Investigation of the hemostatic effects of freeze-dried extracts of selected Kenyan plants

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Abstract. Makunyi EG, Bukachi F, Waweru P. 2019. Investigation of the hemostatic effects of freeze-dried extracts of selected Kenyan plants. Biofarmsi J Nat Prod Biochem 17: 39-46. This study aims to investigate the effect and mechanism of action of freeze-dried extracts of Tridax procumbens, Terminalia brownii, Euphorbia tirucalli, and Asphillia africana on hemostasis. Freeze-dried extract of the selected plants was prepared and dose determined for the study. Twelve male New Zealand white rabbits were randomly allocated to two groups (control and test). Blood was collected under standard procedures. Duke's method was used for the bleeding time while the capillary method was used for clotting time. ACL Elitepro machine was used to do prothrombin time and activated partial thromboplastin time. Thromboelastography was done for the most potent extracts. Data were analyzed using independent t-test and results were presented as mean ± standard error of means. Differences were considered to be significant if P < 0.05. The results showed that the percentage yield of the extract was; Tridax procumbens (0.8%), Terminalia brownii (0.5%), Euphorbia tirucalli (0.2%) and Asphillia pluriseta (1.3%). Bleeding and clotting time: The bleeding time was reduced by freeze-dried leaf extract of Tridax procumbens (p = 0.0068) and by freeze-dried bark extract of Terminalia brownii (p=0.0068). Freeze-dried leaf extract of Asphillia africana increased the bleeding time (p=0.01). The clotting time was reduced by freeze-dried leaf extract of Tridax procumbens (p = 0.038), the freeze-dried bark extract of Terminalia brownii (p=0.043) and by freeze-dried stem extract of Euphorbia tirucalli (p=0.01). Prothrombin and activated partial thromboplastin time: The prothrombin time was reduced by freeze-dried leaf extract of Tridax procumbens (p = 0.004), the freeze-dried bark extract of Terminalia brownii (p<0.001) and Freeze-dried stem extract of Euphorbia tirucalli (p=0.001). Activated partial thromboplastin time was reduced by freeze-dried leaf extract of Tridax procumbens (p<0.001), the freeze-dried bark extract of Terminalia brownii (p<0.001) and by freeze-dried stem extract of Euphorbia tirucalli (p<0.001). The results for thromboelastography showed that four parameters of thromboelastography were tested. Freeze-dried leaf extract of Tridax procumbens reduced the r time (p =0.04), k time (p=0.04) and maximum amplitude (p =0.026) but increased the alpha angle (p =0.01). The freeze-dried bark extract of Terminalia brownii did not have statistically significant differences in thromboelastography variables.

Keywords: Asphillia africana, Euphorbia tirucalli, freeze-dried extract, hemostatic, Terminalia brownii, Tridax procumbens

INTRODUCTION

One in seven deaths is associated with traumatic injury and about a quarter of all trauma admissions present with coagulopathy (Mathew and Richard 2010). It is estimated that more than 1.24 million people die annually as a result of road traffic accidents globally. In addition, fifty million people suffer injuries from these road traffic accidents (NTSA 2016). In the United States of America, 40% of trauma fatalities are due to bleeding (Mathew and Richard 2010).

Hemorrhage (bleeding) causes thirty to forty percent of trauma mortality. It accounts for about fifty percent of death in the first 24 hours following the injuries. On admission, 25% to 35% of trauma patients present with coagulopathy, which is associated with a sevenfold increase in morbidity and mortality. Coagulopathy is a condition in which the blood's ability to clot is impaired (Brazzel 2013).

According to the World Health Organization (WHO), as many as 80% of the world’s people depend on traditional medicine for their primary health care needs (Essiet and Akpan 2013). Plants are an abundant natural source of potential new medicines. The approach to new drugs through natural products has proved to be the single most successful strategy for the discovery of new medicines (Jhample et al. 2009).

The use of herbal preparations for staunching blood flow and reducing the risk of blood disorders is prevalent worldwide (Cordier and Steenkamp 2012). Bleeding in rural setups can be caused by injuries or traditional circumcision. Many plants have been used to prevent bleeding. The plants are either chewed or crushed and then applied on the bleeding sites. Many plants are used, but this study chose the commonly used plants in Eastern Kenya.

The present study investigated the effect and mechanisms of action of Tridax procumbens, Asphillia africana, Euphorbia tirucalli, and Terminalia brownii that have been used traditionally to stop bleeding following cuts and after traditional circumcision by eastern Meru and Embu communities. This study used leaves of Tridax procumbens and Asphillia africana, bark of Terminalia brownii, and stem of Euphorbia tirucalli. The aim of this study is to investigate the effects of freeze-dried extracts of Tridax procumbens, Terminalia brownii, Euphorbia tirucalli, and Asphillia africana on hemostasis.
MATERIALS AND METHODS

Extract preparation

The plants were collected and their identity verified at the University of Nairobi Herbarium, Department of Botany, School of Biological Sciences and voucher specimens of the plants deposited therein. The plants were air-dried after which they were milled and then macerated with distilled water in a weight volume ratio of 1:4. The resulting suspension filtered with cotton wool after which Whatman’s filter paper was used. The resulting filtrate was frozen, using the Hot Point deep freezer. The frozen filtrate was freeze-dried at the International Centre for Insect Physiology and Ecology (ICIPE). The resulting freeze-dried extract was weighed and then stored in the deep freezer.

Animal preparation and welfare

Male adult Zealand white rabbits were locally obtained and used for the study. The male rabbits were used because they have almost constant hormonal levels contrary to females. All the animals weighed 2.0-2.5 kg. They were housed in spacious cages in the animal house, Department of Medical Physiology, University of Nairobi. The room temperature was maintained between 15-25°C and relative humidity of 45-65%, a regular 12 hours dark/12hours light cycle. The animals were handled humanely. Selection of rabbits as the experimental animal model was to ensure adequate blood volume is achieved. Adult male New Zealand white rabbits, 8-12 weeks old, weighing 2.0-2.5 kg and healthy were included in the study while sick male rabbits were excluded. They were kept under standard laboratory conditions as recommended by The Federation of European Laboratory Animal Science Associations (FELASA) guidelines (Weiss et al. 2010).

Experimental protocol

Blood was collected using the standard method of bleeding the rabbit from the ear (Duke 1981). For prothrombin time, activated partial thromboplastin time and thromboelastography, blood was collected in clean citrated bottles and tested within two hours.

The tests were performed using blood from six rabbits for each group (control and test groups). The dose of the freeze-dried extracts was determined using the titration method. The dose of freeze-dried extracts used in most of the tests was 10mg/ml. The dosage for the freeze-dried extract of *Terminalia brownii* for thromboelastography was reduced to 2.5 mg/ml because doses of 10 mg, 7.5 mg and 5 mg/ml were too potent that they only indicated a straight line on thromboelastography. Duke’s method of bleeding time was used (Janzarik et al. 1988). Capillary method of clotting time was used (Kumar et al. 2013). Prothrombin time and APTT were done at Kenyatta National Hospital Hematology laboratory. The freeze-dried extracts of *Tridax procumbens* and *Terminalia brownii*, which were the most potent, were evaluated in the thromboelastography stage.

For thromboelastography, 200 μl of sodium citrate was mixed with 1800 μl of rabbit’s blood. The extract and rabbit blood were mixed at a ratio of 1:4 respectively after which 360 μl of the mixture is loaded into a thromboelastography cup. The ratio is similar to the ratio of the reagents used in thromboelastography. Calcium chloride, 0.2 M, was added, and the test is run for one hour.

Data analysis and presentation

Data were entered into STATA Version 11 and were analyzed using independent t-tests. Results were expressed as means ± standard error of means (SEM). Differences were considered to be significant if P < 0.05.

RESULTS AND DISCUSSION

Extract yield

Table 1 shows the percentage yield of the extract that is; *Tridax procumbens* leaf (0.8%), *Terminalia brownii* bark (0.5%), *Euphorbia tirucalli* stem (0.2%) and *Asphilla africana* leaf (1.3%).

Effect of *Tridax procumbens* on bleeding time

Figure 1 shows that the bleeding time is reduced by freeze-dried leaf extract of *Tridax procumbens* with statistically significant differences in the means (93.6±7.4 (c) vs. 64.2±4.5 (t) seconds, P = 0.0068, t =3.39).

Effect of *Terminalia brownii* on bleeding time

The freeze-dried bark extract of *Terminalia brownii* reduced the bleeding time with a statistically significant difference in the means (100.3±7 (c) vs. 82.6 ±4.3 (t) seconds, p=0.0068, t=3.39) (Figure 2).

Effect of *Asphilla africana* on bleeding time

Freeze-dried leaf extract of *Asphilla africana* increased the bleeding time with a statistically significant difference in the means (107.8±10.5 (c) Vs. 152.8±49.2 (t) seconds, t=3.1 p=0.01) (Figure 3).

Effect of *Tridax procumbens* on clotting time

The result shows that the clotting time is reduced by freeze-dried leaf extract of *Tridax procumbens* with a statistically significant difference in means (88.7±7.8 (c) vs. 64.2±4.5 (t) seconds, P = 0.0068, t =3.39).

Effect of *Terminalia brownii* on clotting time

Freeze-dried leaf extract *Terminalia brownii* reduced the clotting time with a statistically significant difference in the means (88.5±8.7 (c) vs. 64.2±9.3 (t) seconds, P = 0.0068, t =3.39)

Table 1. Extract yield

<table>
<thead>
<tr>
<th>Plant</th>
<th>Weight of dried product (gm)</th>
<th>Weight of extract obtained (gm)</th>
<th>Percentage yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Tridax procumbens</em> leaves</td>
<td>500</td>
<td>4</td>
<td>0.8</td>
</tr>
<tr>
<td><em>Terminalia brownii</em> bark</td>
<td>2</td>
<td>10</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Euphorbia tirucalli</em> stem</td>
<td>1</td>
<td>2</td>
<td>0.2</td>
</tr>
<tr>
<td><em>Asphilla Africana</em> leaves</td>
<td>300</td>
<td>4</td>
<td>1.3</td>
</tr>
</tbody>
</table>
Effect of *Euphorbia tirucalli* on clotting time

Freeze-dried stem extract of *Euphorbia tirucalli* reduced the clotting time with a statistically significant difference in means (88.5±8.7 (c) vs. 57±9.7 (t) seconds, t=2.4 p=0.01) (Figure 6).

Effect of *Tridax procumbens* on prothrombin time

The results show that the prothrombin time is reduced by freeze-dried leaf extract of *Tridax procumbens* with a statistically significant difference in the means (9.4±0.17 (c) vs. 5.3±0.17 (t) seconds, t=5.12, p = 0.004) (Figure 7).

Effect of *Terminalia brownii* on prothrombin time

The freeze-dried bark extract of *Terminalia brownii* reduced the prothrombin time with a statistically significant difference in the means (9.4±0.78 (c) vs. 3.8±0.38 (t) seconds, t=6.4, p<0.001) (Figure 8).

Effect of *Euphorbia tirucalli* on prothrombin time

Freeze-dried stem extract of *Euphorbia tirucalli* reduced the prothrombin time with a statistically significant difference in the means (5.8±0.17 (c) vs. 9.4±0.78 (t) seconds, t=4.6, p=0.001) (Figure 9).

Effect of *Tridax procumbens* on Activated partial thromboplastin time

The results showed that the APTT was reduced by freeze-dried leaf extract of *Tridax procumbens* with the statistically significant difference in the means (25.8±1.3 (c) vs. 8.3±1.6 (t) seconds, t=8.53, p< 0.001) (Figure 10).

Effect of *Terminalia brownii* on activated partial thromboplastin time

The freeze-dried bark extract of *Terminalia brownii* reduced the APTT with the statistically significant difference in means of the treatment group (22.1±0.84 (c) vs. 4.2±0.48 (t) seconds, t=18, p<0.001) (Figure 11).

Effect of *Euphorbia tirucalli* Activated partial thromboplastin time

Freeze-dried stem extract of *Euphorbia tirucalli* decreased the APTT with a statistically significant difference in means (22.1±0.84 (c) vs. 5.7±0.31 (t), t=18, p<0.001) (Figure 12).

Effect of *Tridax procumbens* on r-time

The results indicate that freeze-dried leaf extract of *Tridax procumbens* decreased the time with the statistically significant difference in the means (6.2±1.6 (c) vs. 2.7±0.49 (t) minutes, t=2.08, p =0.04) (Figure 13).

Effect of *Terminalia brownii* on r-time

The freeze-dried bark extract of *Terminalia brownii* did not elicit a statistically significant difference in the means of r time (4.9±1.6 (c) vs. 5.3±0.9 (t) minutes, t=0.17 p=0.86) (Figure 14).

Effect of *Tridax procumbens* on k-time

The results indicate freeze-dried leaf extract of Tridax procumbens decrease the k time with the statistically significant difference in the means (3.7±1.1 (c) vs. 1.4±0.18 (t) minutes, p=0.04 t=2.03) (Figure 15).
**Effect of Terminalia brownii on k-time**

The freeze-dried bark extract of *Terminalia brownii* did not elicit a statistically significant difference in the means of k time (1.3±0.26 (c) vs. 2.2±0.6 (t) minutes, t=1.47, p=0.21) (Figure 16).

**Effect of Tridax procumbens on the alpha angle**

The results indicate freeze-dried leaf extract of *Tridax procumbens* increase the alpha angle with the statistically significant difference in the means (43.3±6.9 (c) vs. 69.9±2.51 (t) degrees, t=3.65, p =0.01) (Figure 17).

**Effect of Terminalia brownii on the alpha angle**

The freeze-dried bark extract of *Terminalia brownii* did not elicit a statistically significant difference in the means of alpha angle (69.1±3.4 (c) vs. 51.3±10.8 (t) degrees, t=1.6 p=0.08) (Figure 18).

**Effect of Tridax procumbens on maximum amplitude**

The results indicated freeze-dried leaf extract of *Tridax procumbens* increased the maximum amplitude with the statistically significant difference in the means (62.4±5.6 (c) vs. 34.8±7.6 (t) mm, t=3.65, p =0.026) (Figure 19).

**Effect of Terminalia brownii on the maximum amplitude**

The freeze-dried bark extract of *Terminalia brownii* did not elicit a statistically significant difference in the means of maximum amplitude (61.5±2.8 (c) vs. 46.9±9.7 (t) mm, t=1.44, p=0.19) (Figure 20).

**Discussion**

Coagulation requires complex interactions of cellular and molecular components that mainly involve platelets, plasma, and red blood cells (Hoffman and Monroe 2007). Initially clotting was seen as involving intrinsic and extrinsic pathways with a common pathway at the end, but lately, it has been noted to be due to a balance between the pro-coagulants and anti-coagulants (Hoffman and Monroe 2007). It involves the interaction of coagulation factors and platelets. Coagulation status can be measured by means of laboratory tests. The hemostatic effects of four Kenyan plant extracts (*Tridax procumbens, Terminalia brownii, Euphorbia tirucalli* and *Asphillia africana*) were elucidated using five laboratory tests; bleeding time, clotting time, prothrombin time, activated partial thromboplastin time (APTT) and thromboelastography. Bleeding time assesses the capillary integrity and platelet function. Clotting time measures the time taken to generate thrombin. Prothrombin time mainly measures the effect on the extrinsic pathway and is more sensitive to factor VII. Activated partial thromboplastin time evaluates the effect of the intrinsic pathway factors.

The freeze-dried leaf extract of *Tridax procumbens* significantly reduced the bleeding time (Fig.1). These findings confirm those of Ikese et al. (2015), who found that a freeze-dried extract of *Tridax procumbens* significantly decreased bleeding time. The results are also similar to the ethanolic extracts of the same plant that were shown to reduce the bleeding time (Manjusha et al. 2014) which reflects platelet function (Kumar et al. 2013). This plant has potential activation effects on the platelets.
Figure 9. Effect of *Euphorbia tirucalli* on prothrombin time

Figure 10. Effect of *Tridax procumbens* on Activated Partial Thromboplastin Time

Figure 11. Effect of *Terminalia brownii* on Activated Partial Thromboplastin Time

Figure 12. Effect of *Euphorbia tirucalli* on Activated Partial Thromboplastin Time

Figure 13. Effect of *Tridax procumbens* on r-time

Figure 14. Effect of *Terminalia brownii* on r-time

Figure 15. Effect of *Tridax procumbens* on K-time

Figure 16. Effect of *Terminalia brownii* on K-time
The freeze-dried bark extract of *Terminalia brownii* reduced the bleeding time (Figure 2). These are new findings on this plant that have previously not been reported. Bleeding time indicates the platelet function (Kumar et al. 2013), and therefore, this plant may have activation effects on the platelets.

The freeze-dried leaf extract of *Asphillia africana* increased the bleeding time (Figure 3). These findings were unexpected because other studies demonstrated that it reduces bleeding time. These findings are contrary to those found by Okoli et al. (2007) that showed that methanol and hexane of *Asphillia africana* extract reduced bleeding time. Bleeding time indicates the platelet function (Kumar et al. 2013), and therefore, this plant may have an inhibitory effect on platelet function.

The freeze-dried leaf extract of *Tridax procumbens* significantly reduced the clotting time (Figure 4). This confirms the results of a similar study in which an aqueous extract reduced the clotting time (Ikese et al. 2015). The results are also identical to those of pet ether extract of the same plant that showed a significant reduction in the clotting time (Manjusha et al. 2014). The results are also similar to those of Kale et al. (2008) that showed that ethanolic leaf extracts reduced the clotting time. Jhample et al. (2015) also demonstrated that the plant extract reduced the clotting time. Clotting time reflects the time taken to generate clotting factors specifically thrombin to form a clot (Hoffman and Monroe 2007), and therefore, this plant may have activation effects to clotting factors that lead to thrombin formation.

The freeze-dried leaf extract of *Tridax procumbens* reduced the prothrombin time (Figure 7). These are new findings that have previously not been reported. Prothrombin time evaluates the extrinsic pathway (Hoffman and Monroe 2007), and therefore, this plant may have some activation effects to clotting factors that lead to thrombin formation.

The freeze-dried bark extract of *Terminalia brownii* reduced the prothrombin time (Figure 8). These are new findings on this plant that have previously not been reported. Prothrombin time evaluates the extrinsic pathway (Hoffman and Monroe 2007), and therefore, this plant extract may have some activation effects on the extrinsic pathway coagulation factor.

The freeze-dried stem extract of *Euphorbobia tirucalli* reduced the clotting time (Figure 5). These are new findings on this plant that have previously not been reported. Clotting time reflects the time taken to generate clotting factors specifically thrombin to form a clot (Hoffman and Monroe 2007), and therefore, this plant could have activation effects to clotting factors that lead to thrombin formation.

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of *Euphorbobia tirucalli* significantly reduced the prothrombin time (Figure 9). These are new findings on this plant. Prothrombin time evaluates the extrinsic pathway (Hoffman and Monroe 2007), and therefore, this plant extract may have activation effects on the extrinsic pathway coagulation factor.

The freeze-dried leaf extract of *Tridax procumbens* reduced the activated partial thromboplastin time (Figure 10). These are new findings that have previously not been reported. Activated partial thromboplastin time evaluates the intrinsic pathway (Hoffman and Monroe 2007), and therefore, this plant extract has an activation effect on intrinsic pathway factors.

The freeze-dried leaf extract of *Terminalia brownii* reduced the activated partial thromboplastin time (Figure 11). These are new findings on this plant that have previously not been reported. Activated partial thromboplastin time evaluates the intrinsic pathway (Hoffman and Monroe 2007), and therefore, this plant extract has an activation effect on intrinsic pathway factors.

The freeze-dried leaf extract of *Tridax procumbens* reduced the activated partial thromboplastin time (Figure 12). These are new findings on this plant that have previously not been reported. Activated partial thromboplastin time evaluates the intrinsic pathway (Hoffman and Monroe 2007), and therefore, this plant extract has an activation effect on intrinsic pathway factors.

Freeze-dried extracts of *Terminalia brownii* and *Tridax procumbens* were noted to be most potent among the group and were used during thromboelastography (Figure 12-20). Thromboelastography generated four variables; r, k times, alpha angle, and maximum amplitude. The r time evaluated the time of initiation of a clot of 2 mm amplitude. At ‘initiation,’ Tissue Factor binds to circulating FVIIa and acts with FV to generate FIXa and FXa. The k time evaluated the time taken for clot amplitude of 2 mm to reach 20 mm. This involves the interaction of clotting factors and platelets. During ‘amplification’ of a clot thrombin triggers reactions on the surface of activated platelets, where more FVIIa is produced. Thrombin activates co-factors FV and FVIII. The alpha angle measured the speed of clot strengthening. This mainly involves the interaction of fibrin and platelets. The maximum amplitude measures the ultimate clot strength. This includes mostly platelet function. In the hypercoagulable state, the r and k times are decreased while alpha angle and the maximum amplitude are increased.

The freeze-dried leaf extract of *Tridax procumbens* significantly reduced the r-time, k-time and maximum clot amplitude but increased the α angle. These are novel findings that have previously not been reported. Thus the plant extract acts on the clotting factors mainly in the extrinsic pathway and does not have much effect on the platelets. The main factor in the initiation of clot formation in the extrinsic pathway is factor VII (Hoffman and Monroe 2007).

Freeze-dried bark extract at a dose of 2.5 mg/ml of *Terminalia brownii* increased the r-time, k-time, and the maximum amplitude, but it decreased the alpha angle through none of the changes were statistically significant. Doses of 10 mg/ml, 7.5 mg/ml, and 5 mg/ml all caused very rapid coagulation that indicated a straight line on thromboelastography. The fact that the lower dose produced opposite results may be due to disproportional interaction of the extract molecules and the coagulation factors. This is the first time that this study has been done on this plant. The limitation of the study was that the platelet aggregation test that would have indicated the effect of *Tridax procumbens* on coagulation was not possible because of time and logistical considerations.

In conclusion, freeze-dried leaf extracts of *Tridax procumbens* and freeze-dried bark extract of *Terminalia brownii* have some activation effect on the platelets and have a stimulatory effect on the capillary muscles. Freeze-dried extract of *Asphilia africana* has an anti-coagulant impact contrary to traditional perceptions of it being pro-coagulant. Freeze-dried leaf extract of *Tridax procumbens*, freeze-dried stem extract of *Euphorbia tirucalli* and freeze-dried bark extract of *Terminalia brownii* have some activation effect on both extrinsic and intrinsic coagulation pathways. Freeze-dried leaf extract of *Tridax procumbens* has more effect on factor VII, which is involved in the initiation of clot formation. *Tridax procumbens*, *Terminalia brownii*, and *Euphorbia tirucalli* are potential plants for the development of drugs that can be used to reduce bleeding. The present research recommends that further studies on *Tridax procumbens* to assess the active molecule in coagulation, which can further be evaluated for drug development. Platelet aggregation test for the freeze-dried extracts should also be carried out to verify the low platelet effect seen in thromboelastography.

**REFERENCES**


