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Hemostatic and Antihemolytic Effects of the Aqueous Extract of Manihot esculenta (Euphorbiaceae) Leaves.

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ABSTRACT

Manihot esculenta leaves (Euphorbiaceae) are reputed to have hemostatic properties. Traditional medicine practitioners used the leaves to arrest bleeding in post-partum hemorrhage cases. This study was undertaken to evaluate the effects of the aqueous extract of *Manihot esculenta* leaves (EAME) on blood coagulation *in vivo*. Twenty rats of both sexes were used in this experiment. Five groups of four rats (200 ± 5 g) received orally distilled water, phytomenadione (15 mg/kg b.w) and *Manihot esculenta* leaves extract (250, 500 and 1000 mg/kg b.w.) for four days and after this period, bleeding time was measured by tail hemorrhage model. Prothrombin time (PT) and platelet count were determined by coagulometer and hematological analyzer respectively. Antihemolytic activity was measured by the methods of 2, 2′-Azobis (2-methylpropionamidine) dihydrochloride (AAPH)-induced hemolysis. EAME at 1000 mg / kg b.w. induced a significant decrease of bleeding time from 421 ± 19, 5 s to 247, 5 ± 25, 6 s (p < 0, 01, n = 4). The platelet count of a group of rat treated with EAME (1000 mg / kg b.w) was not affected. The application of EAME decreased PT in a concentration-dependent manner, but this decrease was not significant (p > 0, 05, n = 4). EAME inhibited AAPH-induced hemolysis with IC_{50} values of 0, 2. 10^{-2} ± 0, 15 mg/ml. EAME exhibits hemostatic and antihemolytic effects. The hemostatic property of EAME *in vivo* justifies the use of *Manihot esculenta* leaves in the treatment of post-partum hemorrhage cases.

Keywords: *Manihot esculenta*, Hemostatic, Antihemolytic, Bleeding time, platelet.

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INTRODUCTION

Manihot esculenta Crantz belonging to Euphorbiaceae family, are consumed in western of Ivory Cost and contribute to proteins, minerals, vitamins, fiber and other nutrients which are usually in short supply in daily diets of the majority of population[1].This plant is a woody shrub, providing a basic diet for around 502 million people[2]. *Manihot esculenta* leaves are leafy vegetables considered as valuable sources of nutrients, having great pharmacological properties[3]. These leafy vegetables have been used against many disorders, such as rheumatism, fever, headache, diarrhea and loss of appetite[4]. Pharmacological activities reported in the literature showed that the leaves of this plant possess antihemorrhoid, anti-inflammatory and antimicrobial activities [5,6]. It has also been proved experimentally that peel extract of *Manihot esculenta* Crantz as a coagulant aid to stabilize leachate[7].

Hemostatic agents stop bleeding from an injured vessel, using different mechanisms[8]. These agents are chemical or physical and could prevent anemia or death associated with blood loss. Physical hemostatic agents such as hemostatic forceps were used to clamp injured vessels. Chemical hemostatic agents, including synthetic and natural agents are often used topically in surgery settings to form the clot. Microfibrillar collagen brings natural platelets together and begins the blood clotting process as it comes in contact with platelets[9]. The hemostatic activity of some natural product has been studied by several authors[10]. Traditional medicine practitioners claimed that *Manihot esculenta* leaves possess hemostatic properties[11]. Some practitioners treat blood loss orally in post-partum hemorrhage cases. A study in Ghana, exhibited hemostatic effects of *Manihot esculenta* leaves in human blood *in vitro*[9].However the hemostatic effects of the aqueous extract of *Manihot esculenta* leaves *in vivo* remains unknown.

This study was *undertaken* to establish scientific basis of its use as a hemostatic *in vivo* in the treatment of post-partum hemorrhage.

MATERIALS AND METHOD

Plant material and extract preparation

Manihot esculenta leaves were collected and dried at room temperature. Dried leaves were powdered and two hundred grams (200g) are macerated in a flat-bottomed flask containing 1.5 L of distilled water for 24 hours under a magnetic stirrer. The mixture obtained is filtered through absorbent cotton. The filtrate is dried in an oven at 40 °C for 48 hours. The extract obtained, is stored at 5˚C and used for different assays. A voucher specimen was deposited at the Herbarium from the National Floristic Center of Felix Houphouet Boigny University (UCJ006192)

Animals and ethics

Rabbits (*Oryctologus cuniculus)* and rats (*Ratus norvegicus)* weighing 2 ± 2.4 kg and 215 ± 5 g respectively were used in our work. Experimental procedures and protocols used in this study were approved by the ethical committee of Health Sciences, University Felix Houphouet-Boigny of Cocody-Abidjan. These guidelines were in accordance with the internationally accepted principles for laboratory use and care (86/609/EEC) [12]. Each species of animals were obtained from the Animal House of the Laboratory of Biology and Health of UFR Biosciences at Cocody University in Abidjan (Côte d'Ivoire). They were housed in a constant temperature room with a light/dark cycle of 14/10 hours. All animals were fed and given water ad libitum until use.

Phytochemical screening

Phytochemical screening of *Manihot esculenta* leaves was performed, to highlight the major chemical group such as alkaloids, saponosids, flavonoids, polyphenols, tannins, quinons, sterols and polyterpens, using standard procedures[13, 14].

Chemicals and reagents

Prothrombin, cephalin-kaolin and Calcium chloride ($CaCl_2$ 0.025 M) were obtained from Cypress Diagnostics (Belgium). Ascorbic acid was purchased from Merck (Germany). 2,2′-Azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2,2-Diphenyl-1-picryl hydrazyl (DPPH), were purchased from Sigma-Aldrich (USA). All other chemicals and reagents used, were of analytical grade.

Blood red cell protective activity

Preparation of rabbit erythrocytes

To prepare the red blood cells, two rabbits were anesthetized with ketamine (100 mg / kg b.w.) and the blood was collected from saphena vein. Erythrocytes were isolated and stored according to the method described by [15] with slight modifications. The blood samples collected were centrifuged at 3000 rpm for 10 min. The erythrocytes were separated from the plasma and Buffy coat and were washed three times by centrifugation (3000 rpm, 5 min).The supernatant and buffy coats of white cells were carefully removed with each wash. The washed erythrocytes were stored at 4 ℃ and used within 6 h for further studies.

Hemolytic assay

The method described by 16 was used to determine the percentage of hemolysis of erythrocytes. *Manihot esculenta* extract (10-1 mg / ml), was added to 20% red blood cell solution. The saline solution NaCl (0.9%) (Positive control) and distilled water (negative control) were also added separately to the 20% red blood cell solution. The mixture of 0.2 ml of 20% red blood cell solution and 0.8 ml of Manihot esculenta was incubated for 30

min at 37 ° C and then centrifuged at 3000 rpm for 10 min. The percentage of hemolysis determined at the longwave of 470 nm, was expressed as.

% Hemolysis = [AE / AC] * 100, with AE: Absorbance of the sample and AC: Absorbance of the control (hypotonic solution).

Antihemolytic activity

Investigation of the antihemolytic effect of the extract of *Manihot esculenta* was carried out according to the AAPH (2, 2-azobis 2 amidino-propane-dihydrochloride) method used by [17]**.** The standard control used for the study is ascorbic acid. Conveniently, it was added to (200 µL) extracts or ascorbic acid at different concentrations (10^{-1}-1mg / mL), 200 µL of 20% red blood cells. The mixture was incubated for 30 min at 37^0C. 400 µl of AAPH (200 mM) was added to the mixture and was incubated again at 37^0C for 2h. Before centrifugation of the mixture at 1200 rpm for 10 min, 3 ml of PBS was added. The percentage of inhibition of hemolysis was determined at the longwave of 540 nm, as follows:

% Inhibition of hemolysis = [1- (AE / AC)] * 100, with AC: Absorbance of the positive control and AE: Absorbance of the sample.

DPPH free radical scavenging activity

The antioxidant activities of the plant extracts against 2, 2-Diphenyl-1-picryl hydrazyl radical were determined by spectrophotometry at a longwave of 517 nm. Radical scavenging activity was measured by a slightly modified method previously described by[18]**.**The following concentrations of the extracts were prepared, 0.001, 0.01, 0.1 and 1 mg/ml in ethanol. Ascorbic acid was used as the antioxidant standard at the same concentrations. 1 ml of the extract was placed in a test tube, and 2 ml of ethanol solution of DPPH (0.1mM) was added. A blank solution was prepared containing the same amount of ethanol and DPPH. The radical scavenging activity was calculated using the following formula: % Inhibition = $[(A_0-A_1) / A_0] \times 100$, with A_0: Absorbance of control at a longwave of 517 nm and A_1: Absorbance of testing substance at a longwave of 517 nm

Hydrogen Peroxide (H_2O_2) scavenging activity

In this experiment, we used a prior method of [19]**,** with some modifications for our experiments. A solution of 43 mM H_2O_2 was prepared in phosphate buffer solution (pH 7.4). Both extract and standard solution were prepared at four different concentrations (10^{-3}-1 mg/mL). 2 ml of different concentrations (10^{-3} -1mg/ml) of *Manihot esculenta* extract were added to a H_2O_2 solution (1 mL, 43 mM). Absorbance was measured at 230 nm by spectrophotometer. A blank was prepared using a sodium phosphate buffer without H_2O_2. The percentage of H_2O_2 scavenging was calculated using the following equation:

% Inhibition = $[(A_0-A_1) / A_0] \times 100$, with A_0: Absorbance of the control and A_1: Absorbance in the presence of the sample.

Hemostatic activity

Preparation of platelet-poor plasma

The effect of aqueous extract of *Manihot esculenta* was carried out with platelet poor plasma. Platelet-poor plasma preparation was realized according the method described by the professional order of medical technologists of Quebec[20]. Platelet-poor plasma (PPP) was separated from citrated rabbit whole blood. At first, the whole blood was centrifuged at 2500 rpm for 15 min. The supernatant obtained, was removed without disturbing the pellet. To be sure that the plasma was devoid of platelet, a second centrifugation was operated at 2500 rpm for 10 min. The new plasma was taken without cellular debris and was stored at −20°C until used.

Prothrombin Time (PT) assay

The prothrombin time assay was realized according to [21] methods with few modifications. To explore the effect of *Manihot esculenta* aqueous extract through the extrinsic pathway of coagulation, 43 µl of plasma was pipetted into clotting tubes and incubated for 2–3 min at 37 °C. Then, 7 µl of distilled water (for control), and of plant extracts (10^{-1}- 1 mg/ml) for the test, were added to clotting tubes. Prothrombin reagent (100 µl) pre-warmed at 37°C for 2-3 min was added to the mixture. The coagulation time was recorded with a coagulometer (CyanCoag, Bruxelles).

Acute toxicity

The goal of this study was to determine the mortality percentage and the behavioral changes of animal. The acute oral toxicity of *Manihot esculenta* extract was performed according the **OECD 423** [22] Guidelines. Nine female Rats were divided into three groups of three animals. The first group received distilled water used as reference, and the two other groups were treated with 2000 and 5000 mg / kg b.w. of the extract. To study the behavioral changes, the treated rats were observed every 30 mins for a period of two (2) hours [23].The mortality of animals was appreciated after 24 hours and the toxicity of the extract was determined.

Bleeding Time analysis

Bleeding time was described by several models. The rat tail hemorrhage model is one of the most commonly used animal models for preclinical efficacy studies of hemostatic agents[24, 25]. *In vivo* hemostatic activity of *Manihot esculenta* extract was investigated as described by[25]. Twenty rats of both sexes, were divided into five groups of four animals. Distilled water was administered per os to the control group. The second group received Phytomenadione (15 mg/ kg b.w, positive control). The three other groups were pretreated orally with 250, 500 and 1000 mg/kg

b.w. of the extract for four days. The treated rats were anesthetized with ketamine (100 mg /kg b.w.) and the tip of their tails was cut to induce bleeding. The site of the bleeding was gently blotted with filter paper every 30 s, till the cessation of bleeding. The observation of time was limited to 20 min. Care was taken, that no pressure was exerted on the tail tips that could affect homeostasis.

Hematology assays

Platelet counts are useful information in hemostasis. Blood samples were collected from rats used to achieve bleeding time assay, into heparinized tubes. Hematology Analyzer (Sysmex XN-1000, Japan) was used to determine platelet counts. This automate was able to measure several others parameters such as white blood cell counts, red blood cell count.

Statistical analysis

Statistics were performed using Graph-pad Prism 5 (Graph-pad Software Inc., USA). The results were expressed as mean ± SEM of four independent measurements. Statistical analysis was determined by using One-way Analysis of Variance (ANOVA), and Turkey's multiple comparison test was also applied. The results were indicated as significant at $p < 0.05$.

RESULTS AND DISCUSSION

Phytochemical screening of *Manihot esculenta* aqueous extract

The phytochemical study of the aqueous extract of *Manihot esculenta* revealed the presence of sterols and polyterpins, polyphenols, flavonoids, saponins, quinones and alkaloids (Table1).

Table 1: **Phytochemical analysis of aqueous extract of *Manihot esculenta***

Phytochemicals	EAME
Sterols and polyterpins	+
Polyphenols	+
Flavonoids	+
Saponins	+
Quinones	+
Alkaloids	+
Tannins	-

"+ means presence and – means absence"

Hemolytic activity of *Manihot esculenta* aqueous extract (EAME)

Erythrocytes of rabbit were used to evaluate the hemolytic activity of *Manihot esculenta* extract (EAME) .EAME induced 14,50 ± 2,2 % of hemolysis at 10^{-1} mg / ml. Water (positive control) and saline solution (NaCl 0,9 %) used as negative control, are shown the percentage of hemolysis of 92,1 ± 11,3 % and of 10,2 ± 1,26 % respectively. The percentage of hemolysis of EAME is very close to that of saline solution (isotonic solution). The extract did not show adverse effects on

erythrocytes. Water (hypotonic solution) used as reference, induced significant hemolysis percentage ($p < 0.001$, n = 4) (**Figure 1**)

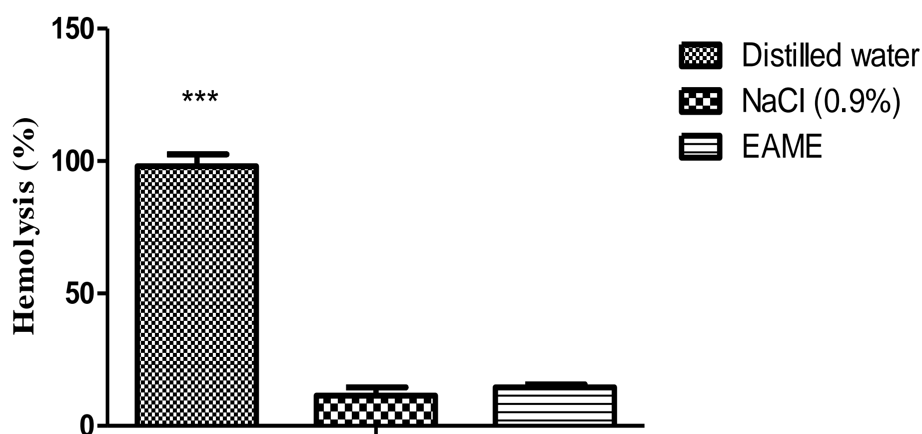

Figure 1: **Hemolytic activity of aqueous leaves extract of *Manihot esculenta* (EAME). EAME showed a weak hemolytic activity compared to that of distilled water (positive control) (***p<0.001).**

Antihemolytic activity of *Manihot esculenta* aqueous extract

2,2′-Azobis (2-methylpropionamidine) dihydrochloride (AAPH) was used to study antihemolytic activity of EAME. *Manihot esculenta* exhibited an antioxidant activity, thereby protecting erythrocytes from hemolysis. The percentage of hemolysis inhibition of EAME was increased in a concentration-dependent manner, with IC_{50} values of 0, 2. 10^{-2} ± 0, 15 mg / ml compared with 0, 25.10^{-2} ± 0.4 mg / ml for ascorbic acid, which served as positive control. Ascorbic acid showed significant inhibition of hemolysis ($p < 0.001$, n = 4). The antihemolytic effects of EAME and ascorbic acid are summarized in Figure 2.

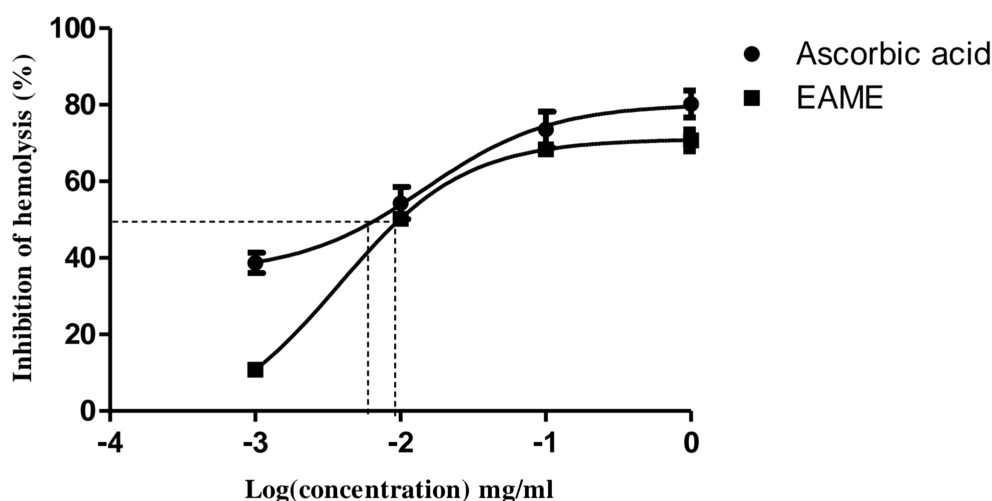

Figure 2: **Antihemolytic activity of aqueous leaves extract of *Manihot esculenta* (EAME). EAME showed an antihemolytic activity very close to that of Ascorbic acid (positive control).**

DPPH radical scavenging activity of *Manihot esculenta* aqueous extract

The aqueous extract of *Manihot esculenta* (EAME) was shown to have significant concentration-dependent DPPH radical scavenging activity, with a concentration required to inhibit radical formation by 50 % (IC_{50}) value of 1, 03 ±0, 4 mg/ ml. In comparison, ascorbic acid, as control, was used and IC_{50} values obtained was 0, 5.10^{-3} ± 0.1 mg / ml (Figure 3).

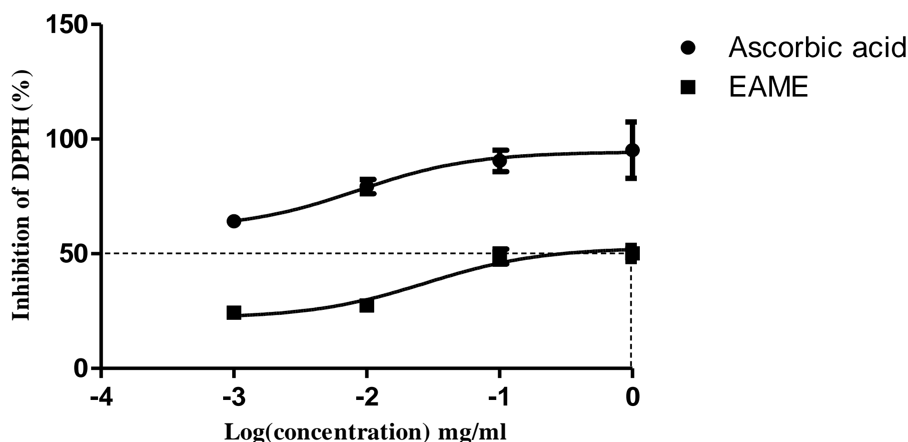

Figure 3: **DPPH scavenging activity of aqueous leaves extract of *Manihot esculenta* (EAME). EAME and Ascorbic acid (positive control) showed IC_{50} values of 1, 03 ±0, 4 mg/ ml and 0, 5.10^{-3} ± 0.1 mg / ml respectively**.

Peroxide scavenging activity of *Manihot esculenta* aqueous extract

The extract scavenged hydrogen peroxide in a concentration-dependent manner with the IC_{50} value of 1, 01± 0.5 mg/ ml compared with IC_{50} values of 0, 1. 10^{-1} ± 0, 1 mg / ml for ascorbic acid (Figure 4).

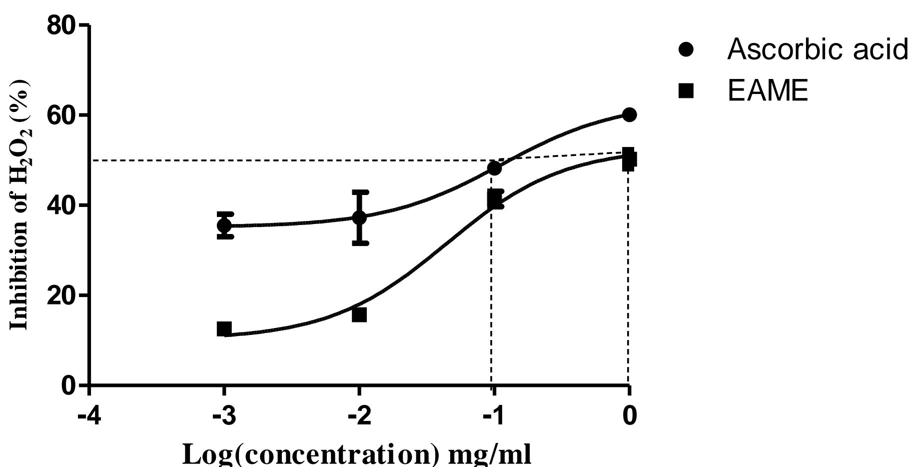

Figure 4: **Hydrogen peroxide scavenging activity of aqueous leaves extract of *Manihot esculenta* (EAME). EAME and Ascorbic acid (positive control) showed IC_{50} values of 1, 01± 0.5 mg/ ml and 0, 1.10^{-1} ± 0, 1 mg / ml respectively**.

Effects of *Manihot esculenta* aqueous extract on prothrombin time (PT)

The plasma poor platelet was used to study the hemostatic effects of *Manihot esculenta*. Aqueous extract of *Manihot esculenta* caused the decrease of prothrombin time in a concentration-dependent manner. At the concentration of 1mg /ml, EAME induced decrease the PT extract/PT control ratio of 1.01 ± 01 to 0.49 ± 0, 4. But these decreases were not significant compared to the control which was distilled water (p > 0, 05, n = 4). The outcomes are summarized in Figure 5.

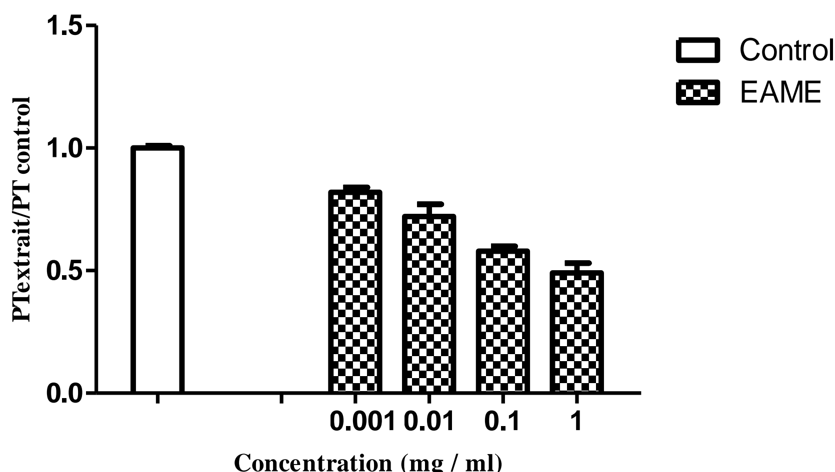

Figure 5: **Effects of *Manihot esculenta* aqueous extract (EAME) on prothrombin time. EAME decreased concentration- dependent manner prothrombin time. The decrease was not significant.**

Acute oral toxicity of *Manihot esculenta* aqueous extract

EAME, at the dose of 5000 mg/ kg b.w., did not induce any mortality in rat .However, at the same dose, EAME produced a general modification of rat behavior compared to the control group. They became very quiet, and remained in groups at the corner of the cage. Their spontaneous locomotion decreased to 2 hours.

Effects of *Manihot esculenta* aqueous extract on bleeding time

The effects of aqueous extract of *Manihot esculenta* were determined on bleeding time. Bleeding time was shortened in rats treated with different doses (250, 500 and 1000 mg /kg b.w.) of the aqueous extract of *Manihot esculenta* compared to the control group of rat. The decrease of bleeding time was dose-dependent. Rat groups treated with EAME (1000 mg / kg b.w) and Phytomenadione (15 mg / kg b.w) were shown to reduce significantly bleeding with the time values of 247, 5 ± 25, 6 s and 128, 7 ± 27, 8 s respectively. A rat group treated with distilled water, used as negative control, showed a bleeding time value of 421 ± 19,5 s (p < 0.01, p < 0.001, n = 4). The results are shown in Figure 6.

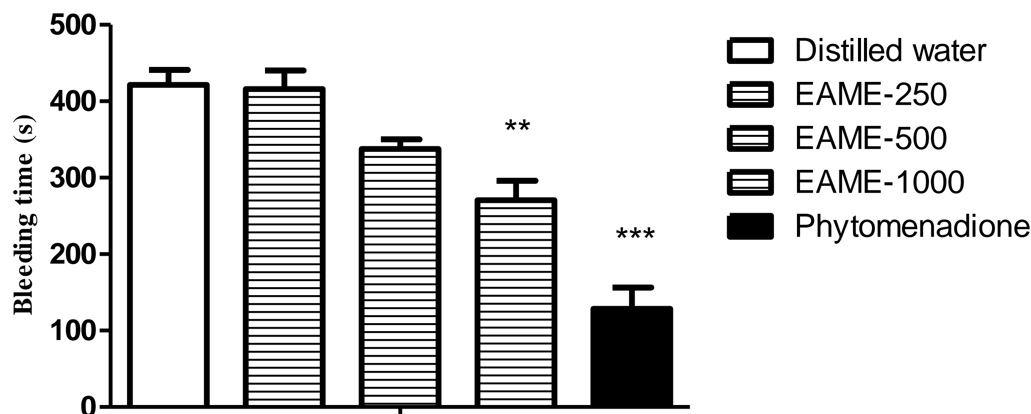

Figure 6: **Effects of *Manihot esculenta* aqueous extract (EAME) on bleeding time. EAME and Phytomenadione decreased dose- dependent manner bleeding time. The decrease was significant ($p < 0.01$, $p < 0.001$, n = 4).**

Effects of *Manihot esculenta* aqueous extract on platelet count

The effects of aqueous extract of *Manihot esculenta* were evaluated on platelet count. Platelet count was not affected in rats treated with different doses (250, 500 and 1000 mg / kg b.w.) of the aqueous extract of *Manihot esculenta* compared to the control group of rat. A rat group treated with Phytomenadione (15 mg / kg b.w) was shown to increase significantly platelet count, compared to control group ($p < 0.001$, n = 4). Outcomes are shown in Figure 7.

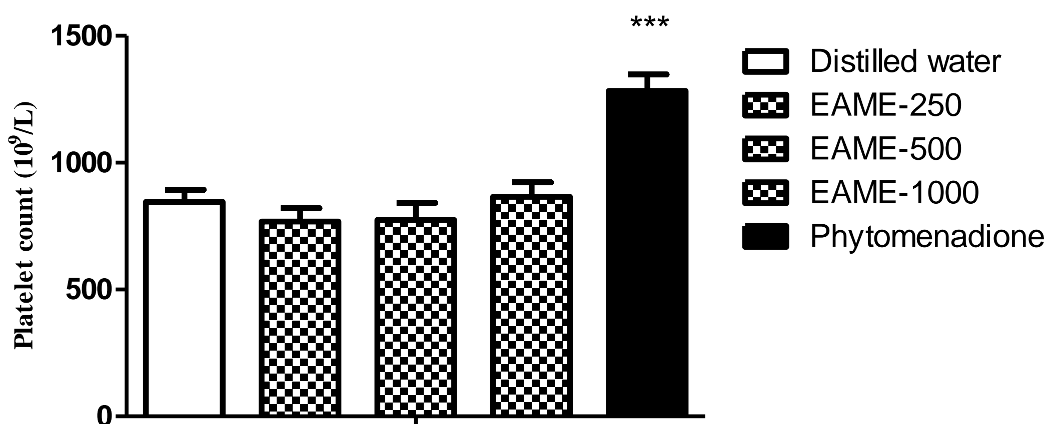

Figure 7: **Effects of *Manihot esculenta* aqueous extract (EAME) on platelet count. EAME was not affected platelet count. Phytomenadione showed a significant increase of platelet count compared to that of distilled water used as negative control ($p < 0.001$, n = 4).**

In this study, *in vivo* and *in vitro* experiments were carried out on blood coagulation. The aqueous extract of *Manihot esculenta* leaves shortened bleeding time whereas platelet count was not affected in rats. The bleeding time is an *in vivo* screening examination for the interaction between platelets and blood vessel wall. A low platelet counts lead to loss of blood [26]. Platelet aggregation

is a useful procedure for thrombus formation following the adhesion of platelets to the site of damaged vessel [27]. According to [28]**,** different steps of platelet aggregation are adhesion, extension and cohesion/aggregation. The aqueous extract of *Manihot esculenta* leaves could stimulate the different steps of platelet aggregation to shorten the bleeding time. However, further study is necessary to evaluate the effects of *Manihot esculenta* on platelet aggregation.

This leafy vegetable aqueous extract reduced prothrombin time, but this decrease was not significant. The extract could not act mainly by extrinsic pathway. Studies realized in Ghana, showed a significant decrease of prothrombin time when cassava leaves extract was applied on Human blood *in vitro*[9]**.** Leafy vegetable extract of *Manihot esculenta* exhibited hemostatic properties. The hemostatic activity of plant extract is attributed to several mechanisms, including activation via increasing the factor XII activity, plasma fibrinogen levels and platelet aggregation[29].

It is well documented that hemolytic samples are unsuitable for coagulation evaluation [20]. Thus, the capacity of *Manihot esculenta* to protect erythrocytes against hemolysis was studied. The aqueous extract of *Manihot esculenta* leaves showed strong antihemolytic activity. This antihemolytic property may be due to the ability of the extract to scavenge free DPPH radical and hydrogen peroxide, elucidated in our work.

Phytochemical screening of *Manihot esculenta* leaves showed the presence of polyphenols, flavonoids. Polyphenols play crucial role in pharmacological activities. Phenols and polyphenolic compounds, such as flavonoids, are widely found in leafy vegetable and show significant antioxidant activity[29]. Also, these compounds can donate electrons to hydrogen peroxide (H_2O_2), thus neutralizing it to water (H_2O). *Manihot esculenta* leaves are not toxic and protect cells against free radical. These effects could justify its use as leafy vegetable and also as a medicinal plant to manage diseases related to oxidative damage of cellular components such as cancer, Parkinson and Alzheimer diseases.

CONCLUSION

The aqueous extract of *Manihot esculenta* leaves exhibits hemostatic effect *in vivo* and protects erythrocytes against hemolysis, scientifically validating the traditional use of *Manihot esculenta* leaves to stop bleeding. However, studies are necessary to evaluate its effect on platelet aggregation.

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AUTHORS'S CONTRIBUTIONS

BLEU GM carried out statistics analysis and FOFIE NMY supervised the work.

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