of milk was added in each tube. They were homogenized and allowed to stand for 03 minutes, then centrifuged for 01 minute at 3000 rpm. The presence or absence of pellets was noted.

Table 1. Plant extracts effect on milk

	Control	EHA_s	EHN_L	EHC_f	EHC_m
Observations					
Results	-	+	-	-	-

(+) = positive result ; (-) = negative result ; EHA_S = Hydro alcoholic extract of leaves of A. senegalensis; EHN_L = Hydro alcoholic extract of leaves of N. leavis; EHCf = Hydro alcoholic extract of aerial part of C. filiformis; EHC_m = Hydro alcoholic extract of aerial part of C. mucronata

Study of pro-coagulant properties

Measurement of plasma re-calcification time (PRT): For each plant extract, there were four glass hemolysis tubes. 10 µl of the extract solution to 10% was added in two hemolysis tubes. Two empty tubes were used as controls. The tubes were kept in a water bath at 37 °C. 200 µl of plasma, 200 µl of calcium chloride ($CaCl_2$) 0.025 M were introduced into each tube placed in water bath at 37°C and were quickly shaken. The tubes (test and contril) from the water bath were left inclined at an angle of 45° to be viewed every 30 seconds at first, more frequently thereafter, until one observed caking of the mixture (Aouissa, 2002). For each sample, the test was repeated five times, and the average time was calculated.

Effect of plant extracts on coagulation pathways

Effect of plant extracts on the extrinsic pathway of coagulation: Determination of Prothrombin Time (PT): Two glasses of hemolysis tubes, one for testing and one for the witness were used. 100 µl of plasma was put in each tube. Then 10 µl of extract solution to 10% was added in the test tube and 10 µl of distilled water was added to the control tube. The mixture was incubated for 1 minute at 37°C. Then added to each tube was 200 µl of calcium thromboplastin. The clotting time was measured by tilting the tubes at an angle of 45° every 30 seconds to recognize the presence or absence of coagulum (Schved and Biron-Andréani, 2006). For each sample, the test was repeated five times and the average time was calculated.

Effect of plant extracts on the intrinsic pathway of coagulation: Determination of activated partial thromboplastin time (APTT): Two glasses of hemolysis tubes, one for testing and one for the witness were used. 100 µl of plasma was put in each tube and then 10 µl (1 mg) of extract solution was put in the test tube whereas 10 µl of distilled water was put in the control tube. Then to 100 µl of cephalin at 1/10 was added to each tube. After 2 min of incubation at 37°C, 100 µl of calcium chloride ($CaCl_2$) 25 mM was added and the time of appearance of

Table 2. Determination of PRT

	EHN_L	EHA_s	EHC_f	EHC_m
Control time (min)	4.8 (1.292 - 8.308)	5.4 (1.836 - 8.964)	4.5 (1.65 - 7.35)	4.4 (1.346 - 7.454)
Test time (min)	4.3 (1.334 - 7.266)	3.3 (1.626 - 4,974)	3.1 (1.46 - 4.74)	4.1 (1.066 - 7.134)
Reduction percentage	10%	39%	31%	7%
Test t	P = 0.639	*P = 0.044	P = 0.094	P = 0.763

**P < 0.05 = statistically significant difference ; PRT = Plasma Re-calcification Time; EHA_S = Hydro alcoholic extract of leaves of A. senegalensis; EHN_L = Hydro alcoholic extract of leaves of N. leavis; EHCf = Hydro alcoholic extract of aerial part of C. filiformis; EHC_m = Hydro alcoholic extract of aerial part of C. mucronata*

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Table 3. Identification of chemical groups contained in the plant species

Chemical groups	*C. mucronata*	*A. senegalensis*	*C. filiformis*	*N. leavis*
Alkaloids	+	-	+	-
Tannins	+	+	+	+
Cathetics Tannins	-	+	+	+
Gallic Tanins	+	+	+	+
Flavonoids	-	-	-	-
Anthocyanins	-	-	-	-
Leuco-anthocyanins	-	-	-	-
Quinone derivatives	-	-	-	-
Saponins	-	-	-	-
Triterpenoids	-	-	-	+
Steroids	-	-	-	-
Derived cyanogenic	-	-	-	-
Mucilages	-	+	+	+
Coumarins	-	-	-	-
Reducing compounds	-	+	-	+
Anthracene	-	-	-	-
O-heteosides	-	-	-	-
C-heterosides	-	-	-	-
Cardiac heterosides	-	-	-	-

(+) = positive ; (-) = negative

the clot were measured by tipping the tube at an angle of 45 ° every 30 seconds (Schved and Biron-Andréani, 2006) For each sample, the test was repeated five times and the average time was calculated.

Phytochemical analyses

This is a qualitative analysis based on reactions of dyes and / or differential precipitation of the major groups of chemical compounds in plants. This was done on the dry plant material using the methodology described by Houghton & Raman (1998).

Statistical analyses

The data obtained were expressed as means ± standard deviation (S.D.). To check if the time difference between the test and control is significant, Student test was used and the significance level was set at $p< 0.05$. These various operations were performed using the software Excel 2007 and XL-2011 version of Stata.

Results

Research on astringent plant extracts shows that only EHAs precipitated milk proteins (presence of precipitate at the bottom of the tube) (Table1). The results of the determination of plasma re-calcification time (PRT) are reported in Table 2. From this Table, it appears that all plant extracts decreased both the TRP but at different percentages. The best percentage reductions were observed with the EHAs, EHCf and respectively 39% and 31%. Only the reduction obtained with the EHAs is statistically significant. Fig.1 shows results of the measurement of activated partial thromboplastin time (APTT). This graph shows that the APTT obtained with plant extracts is identical to that of the control. This shows that extracts of plant had no effect on intrinsic pathway (factors I, II, V, IX, X, XI and XII) of coagulation.

Fig. 2 presents the results of measuring prothrombin time (PT). This graph shows that the PT obtained with EHCf is identical to that of the control. The EHAs caused a prolongation of PT but with a no significant difference ($p > 0.05$). These extracts plant had no effect on extrinsic pathway (factors I, II, V, VII, X) of coagulation.

The results of the phytochemical analyses are reported in Table 3. It appears that tannins in general and specifically gallic tannins are present in 100% of the species; mucilages are present in 75% of species (*A. senegalensis*, *C. filiformis* and *N. leavis*) alkaloids in 50 % of species (*C. mucronata*, *C. filiformis*); reducing compounds in 50% of species (*A. senegalensis* and *N. leavis*) and triterpenoids in 25% of species (*N. leavis*). We noted the absence of flavonoids, anthocyanins, leuco-anthocyanins, quinone derivatives, saponins, steroids, cyanogenic derivatives, anthracene derivatives and cardiac glycosides in all plants.

Discussion

In this study, hydro-alcoholic extracts of different plants were preferred due to their reported use in traditional medicine as maceration or decoction (Klotoé, 2011). *A. senegalensis* precipitated milk proteins imply the activity of this plant as an astringent. It is important to emphasize here that this astringent activity favors vasoconstriction, which is an important parameter in hemostasis. This vasoconstriction is due to the presence of tannins in this plant. These observations are comparable to Aouissa (2002) who found that the astringent property of the leaves of *Mangifera indica* was related to their rich tannins. Indeed, the tannins have a haemostatic and vasoconstrictor effect on the small vessels, thus their use in trexing varicose veins and hemorrhoids (Bruneton, 1999). According to author, the tannins used orally, are vasoprotective; they limit fluid loss and promote regeneration of tissues in cases of superficial wound or burns. All the plant extracts reduced the plasma re-calcification time, which favors pro coagulating activity of these plants.

It is important to emphasize here that the addition of mineral elements, namely kaolin in medicinal preparations, indicated by herbalists has not been considered in this study. Indeed, kaolin causes *in vitro* activation of factor XII in the presence of high molecular weight kininogen (KHPM) and kallikrein (Javot, 2009). The presence of kaolin could therefore have a potentiating action on the hemostatic activity of these plant extracts. It shall also be noted that these plants are also used in combination with other plants. The combined

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Fig. 1. Measurement of activated partial thromboplastin time (APTT)

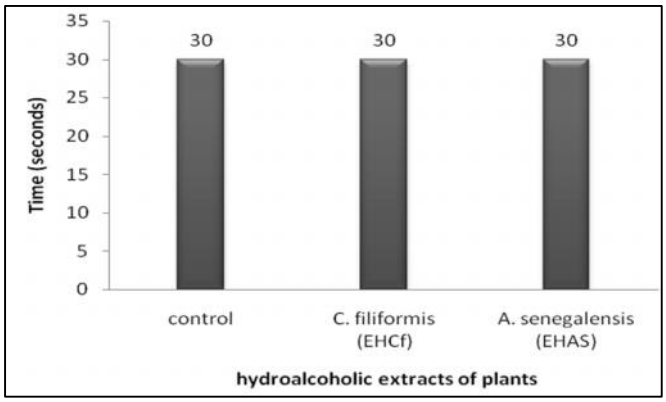
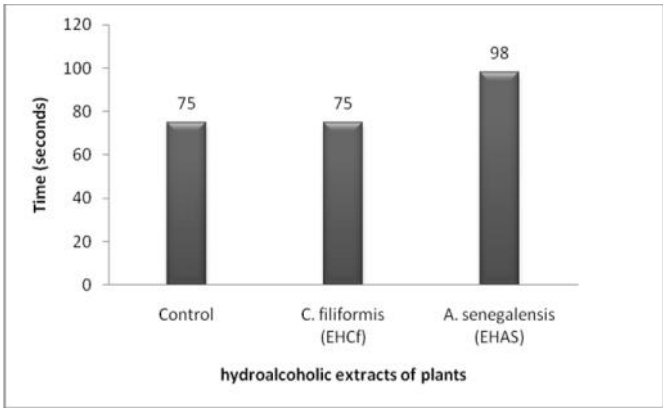


Fig. 2. Measurement of prothrombin time (PT)



use could lead to a synergy of these hemostatic plants. This synergy of activity has already been proved in Turkey by a study of the hemostatic properties of Ankaferd Blood Stopper (ABS) which is a standardized mixture of five plants (*Thymus vulgaris, Glycyrrhiza glabra, Vitis vinifera, Alpinia officinarum* and *Urtica dioica*) (Duz *et al.,* 2010).

Prolongation of PT observed with the extract of *A. senegalensis* is explained by the activity of this plant which is astringency, by precipitating the proteins of the extrinsic pathway of coagulation.

Phytochemical analyses of *A. senegalensis* revealed the presence of tannins, mucilages and reducing compounds. Tannins have been found in this plant by Ouattara (2005) in Mali, Ajaiyeoba *et al.* (2006) in Nigeria, Dodehe *et al.* (2011) in Ivory Coast. The reducing compounds identified in this plant were also revealed by Oumar (2005) in Mali. The same author has shown flavonoids in *A. senegalensis*. Oumar (2005), Dodehe *et al.* (2011) identified coumarins and triterpenoids in this plant. Oumar (2005); Ajaiyeoba *et al.* (2006) have identified saponins; polyuronides, sterols and cardiac glycosides were identified by Oumar (2005) while alkaloids were reported by Ajaiyeoba *et al.* (2006) in this plant.

The phytochemical analyses of *C. filiformis* revealed the presence of alkaloids, tannins and mucilages. Alkaloids have already been demonstrated by Chang *et al.* (1998) in China and Stévigny *et al.* (2002) in Belgium. Tsai *et al.* (2008) in China have also shown flavonoids in these plants.

The presence of tannins in *N. leavis* by our study was also revealed by Usman and Osuji (2007) in Nigeria; Azando *et al.* (2011) in Benin. Our analyzes also revealed the presence of triterpenoids like Usman and Osuji (2007) but in addition their study also revealed the presence of flavonoids, steroids and cardiac glycosides, which were not detected in our analysis. Azando *et al.* (2011) also identified alkaloids and flavonoids which could not be found in our study however mucilage and reducing compounds were confirmed by our study.

Phytochemical analyses of *C. mucronata* showed presence of alkaloids and tannins. However, Tanko *et al.* (2007) identified in addition to tannins, reducing sugars, cardiac glycosides, resins, saponins, flavonoids, glycerin and steroids but they did not detect alkaloids. It follows from the analysis of these plants that the various compounds present especially tannins, may be responsible for the pro-coagulating effect observed with these plant extracts.

Conclusion

The results of these tests have shown that *A. senegalensis* has an astringent property, so it could act on primary hemostasis through vasoconstriction. All the plant extracts showed pro-coagulant action through a reduction in the PRT but in varying percentages. *C. filiformis* and *A. senegalensis* showed the best percentages. These results confirm the therapeutic indications of these plants by herbalists as antihemorrhagic. The extracts did not significantly affect the intrinsic and extrinsic pathway of coagulation, their mode of action would be different from the classical mode of action of coagulation and appears to be related to the effect of certain organic compounds (secondary metabolics), including tannins, on plasma proteins. These plants could probability be developed as Traditional Medicine (TM) for the treatment of bleeding.

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