Mitigating Thrombotic and Inflammatory Events in Sickle Cell Disease Using a Polyherbal Formulation

Akinbo B. David [1*] • Bamisaye E. Oluwaseyi [1] • Akinbo S. Yetunde [2] • Akanni E. Olufemi [3]

[1] Department of Medical Laboratory Science, College of Medicine and Health Sciences, Afe Babalola University, Ekiti, Nigeria
[2] Department of Human Biology, Brigham Young University, Idaho, United States of America
[3] Department of Medical Laboratory Science, College of Health Sciences, Ladoke Akintola University of Technology, Osun, Nigeria
dakinbo@abuad.edu.ng

ABSTRACT

Abstract. The hypercoagulability, thromboembolic and endothelial complications associated with hemoglobinopathies often results in vascular blockage and occasionally death; which has, therefore, become a challenge to the physician over time. This study aimed at evaluating the protective effects of a polyherbal formulation on endothelial dysfunction, venous thrombosis, coagulation indices; D-dimer and inflammation using 2-butoxyethanol (2BE) -induced experimental model of thrombosis and vaso-occlusive disorders in female albino rats. Forty-two (42) healthy twelve-week old female albino rats were randomly selected for this study and divided into six groups, the rats were administered 2.5mL/kg of 2-BE p.o. and 1mL/Kg of 100mg/mL polyherbal formula for 15 days respectively pre- and post-2-BE administration. The levels of coagulation indices, D-dimer and inflammation marker were thereafter evaluated. Histological assessment of the liver was used to evaluate the expression of microvascular occlusion and ischemia. Polyherbal formulation treatment significantly ($p<0.05$) reduced circulating D-dimer and C-reactive protein levels, as well as the platelet dyscrasias, associated endothelial dysfunction and venous thrombosis in the vessels by decreasing the levels of coagulation indices of the treated groups. There was also down-regulation of the expression of tissue necrosis in the liver of rats treated with 2-BE as a result of Polyherbal treatment. The polyherbal formula, therefore, shows potential for use in the management of conditions associated with thrombotic, thromboembolic disorders and the associated endothelial dysfunction. Molecular investigation of the bioactive components is however expedient to better understand the specific mechanisms of action.

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Keywords: 2-butoxyethanol, Anti-thrombotic, D-dimer, Haemoglobinopathy, Polyherbal Formulation.

1. Introduction:

Haemoglobinopathy has been described as a chronic hypercoagulable state with the patients having an increased predisposition to thrombotic complications, such as venous thromboembolism, inflammation, cardiovascular problems, and stroke, all of which are prominent features of the disease thereby causing a high rate of hospital admission among this group of patients also. Among these disorders, sickle cell disease (SCD) represents a larger proportion of the type of hemoglobinopathy that is predominant in Sub-Saharan Africa and Nigeria has the highest recorded incidence of SCD in the world; with a total of 150,000 babies with SCD being recorded annually. Varied clinical heterogeneities are prominent features of hemoglobinopathies that can be linked to two main pathologic developments which are chronic hemolysis and high viscosity or obstruction within the blood vessels (Brittain and Parise, 2008).

Amplified adhesiveness of the white blood cells, platelets, as well as other sickled cells to the endothelium of blood vessels is a leading cause for hospital admission among patients with hemoglobinopathies (Akinbo et al., 2018). Prior researches in animal models have been able to reveal accelerated thrombus formation in the cerebral microvessels of sickle cell (SS) mice.

The hypercoagulability, thromboembolic complications and cardiovascular problems associated with hemoglobinopathies result from the hemostatic failure thereby causing vascular blockage and occasionally leading to death; which has thus become a challenge to the physician over time (Anwar et al., 2011). Attempts to treat thrombosis with the use of thrombolytic agents have failed to demonstrate practical benefits in patients, as the use of thrombolytic agents such as the alteplase, anistreplase, streptokinase, urokinase, and tissue plasminogen activator are associated with potentially life-threatening side effects

which include anaphylactic reactions, systemic fibrinolysis and tendency for hemorrhage (Mannan et al., 2011; Mahmud et al., 2015). Attention is therefore often focused on managing the associated complications of these thrombolytic drugs by aiming at reducing the duration of thrombotic events and the synthesis of improved therapeutics and recombinant variants of thrombolytic agents for atherothrombotic diseases, sickle cell disease, and other hemoglobinopathies.

Herbal preparations have been employed in the treatment and prevention of several diseases and are renowned for their safety and "natural" abundance in the environment (Anwar et al., 2011). Previous epidemiological studies have also reported some culinary herbs with anti-thrombotic and thrombolytic activities which are now increasingly being studied (Basta et al., 2004). Polyherbal preparations are a combination of various herbs which are now popularly used for their ability to exert their therapeutic effects via concerted efforts of different phytoconstituents in them when compatibly formulated together in the solution (Parasuraman et al., 2014). The polyherbal formulation used in this study comprised of five plants which are; Hunteria umbellate, Calliandra portoricensis, Kigelia Africana, Lagenaria breviflora, and Nauclea latifolia. Each of these plants has been reported to exert analgesic, antispasmodic, antipyretic, immuno-stimulatory, antihyperlipidemic, anti-thrombotic, anti-inflammatory, antioxidant activities and confer cardiovascular protection (Saba et al., 2009; Onasanwo et al., 2011; Adeneye et al., 2011). Hence, a combination of these plant compounds that possess anti-thrombotic, thrombolytic, anti-inflammatory, antispasmodic and antioxidant properties could be useful in preventing thrombotic-induced complications. This study aimed at evaluating the protective effects of a polyherbal formulation on endothelial dysfunction, venous thrombosis, coagulation indices; D-dimer and inflammation using 2-butoxyethanol (2BE) -induced experimental model of thrombosis and vaso-occlusive disorders in female albino rats.

2. Methodology:

2.1. Chemicals and Plants Extraction

The 2-Butoxyethanol solution was a product of Sigma-Aldrich GmBH; Steinheim, Germany while the plant materials were gotten from a local market in Osun State, dried and powdered with a mechanical grinder. 20g of each plant was extracted separately by soaking in 200ml of methanol, allowed to stand overnight and filtered; the filtrate concentrated under reduced pressure at a temperature below 50˚C using rotary evaporator. The residues were resuspended in an equal volume of methanol for 48 hours, the whole extracts of individual plants collected into conical flasks, filtered and the dried extracts from various herbs reconstituted with distilled water.

2.2. Animals and Experimental Protocol

Forty-two (42) twelve-week old female albino rats with average weights of 200 ± 50g were used for the experiment. Animals were housed in solid-bottom aluminum cages, subjected to standard 12-hour light and dark cycle and were fed with commercial pellets and water *ad libitum*. The design and conducts of the experiments were in accordance with ethical norms approved by the Afe Babalola University Medical Research Ethical Committee; all instructions, principles of laboratory animal care and protocols regarding treatment during the experiments were conducted in compliance with the National Institute of Health Guide for Care and Use of Laboratory Animals (1985). The rats were randomly distributed into six (6) groups comprising of 7 animals per group according to the treatment received, acclimatized to their environment for 2 weeks under same conditions and examined to be free of wounds, swellings, and infections prior to the experiments.

2-BE was made up to 2.5mLs/kg b.w using water and administered orally as previously described (Akinbo *et al*., 2018), while the rats were allowed to grow under observation and tested periodically until the thrombotic features developed in significant numbers of them and they were later separated for further experimental treatment protocol as detailed below. 100 mg/mL of the polyherbal formula was thereafter administered orally to all the groups except the negative and 2-BE groups, which received only distilled water and 2-BE orally respectively. These concentrations are sufficient to produce an accurate model without resulting in animal mortality.

The experimental groups included the following:

Negative control (n=7): Received 2.5mL/Kg of distilled water p.o. daily.

2-BE alone (n=7): Received 2.5mL/kg of 2-BE p.o. daily for 2 days.

Prophylactic (n=7): Received an aliquot of 100 mg/mL polyherbal formula p.o. for 15 days followed by 2.5mL/kg of 2-BE administration p.o. for 2 days.

Therapeutic (n=7): Received 2.5mL/kg of 2-BE p.o. for 2 days followed by 100 mg/mL polyherbal administration p.o. for 15 days.

Therapeutic 2 (n=7): Received 2.5mL/kg of 2-BE for 3 days followed by 100 mg/mL polyherbal administration p.o. for 15 days.

Adverse (n=7): Received 100 mg/mL polyherbal p.o. for 15 days.

2.3. Sample Collection

At the end of the experiment, the rats were sacrificed after being fasted overnight under light ether anesthesia and blood was collected from the inferior vena cava using tri-sodium citrate and ethylene diamine tetra-acetic acid (EDTA) anticoagulant bottles, gently mixed and appropriately labeled. The freshly harvested liver was fixed in 10% neutral-buffered formalin for histological analysis.

2.4. Clotting Profile

PT and aPTT were evaluated using DiaPlastin and DiaClin kits (DiaMed GmbH, Switzerland) a previously described method (Cheesebrough, 2006).

2.5. D-dimer Analysis

Plasma concentrations of D-dimer were determined by an immunoturbidometric method using the Roche Cobas Quantitative Integra 400 Analyzer (F. Hoffman-La Roche Ltd., Switzerland). The plasma samples, calibrators, and quality controls were added to the latex particles of uniform sizes which were already coated with monoclonal antibodies (F(ab')2 fragments) to the D-dimer epitopes. The antigen/antibody complexes produced by the mixture yields an increase in the turbidity of the test reactants and change of absorbance with time is dependent on the concentration of the D-dimer epitopes in the sample. The precipitate is then determined turbidimetrically.

2.6. Determination of CRP

Peripheral C-reactive protein (CRP) was assessed in the plasma sample using rat CRP ELISA kit purchased from Hangzhou East Biopharma Co. China according to the instructions of the manufacturer.

2.7. Histopathological Studies

Tissues from the various treatment groups were processed after being harvested, paraffin embedded, sectioned and finally stained with Hematoxylin and Eosin for microscopic examination to determine the definitive evidence of major thrombosis and histopathological changes during the experimental period (Igho and Afoke, 2014; Afolabi et al., 2017).

2.8 Statistical Analysis

Analysis of variance (ANOVA) was employed to determine variance at $p<0.05$ among the study groups and pairwise comparisons were tested with post-hoc tests. Data were expressed as mean ± SEM of a replicate in each group.

3. Results:

aPTT values were significantly increased ($P < 0.001$) in the 2-BE treated group compared to the negative control, treatment with the polyherbal however significantly reduced ($P< 0.001$) the aPTT in comparison to the 2-BE alone group. Plasma CRP levels and PT-INR values were also significantly increased ($P< 0.05$) in the positive control group than the negative control group. The polyherbal co-treatment resulted in a significant reduction ($P<0.001$) in the plasma levels of CRP of respective treated groups. The levels of D-dimer in the plasma of groups treated with polyherbal were significantly reduced ($p<0.005$) when compared to the 2-BE alone group (Table 1).

2-BE treatment resulted in prominent disruption of the liver morphology during histological examinations of the liver of the experimental animals. The 2-BE alone group showed moderate necrosis with congested blood vessels (indicated by arrows) in the triad and disruption of cellular integrity. In contrast, such necrosis and structural disruptions were not observed in the liver of polyherbal treated groups (Figure 1).

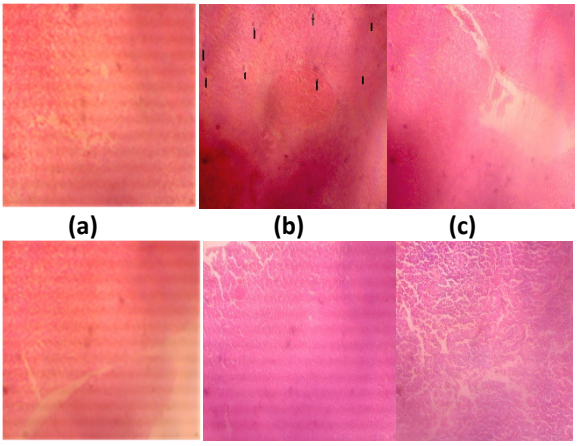

Figure 1. Hepatic sections of all groups (H and E staining); (a) Staining revealed normal features of the liver with

Table 1: *Effect of polyherbal treatment on coagulation indices, D-dimer and CRP*

Variables	Group A	Group B	Group C	Group D	Group E	Group F
PT-INR	0.9 ± 0.01	1.5 ± 0.20*	1.03 ± 0.11[a]	1.13 ± 0.18*[a]	1.03 ± 0.09[a]	0.87 ± 0.03[a]
aPTT (Sec)	41.33 ± 2.4	105.67 ± 7.5*	50.67 ± 4.1*[a]	64.00 ± 7.2*[a]	59.67 ± 3.6*[a]	40.67 ± 4.1[a]
D-Dimer (ng/mL)	491 ± 28.7	685 ± 42.4*	520 ± 37.1*[a]	555 ± 40*[a]	531 ± 50.4*[a]	489 ± 11.3[a]
CRP (ug/mL)	6.26 ± 0.8	14.75 ± 2.7*	9.45 ± 1.2*[a]	10.71 ± 1.9*[a]	11.32 ± 2.1*	8.00 ± 1.2*[a]

All values are expressed as mean ± SD (n=7) and significantly different at $p<0.05$, * Significantly different from group A at $p<0.05$, a Significantly different from group B at $p<0.05$.

evident hepatic portal veins and triad, (b) Staining revealed moderate necrosis with congested blood vessel (indicated by arrows) in the triad and disruption of cellular integrity, (c,d,e) Staining's revealed slight necrosis and restoration of structural integrity of the liver, (f) Staining revealed areas of mild discoloration

4. Discussion:

The effect of polyherbal formulation on some basic biomarkers of endothelial dysfunction and venous thrombosis was analyzed by evaluating coagulation indices and circulating levels of inflammation, D-dimer in the plasma and liver histology of control and treatment groups. 2-butoxyethanol models have been used to investigate thrombotic and thromboembolic presentations having close similarities to the complications associated with sickle cell disease and thalassaemias pathologies (Lewis et al., 2006; Ataga and Key, 2007; Charneski and Congdon, 2010). The decrease in the coagulation indices of polyherbal treated groups suggests that this polyherbal formula possess the ability to enhance clearance of induced thrombosis and microvascular occlusions. Numerous studies have revealed the widespread use of medicinal plants in the treatment of specific hemoglobinopathies such as the sickle cell disease and its complications (Meselhy et al., 2012); the knowledge of their anti-thrombotic and anti-inflammatory activities albeit being currently limited. It is therefore possible that a decrease in the activity of endothelin, cellular adhesion and hemostasis by polyherbal might account for this observation.

The reduction in the coagulation indices observed in rats treated with polyherbal compared to the negative control group could have accounted for the use of these herbs for pro-thrombotic and thromboembolic conditions in folkloric medicine (Charneski and Congdon, 2010; Afolabi et al., 2017). Coagulation indices are considered one of the best biomarkers for endothelial dysfunction and thrombosis (Akinbo et al., 2018). Previous researches have revealed the phytochemistry of some of the experimented plants to contain cardiac glycosides, alkaloids, and flavonoids and they have all been associated with anti-inflammatory, anti-thrombotic and antioxidant actions when combined together (Ayoola et al., 2008). Flavonoids' activities have been linked to their ability to complex with extracellular and soluble proteins that are also found to be effective antioxidants and antiplatelets (Gong et al., 2011). Numerous studies have also shown glycosides to down-regulate blood pressure (Mahmud et al., 2015).

Excessive production of thrombopoietic factors which signals the precursors of megakaryocytes and the megakaryocytes have been reported in infectious and inflammatory states (Thomas and Storey, 2015). CRP and IL-6 are pro-inflammatory thrombopoietic factors which have been implicated in cases of reactive thrombocytosis having elevated plasma levels capable of inducing increased thrombopoietin and consequent reactive thrombocytosis (Heit, 2007). The present study showed a significant increase in the serum CRP expression as a pro-inflammatory biomarker in the 2-BE treated rats compared to the control group. One mechanism of 2-BE induced thrombosis is via the direct alteration of the endothelial lining by the pro-inflammatory markers subsequently resulting in the expression of tissue factor thereby initiating the production of thrombin and thus promoting coagulation. Tissue factor has also been reported to be a proinflammatory mediator (Qari et al., 2012).

However, there is a co-occurrence of erythrocyte adhesion with endothelial dysfunction during systemic inflammation (Rotimi et al., 2016). Peripheral CRP expression in circulation was reduced in the polyherbal treated groups compared to the thrombotic 2-BE positive group. Flavonoids aim for the prostaglandins involved in the late phase of acute inflammation and pain perception and its presence can thus be suggested to be influential in the anti-inflammatory and anti-thrombotic properties of the Polyherbal (Chakraborty et al., 2004). This is consistent with earlier reports which demonstrated the vascular protective activities of the polyherbal phytoconstituents as a major mechanism through which it combats endothelial dysfunction associated with inflammation (Onasanwo et al., 2011; Taïwe et al., 2011; Akanni et al., 2017).

Diseases such as sickle cell disease, venous thrombosis and thrombo-embolism characterized by thrombosis/disseminated intravascular coagulation have been reported to result in elevated D-dimer levels associated with hemostatic dyscrasias (Fakunle et al., 2012; Ekwere et al., 2013). It is however remarkable that treatment with the polyherbal also improved markers of endothelial complications investigated in this study. D-dimers are specific plasmin cleavage products of fibrin degradation which are up-regulated in certain conditions characterized by blood clot formation and breakdown (Kusfa et al., 2017). In this study, 2-BE caused a significant increase in the plasma level of the D-dimer compared to the negative control group which could be indicative of thrombi formation involving multiple sites with varying severity in the positive control rats (2-BE alone). Polyherbal reduced the circulating levels of D-dimer in the treated groups, this could indicate the reinstatement of hemostatic balance by the phytoconstituents via deactivation of the tissue factor and decrease in red blood cells adherence capable of inducing thrombophilia and disseminated thrombosis in the 2-BE treated rats (Koshkaryev et al., 2003). This might also contribute to its property as a thrombolytic and vascular protective agent and one of the major mechanisms through which it combats endothelial dysfunction associated with inflammation. This finding correlates with other studies on the modulatory effect of the herbs in this polyherbal formulation (Akinbo et al., 2018).

Random multifocal hepatocellular necroses were seen in the liver histology of the 2-BE group with

congested blood vessels. These necroses and vascular congestions result from intravascular thrombi and thrombotic dissemination along the microvascular circulation of the rats consistent with previous reports (Afolabi et al., 2017). In this study, 2-BE induced microvascular thrombosis was also accompanied with severe triaditis sequel to the continuous blockage of the microvascular environment by thrombogenic processes having a semblance to the complications of hemoglobinopathy (Westerman et al., 2008). These hepatic complications were however prevented by treatment with polyherbal and the architectural structure of the liver restored to normal. This implies that the polyherbal not only combats thrombosis and endothelial dysfunction but also promotes the restoration of tissue structure by depleting free radicals and reactive oxygen species. This finding also supports previous studies on the reversal of increased thrombogenesis as the active phytochemical constituents of individual plants in the polyherbal has been associated with anti-thrombotic activities on humans and animal models (Oridupa et al., 2011; Adeneye et al., 2011; Akinbo et al., 2018).

5. Conclusion:

The polyherbal formula demonstrated thrombolytic and anti-inflammatory activities in this study, thus proffering more insights into the popular folkloric usage of the polyherbal in Nigeria and some sub-Saharan African countries. The polyherbal formula, therefore, shows potential for use in the management of conditions associated with thrombotic, thromboembolic disorders and the associated endothelial dysfunction. Molecular investigation of the bioactive components is however expedient to better understand the specific mechanisms of action.

6. Conflict of Interests:

Authors have declared that no competing interests exist.

7. Acknowledgments:

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Corresponding Author:
Akinbo B. David, Ph.D.
Department of Medical Laboratory Science, College of Medicine and Health Sciences, Afe Babalola University, Ekiti, Nigeria
E-mail: dakinbo@abuad.edu.ng

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