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Hemostatic activity screening and skin toxicity of sap of *Jatropha multifida* L. (Euphorbiaceae) used in traditional medicine (Benin)

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ABSTRACT

Objective: To evaluate the hemostatic potential of *Jatropha multifida* (*J. multifida*)'s sap as traditional medicine in Benin attributed hemostatic properties to this substance. **Methods:** Several hemostatic tests such as blood coagulation time, Quick time, activated cephalin Time, test of milk precipitation and dosage of total proteins of blood samples were performed at 37 °C. Skin toxicity tests were also realized on 14 Wistar rats. **Results:** Prothrombin time revealed that prior to thromboplastin's addition, there was precipitation of the plasma in all tubes except for the control tube (T_0). After addition of thromboplastin, plasma in control tube coagulated in 78 seconds while plasma in tests tubes were not coagulated (>15 min) ($P<0.05$). The same observations were made for the activated cephalin time for which prior to addition of cephalin activator, there was precipitation of plasma in all tubes except for the control tube (T_0). After addition of cephalin activator, plasma in control tube was coagulated within 43.33 seconds while plasma in tests tubes were not coagulated (>15 min) ($P<0.05$). Blood coagulation time decreased regardless of the administered dose of sap. Time of milk's precipitation showed the sap rushes milk proteins. This was confirmed by the determination of total proteins in serum, proteins which decreased by over 40% with 1/4 dilution for example ($P<0.05$). Sap hasn't also any irritant effect on the skin of animals (the irritation score obtained was 0.00). **Conclusions:** It has been proved that using sap of *J. multifida* has some effects on hemostasis, so its usage in traditional medicine is justified. Moreover, it has no skin toxicity so its usage as local hemostatic is recommended.

1. Introduction

Since 1978, WHO has developed a global classification of traditional medicine[1]. Therefore, the pharmacological properties of several plant extracts have been demonstrated. No less than 170 000 bioactive molecules have been

identified from plants[1]. Despite these results, few plants have been studied for their therapeutic properties, and some pharmacological activities such as hemostatic effects remain unexplored. In Benin, for example, 500 plants were studied among the 3 000 species of plants[2,3]. Only antimicrobial activities, anti-infective and anti-inflammatory drugs are the most popular[4]. No studies have looked specifically at the inventory of hemostatic plants. The few existing datas are from ethnobotanical surveys on the flora as a whole[5]. However, there are some hemostatic plants including *Jatropha multifida* (*J. multifida*) which is used in Vodou ritual practices to stop external bleeding.

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Interest for hemostatic plants is justified by the fact that hemorrhage is the first cause of early death in surgery[6]. It is now a leading cause of maternal mortality worldwide[7–9]. Hemorrhages are unpredictable in 84% of cases[10]. Although it often stops on its own (minor injuries), in many situations, use of mechanical barriers, thermal and hemostatic drugs is essential[11,12]. Thus, the variability in the severity of bleeding today justifies the existence of a variety of hemostatics. Some of them are human, animal, vegetable or synthetic. These substances are administered topically, orally or by injection[13]. But whatever their status or origin, hemostatic products that currently exist are not always efficient[14]. It is therefore important for African researchers to investigate plants known for their therapeutic properties by the legends and traditions. The identification of efficient hemostatics could improve the management of bleeding in all medical disciplines.

J. multifida, in traditional medicine, is used as a hemostatic, usually to treat wounds. It is also used as herbal tea to treat microbial infections. However, in Benin, no scientific study has proved hemostatic potential of this plant. It is then what justifies the study entitled “Hemostatic activity screening and skin toxicity of sap of *Jatropha multifida* L. (Euphorbiaceae) used in traditional medicine (Benin)”.

2. Materials and methods

This study took place in Abomey–Calavi (Benin). Hemostatic tests and skin toxicity tests were conducted at the Laboratory for Research in Applied Biology of Polytechnic School of Abomey–Calavi located at the University of Abomey–Calavi (UAC).

2.1. Study material

2.1.1. Plant material

Plant material is made from the sap of *J. multifida*. It was collected directly into Eppendorf tubes after leaf cutting (Figure 1). Samples were kept refrigerated at 4 °C.

2.1.2. Animal material

Fourteen male albino Wistar rats of 16–20 weeks and weighing between 240 g and 270 g were kept in the Research Laboratory in Applied Biology at constant temperature of (22±1) °C with a 12 h on light and 12 h in the dark. They were fed with pellets and water *ad libitum*.

2.1.3. Other materials

Eppendorf tubes, gloves, alcohol–iodine, glassware (flasks, pipettes, stove) for hemostatic tests, among other things were used. Blood samples were also used. Indeed, they were



Figure 1. Collection from the sap of *J. multifida*.

obtained by venipuncture from adults human volunteers after informed consent. Blood was collected in dry tubes and tubes containing sodium citrate (0.109 mol). Serum and plasma were obtained after centrifugation of these tubes at 3500 towers.

2.2. Methods of study

This work is a prospective experimental study which investigated the effect of applying *J. multifida*'s sap on blood coagulation time, Quick time, activated cephalin time, test of milk precipitation and total proteins of blood samples. All tests were performed at 37 °C. Each test was repeated five times and the average was calculated. A control tube was performed in each case by replacing the sap of *J. multifida* by physiological water.

2.2.1. Measure of blood coagulation time

Five tubes were numbered T₀, T₁, T₂, T₃ and T₄. T₀ served as control and T₁, T₂, T₃, T₄ were respectively given 10, 25, 50 and 100 µL of sap. After passing a water bath for one minute, 500 µL of blood were added to each tube. The timer is immediately started and blood coagulation time of each tube was noted.

2.2.2. Measure of Quick time

Two glasses hemolysis tubes were needed, one for testing and one for the control: 100 µL of plasma was taken in each tube and 10 µL of extract solution was added into the test tube while 10 µL of physiological water was added into the control tube. The mixture was incubated for 1 min at 37 °C and then to each tube 200 µL of calcic thromboplastin was added. The coagulation time corresponding to the Quick time was measured.

2.2.3. Measure of activated cephalin time

Two glasses hemolysis tubes were used, one for testing and one for the control: 100 µL of plasma was put in each tube then 10 µL of extract solution was added to the test tube

while 10 µL of physiological water was put in the control tube, To each tube 100 µL of cephalin with 1/10 dilution was added, After 2 min of incubation at 37 °C, 100 µL of calcium chloride (CaCl₂) was added, The time of appearance of the clot which is the activated cephalin time was then measured.

2.2.4. Precipitation of milk test

Two glasses test tubes were used, one for testing and one for the control. 1mL of plant extract was taken in the test tube and 1 mL of physiological water in the control tube. 100 µL of milk was then added in each of the two tubes. After homogenization, they left to stand for 3 min and centrifuged for 1 min at 3000 r/min. The presence or absence of pellet were noted.

2.2.5. Determination of total proteins

The sap of *J. multifida* was diluted with a pool of fresh normal human serum in a dilution range from 0% to 50% and total proteins were sought in the supernatant after centrifugation. Total proteins were assayed spectrophotometrically using the Biuret method.

2.2.6. Skin toxicity tests

Tests were performed following the guideline of Organisation for Economic Co-operation and Development[15] for testing of chemicals. 24 h before the test, animals' furs of dorsal trunk were shaved. This operation was performed with care avoiding skin injury which could alter its permeability. An area of 40 mm×30 mm was generated (Figure 2). At the beginning of the experiment, rats were placed in individual cages.



Figure 2. Skin toxicity testing: application of the sap to the shaved skin.

The rats were randomly divided into two groups of seven rats: Test group–rats treated with sap; Control group–rats treated with distilled water.

For each group of rats, sap or distilled water was uniformly applied on a free surface of the skin. It was held in contact with the skin with a porous gauze dressing and non-irritating tape for a period of 4 h. At the end of exposure, any residual sap was removed using a buffer of distilled

water. Skin reactions at the portion of the treated skin were visually evaluated at 1, 24, 48, 72 h, 7 days and 14 days after treatment. Scores corresponding to the skin reactions were attributed according to the scoring system described by Draize and Spinel[15].

2.2.7. Statistical analyses

Consider a set of *n* measurements X_1, X_2, \dots, X_n . It is possible to have an overall idea about the distribution of measures by calculating two parameters that are the average and standard deviation[16]. The average is a statistic parameter of central tendency. Indeed, it lets you know the magnitude of measures of a given type. The values are mean ±SD. If the SD is low, the sample values are clustered around the average. While it is important, they are nevertheless widely dispersed. It is calculated using the formula below[17]:

$$\sigma = 1/\sqrt{\sum n (X_i - X)^2}$$
 ranging from $i=1$ to n

Comparisons of comparing two by two the average using the Student *t* test. The softwares used are Microsoft Excel 2010 and 2011 XL Stat.

3. Results

3.1. Measure of blood coagulation time

Averages obtained using sap of *J. multifida* are presented in Figure 3. The significative result has been obtained with the dose of 100 µL (T₄).

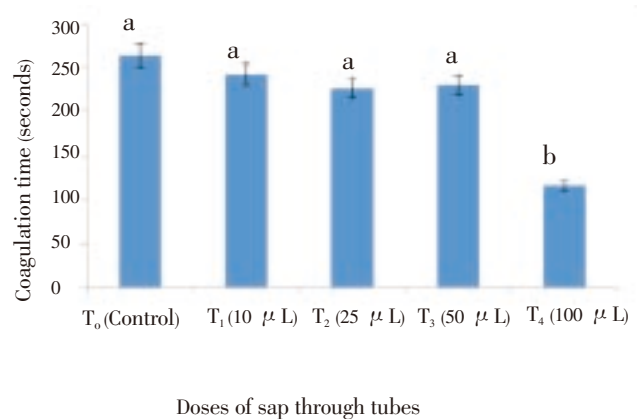


Figure 3. Coagulation time variation depending on the dose of sap. a: P>0.05; b: P<0.05.

3.2. Measure of Quick time

About values of Quick time obtained, before addition of thromboplastin, there was precipitation of the plasma in all tubes except the control tube (T₀). After addition of thromboplastin, plasma in control tube coagulated with

Table 1

Variation of proteins based on dilutions of sap with the serum pool.

Dilutions of sap with the serum pool		0%	5%	10%	15%	20%	25%	40%	50%
Total proteins	Test	77.7±0.1a	72.7±0.2a	69.3±2.5a	52.3±1.4a	46.6±1.2a	25.6±0.2a	25.5±0.1a	25.6±0.4a
	Control	77.7±3.4a	73.8±0.5a	69.9±2.3a	66.1±1.2a	62.2±0.9a	58.3±0.1b	46.6±0.2b	38.9±0.3b

Means with the same letters are not significantly different at significance level $\alpha = 0.05$ (a: $P < 0.05$; b: $P > 0.05$).

78 seconds (Figure 4) while plasma in test tubes were not coagulated (Quick time superior to 15 min) $P < 0.05$ (Figure 5).

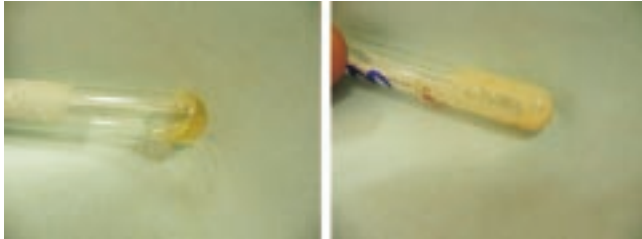


Figure 4. *In vitro* coagulation observed in the control assay.

Figure 5. *In vitro* precipitation observed in the test tube.

3.3. Measure of activated cephalin time

Before addition of cephalin activator, there was precipitation of the plasma in all tubes except the control tube (T_0). After addition of cephalin activator, plasma in control tube was coagulated with 43.33 seconds while plasma in tests tubes were not coagulated, $P < 0.05$ (Figure 6).

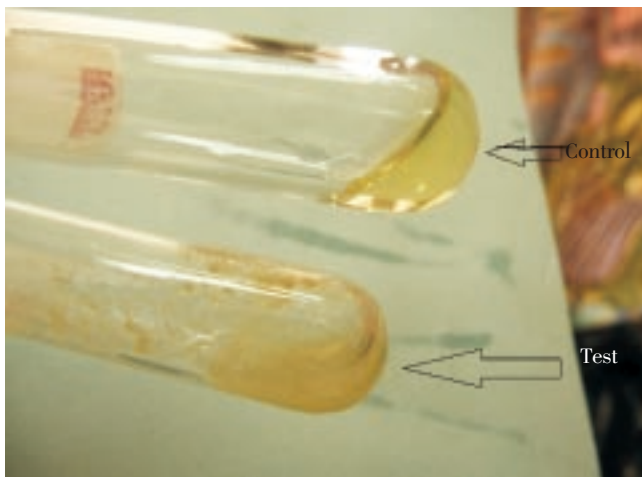


Figure 6. Coagulation and precipitation levels observed in test tube and control tube.

3.4. Test of milk precipitation

Protein's precipitation was noted in less than 1 min in the test tube (Figure 7).

3.5. Determination of total proteins

The addition of the sap of *J. multifida* to serum showed



Figure 7. Precipitation milk test.

a significant decrease of total proteins ($P < 0.05$). Serum proteins decreased by over 40% with $\frac{1}{4}$ dilution. Averages are presented in Table 1.

3.6. Skin toxicity

At the end of first, 24, 48, 72 h, 7 days and 14 days after treatment, rats of the test group and those of the control showed no skin lesion. The irritation score obtained was 0.00 and sap of *J. multifida* is a non-irritating substance.

4. Discussion

Using of the sap of *J. multifida* in traditional medicine has prompted the desire to assess its hemostatic properties *in vitro*. Thus, different tests were performed. Quick time of 78 seconds obtained in the control tube has known indefinite prolongation (more than 15 min) in tests tubes ($P < 0.05$). Before addition of the reagent (thromboplastin), precipitation of plasma was noted in all test tubes. This could be explained by the fact which as blood proteins have been precipitated (conversion of fibrinogen to fibrin), there is no longer sufficient fibrinogen to continue the clotting. This situation was also observed when measuring activated cephalin time. Indeed, there was coagulation in the control tube after 43.33 seconds unlike tests tubes ($P < 0.05$). Quick time and activated cephaline time are indicators of coagulation, it seems that sap of *J. multifida* is not acting on the classical cascade reaction of coagulation neither by the intrinsic pathway nor the extrinsic pathway. The action of the sap of *J. multifida* would be independent of individual clotting factors (II, V, VII, VIII, IX, X, XI, XII and XIII)[18]. This mechanism of action of the sap of *J. multifida* is a particularity of the sap over other hemostatic from plants which acting on the cascade reaction of coagulation[19].

Fibrinogen levels fell significantly immediately after addition of the sap. This justified the protein nature of this substance. It was noticed that the sap of *J. multifida* precipitated all plasmatic macroproteins. A similar mechanism of action has been described for Ankaferd blood stopper, a hemostatic composed of extracts of five plants^[20–22].

The results of blood coagulation time have shown that the more amount of sap is administered, the more rapid clotting is. This might suggest that the effect of sap on coagulation depends on the dose. But comparing these averages to the control value (265 seconds), it has been noticed whatever the dose, the sap has had a reduction of blood coagulation time. Given all of these, it would be rather appropriate to say that the sap of *J. multifida* has a better efficacy on blood coagulation when the application rate is high. Moreover, these tests confirm that even if the sap does not act through intrinsic or extrinsic pathway, it has an undeniable effect on blood coagulation, which demonstrated the specificity of this sap. Decreased coagulation time by this extract is beneficial in case of bleeding.

The study of astringent properties revealed that the sap of *J. multifida* strongly precipitated milk proteins. This protein precipitation could suggest an astringent activity of this plant. It is important to note that the activity of an astringent substance is responsible for vasoconstriction, an important parameter of hemostasis in vascular breakage. This vasoconstriction is certainly due to the presence of tannins and flavonoids in this plant.

The total proteins confirmed the precipitation of plasma proteins. This interaction with the sap and proteins could be explained by the probable action of tannins on blood proteins. Indeed, in the presence of tannins, some soluble proteins are converted into insoluble because of the chemical bonds that develop between them and the tannins^[23]. They are bristling with phenolic hydroxyl groups which are capable of reacting with strong hydrogen bonds with the atoms of peptide binding proteins^[24].

Blood cells quickly joined the protein network and formed with it a cell aggregation comparable to platelet thrombus that forms during the normal process of hemostasis^[18]. The aggregation of red blood cells would be facilitated by the appearance in plasma of high molecular weight proteins (such as fibrinogen, immunoglobulins and albumin) which became insoluble under the action of the sap of *J. multifida*^[25]. Indeed, the insoluble proteins increases blood viscosity and inhibit the movement of red blood cells^[26]. Like fibrin, it is possible that the protein network formed behaves like a net which traps red blood cells but also platelets and leukocytes^[27]. The cell aggregation has significant effects on in vivo hemodynamic^[26]. Increased aggregation produced locally in each capillary will immediately disrupt blood flow^[28]. This could lead to a decrease in bleeding time.

The skin allergy to a substance is a state of hypersensitivity of the skin, immune response to an antigen that appears so excessive or inappropriate, and is also manifested as

erythema and edema^[29]. The absence of these reactions reflects the non-irritant status of *J. multifida* sap. These results are different from those of which showed in his study that volatile toxic may have some skin toxicity^[30]. As the sap of *J. multifida* belongs to the family of Euphorbiaceae^[17], it does not have volatile properties. Works on chitosan, which is also a hemostatic substance, has been reported with no instances of toxicity^[31,32].

Traditional medicine deserves to be appreciated with the numerous studies that were performed on traditional plants around the world^[33–37]. This enhancement involves the production of improved traditional medicines. After this study, it has been proved that the traditional use of the sap of *J. multifida* as a hemostatic is justified. Its effects on various hemostatic parameters were investigated and no skin toxicity was revealed. The mechanism of blood coagulation by the sap of *J. multifida* results from the formation of protein network which constitutes a basis for cell aggregation, independently of coagulation factors. It offers interesting perspectives in therapy because the sap of *J. multifida* could be used as a local hemostatic both for normal subjects as well as those with coagulation disorders such as hemophilia.

Conflict of interest statement

We declare that we have no conflict of interest.

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