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Research Article

Haemostatic Parameters in Normal and Anemia-Induced Rabbits Treated With Anti-Sickling Polyhedral Mixture

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ABSTRACT

Background: Plant based medication serves as one of the best source of variety of drugs and is incredible sources of essential nutrients and phytochemicals and several have been known to manage sickle cell anemia.

Aim: To assess haemostatic parameters of both apparently healthy and Cadmium+Phenylhydrazine induced anemic rabbits treated with anti-sickling polyherbal mixture (*Momordica charantia, Sorghum bicolor, Securidaca longependunculata, Uvaria afzelli, Phyllanthus amarus and Dialium guineense*).

Method: Sixty (60) New Zealand white hybrid rabbits were used and grouped into four (A-D) consisting of five (5) rabbits each. Each group was given a different concentration of the anti-sickling polyherbal mixture (Saline substitute, 250 mg/kg, 500 mg/kg, 750 mg/kg) respectively which lasted for 3 months. Five mililitres (5 ml) of venous blood was collected from the marginal ear vein at the end of each experiment into trisodium citrate bottles to assess the Prothrombin Time (PT), Activated Partial Thromboplastin Time (APTT) and D-dimer level and Ethylene Diamine Tetracetic Acid (EDTA) bottle to assess the platelet count and Erythrocyte sedimentation rate using standard methods.

Results: The results showed that the treatment with anti-sickling polyherbal mixture significantly ($p<0.05$) caused a shortened prothrombin time (10 ± 0.61 and 9.5 ± 1.3) compared to control group (12.5 ± 0.54) of apparently healthy groups, but caused a significant ($p<0.05$) increase in other parameters (APTT=34 ± 1.68 and 35 ± 1.71; ESR=2.8 ± 0.18 and 3.5 ± 0.1; platelet count=253 ± 17.71 and D-dimer level=1.52 ± 0.12 and 1.58 ± 0.83) compared to their control values (27.6 ± 1.34, 2.4 ± 0.12, 244 ± 9.76 and 1.46 ± 0.07) respectively. Upon the induction of anemia and the administration of the anti-sickling polyhedral mixture, there was a significant ($p<0.05$) decrease in the values (PT=14.3 ± 0.78 and 16.8 ± 0.81; APTT=37 ± 1.93; ESR=11.6 ± 0.72 and 14.5 ± 0.82; Platelet count=84 ± 5.88 and D-dimer levels=2.34 ± 0.23 and 1.98 ± 0.34) compared to control groups (19.6 ± 1.2, 48.1 ± 2.34, 8.4 ± 0.65, 115 ± 4.6 and 3.21 ± 0.15) respectively.

Conclusion: The deviation from baseline values of all haemostatic parameters after the administration of the anti-sickling polyhedral mixture indicates that the mixture does in fact has an effect on hemostasis in both apparently healthy and anemic rabbits and the effects observed are dose dependent.

Keywords: Phytochemicals; Sickle cell anaemia; Haemostatic; Cadmium; Phenylhydrazine

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INTRODUCTION

Plants are being used in most developing countries for medicinal purposes and many synthetic drugs are of plant origin. Plants were and are still being used in processed forms or as raw materials for treatment of many diseases. They are known to be important sources of essential nutrients that have significant effects on various biological activities. The beneficial effects observed when these plants are used are attributed to important phytochemicals (also referred to as secondary metabolites) such as alkaloids, phenols, flavonoids, tannins, saponins, glycosides, terpenoids etc. The active phytochemical constituents of individual plants are sometimes insufficient to achieve the desirable therapeutic effects but when the multiple herbs are combined in a particular ratio, it will give a better therapeutic effect and reduce the toxicity [1-6].

Sickle Cell Disease (SCD) is an inherited disease which results when there is substitution of glutamic acid with valise at the sixth position of the beta-globin chain of the hemoglobin causing the cell to be sickle in shape. Sickle cell disease is usually accompanied with recurrent, painful symptoms, acute illness and progressive organ damage and these phenotypic expressions vary greatly among patients. Hypercoagulability, increased risk of cardiovascular complications, increased risk of pulmonary hypertension, stroke or even complications that may arise from pregnancy, which may occur as a result of thrombotic vascular occlusion (thromboembolism) are prominent complications of most haemoglobinopathies commonly the SCD. The mechanism of this is still being studied [7-9].

Hemostasis is a complex process that involves various physiological pathways and mechanisms that interplay to cause bleeding to cease. In homeostasis, there is always equilibrium between coagulation and fibrinolysis systems. Platelets have a critical role in homeostasis hence a decrease in count is demonstrated in bleeding problems like thrombocytopenia. Upon the occurrence of a vascular or tissue injury, there is a removal of the anti-thrombotic endothelial cell layer and exposure of matrix molecules to varying degrees and varying according to the depth of the injury. In response to thrombopoietin, Platelets are synthesized in the bone marrow and smooth muscle cells and undergo different steps (platelet adhesion, platelet activation and platelet aggregation.) to ultimately form a platelet plug when such injuries occurs. This process is supported by various platelet components like the platelet-derived growth factor, fibronectin, transforming growth factor, platelet factor IV, fibrinogen, factors V and VIII, and Von Willebrand Factor (vWF), calcium ions, serotonin, amongst others. Haemostatic parameters constitute measurable indices in the haemostatic system used to assess the functionality of the coagulation system of an individual to establish a state of health or disorder. They include bleeding time, clotting time, Thrombin Test (TT), Prothrombin Time (PT), fibrinogen level, Activated Partial Thromboplastin Time (APTT) among others. The laboratory analysis of the Activated Partial Thromoboplastin Time (APTT) gives a reflection of the intrinsic pathway and Prothrombin Time (PT) is a reflection of extrinsic pathway of the coagulation cascade. However, these screening coagulation tests are abnormal when there is a deficiency or dysfunction in one or more of the soluble coagulation factors and this alone will not provide information as to bleeding risk. D-dimers are products gotten from degradation of cross-linked fibrin. Its assay is commonly used to rule out venous thromboembolism for the diagnostic and prognostic purposes. D-dimer is considered to be a specific end product in the process of thrombosis, and its elevated level indicates that thrombi have been formed and degraded in the body.

Plants are used ethnomedicinally for use in blood-related treatments as blood tonics, to prevent excessive bleeding, to treat hemorrhoids and as wound dressing to staunch blood flow. Various models exist to screen for activity, which include effects on Prothrombin Time (PT), Activated Partial Thromboplastin Time (aPTT), and Thrombin Time (TT) [10].

Uvaria, afzelii, Securidaca longipedunculata, Sorghum bicolor, Momordica charantia, Phyllanthus amarus and Dialium guineense have been attributed with having anti-sickling and anti-anaemic effects. Several researches have also demonstrated that the polyherbal mixture of these six medicinal herbs can alter haematological parameters but it is not well established if these herbs also have effects on the haemostatic (coagulation) parameters. Therefore a study on the effect of these herbs on haemostatic (coagulation) parameters using rabbits may provide useful information relevant to human health. Thus the objective of the study is to; determine the haemostatic indices in apparently healthy rabbits administered with the anti-sickling polyherbal mixture and to determine the haemostatic indices in cadmium and phenylhydrazine induced anaemic rabbits administered with the anti-sickling polyherbal mixture [11-13].

MATERIALS AND METHODS

Animal and experimental design

Sixty (60) rabbits were used for this experiment. The animals were housed in solid-bottomed stainless steel cages, subjected to standard 12 hours light and dark cycle and were fed with Standard rat chow which was prepared at ABUAD farm. The rabbits were allowed to acclimatize to their environment for 2 weeks before the commencement of the experiments that lasted for 3 months. The design and conducts of the experiments were in accordance with the Medical Research Ethical Committee guidelines for clinical and Experimental Researches in Afe Babalola University, Ado-Ekiti [14].

Preparation of the herbal mixture

The plants used in this study are *Uvaria afzelii, Phyllantus amarus, Securidaca longedunculata, Dialium guineense,* Sorghum bicolor and *Momordican charantia.* Each of the plants were handpicked and dried after which they were blended into fine powder and weighed in equal proportions of 100 g and mixed in equal proportions into a 600 g polyherbal mixture [15].

Grouping and Treatment

Experiment A: The rabbits we assigned into four groups of five (5) animals each consisting of three (3) males and two (2)

females. They were acclimatized for a period of two (2) weeks, after which they were treated as follows (Table 1):

Table 1: The rabbits we assigned into four groups of five (5) animals.

Groups	Number of rabbits (n)	Polyhedral Mixture (mg/kg)
Group A	5	Saline substitute
Group B	5	250
Group C	5	500
Group D	5	750

Experiment B: The rabbits we assigned into four groups of five (5) animals each consisting of three (3) males and two (2) females. They were acclimatized for a period of two (2) weeks, after which they were treated as follows (Table 2):

Table 2: The rabbits we assigned into four groups of five (5)animals.

GROUPS	Number of Rabbits (n)	Polyherbal Mixture (mg/kg)	Cadmium + Phyenylhydrazine
Group A	5	Saline substitute	Cadmium (2 mg/kg) +10 mg/Kg body weight PHZ (15 days)
Group B	5	250	Cadmium (2 mg/kg) +10 mg/Kg body weight PHZ (15 days)
Group C	5	500	Cadmium (2 mg/kg) +10 mg/Kg body weight PHZ (15 days)
Group D	5	750	Cadmium (2 mg/kg) +10 mg/Kg body weight PHZ (15 days)

Sample collection

Five milliliters (5 ml) of venous blood was collected from the marginal ear vein of each rabbits at the end of each experiment into disodium citrate bottles to assess the Prothrombin Time (PT), Activated Partial Thrombin Time (APTT) and D-dimer level and Ethylene Damien Tetra etic Acid (EDTA) bottle to assess the platelet count and Erythrocyte sedimentation rate using standard methods. The experiment was done in duplicate and an average was taken [16-20].

Estimation of prothrombin time and activated partial thromboplastic time

PT and APTT were measured using calcium rabbit brain thromboplastin and kaolin platelet substitute techniques (DIAGEN DIAGNOSTIC REAGENT LTD, OXON, U.K) with lot numbers KC340 and T86 respectively. PT and APTT were assayed according to manufacturer's instructions.

PT Principle: citrated plasma is added to a calcium thromboplastin reagent 37°C and the time taken for blood to clot is called the prothrombin time.

APTT Principle: kaolin (surface activator) and platelet substitute (phospholipid) are incubated with citrated plasma. Upon addition of calcium chloride initiates the clotting. The time it required for the plasma to clot is the APTT.

Estimation of platelet count

Principle: The diluent (1% ammonium oxalate) for platelet count lyses red cells and preserve the platelet which may be seen as retractile cells while counting [21].

Estimation of D-dimer

D dimer was analysed with Elabscience ELISA kit based on the principle of competitive inhibition enzyme immunoassay technique. A monoclonal antibody specific to D-dimer (D2D) has been pre-coated onto a microplate. A competitive inhibition reaction is launched between biotin labeled D2D and unlabeled D2D (Standards or samples) with the pre-coated antibody specific to D2D. After incubation the unbound conjugate is washed off. Next, avidin conjugated to Horseradish Peroxidase (HRP) is added to each micro plate well and incubated. The amount of bound HRP conjugate is reverse proportional to the concentration of D2D in the sample. After addition of the substrate solution, the intensity of color developed is reverse proportional to the concentration of D2D in the sample [22-25].

Estimation of erythrocyte sedimentation rate

ESR was measured using DISPETTE™ 2 ESR Test Kit with lot number 2019042601.

Data analysis

The statistical analysis was done using SPSS version 16.0 software package and Excel Spreadsheet Software (Microsoft Office, 2010). The results were expressed as Mean ± Standard Deviation (SD). The level of statistical significance was set at a p-value of less than 0.05.

RESULTS

This present study assessed the effect of an anti-sickling polyhedral mixture (*Momordica charantia, Sorghum bicolor, Securidaca longependuncula, Uvaria afzelli, Phyllanthus amarus* and *Dialium guineense*) on some haemostatic parameters of both apparently healthy and Cadmium+Phenyl hydrazine induced anaemic rabbits. This study involved two sets of experiments. Experiment 1 shows the effect of the anti-sickling polyherbal mixture on coagulation variables in apparently healthy rabbits, while Experiment 2 shows the effect of anti-sickling polyhedral mixture on coagulation variables in induced anaemic rabbits. The results obtained from this study were presented using bar graphs and tables.

The results of this study showed a deviation from the control values for all haemostatic parameters (both apparently healthy and anemic groups) as seen in Tables 3 and 4 below.

Table 3: Mean ± SD of blood coagulation variables in control and experimental groups of apparently healthy groups administered different doses of Anti-Sickling Polyherbal Mixture (APHM).

	PT(s)	APTT(s)	ESR(mm/hr)	PLT(× 109 /L)	D-DIMER
A	12.5 ± 0.54	27.6 ± 1.34	2.4 ± 0.12	244 ± 9.76	1.46 ± 0.07
B	10 ± 0.61*	26.8 ± 1.4	1.9 ± 0.08	286 ± 17.16	1.52 ± 0.12*
C	9.5 ± 1.3*	34 ± 1.68*	2.8 ± 0.18*	253 ± 17.71*	1.58 ± 0.83*
D	11.7 ± 0.52	35 ± 1.71*	3.5 ± 0.15*	226 ± 18.08	1.43 ± 0.15

Key: A: control B: 250 mg/kg C: 500 mg/kg D: 750 mg/kg

*=P< 0.05 (there is significant difference)

Table 4: Mean ± SD of blood coagulation variables of all groups following induction of anemia with Cadmium+phenyl hydrazine treatment.

	PT(s)	APTT(s)	ESR(mm/hr)	PLT(× 109/L)	D-DIMER
A	19.6 ± 1.2	48.1 ± 2.34	8.4 ± 0.65	115 ± 4.6	3.21 ± 0.15
B	14.3 ± 0.78*	37 ± 1.93*	11.6 ± 0.72*	76 ± 4.56	2.34 ± 0.23*
C	16.8 ± 0.81*	41.6 ± 2.61	12.4 ± 0.58	84 ± 5.88*	1.98 ± 0.34*
D	17.4 ± 1.4	43.5 ± 2.41	14.5 ± 0.82*	65 ± 5.2	3.33 ± 0.16

Key A: control for anaemic group B: 250 mg/kg C: 500 mg/kg D: 750 mg/kg

*=P< 0.05 (there is significant difference)

DISCUSSION

This study assessed haemostatic parameters of apparently healthy and Cadmium+phenyl hydrazine induced anemic rabbits treated with Anti-Sickling Polyhedral Mixture. (APHM)

In this study, we observed a general reduction in PT and a prolonged APTT compared to control groups (Table 1) in apparently healthy rabbits. The difference is statistically significant (p<0.05). The values of each parameter in this study changes as the concentration of Anti-sickling polyhedral mixture changes, indicating that the effect is dose dependent. Reported similar findings in a study using different concentration of Momordica charantia (one of the plants used in this present study) where a deviation of coagulation profile from baseline value was observed after the administration of the extract. The study suggested that the seed extract of M. Chianti exhibit strong anticoagulant effect by interfering with the plasma coagulation cascade, specifically the intrinsic pathway, which ultimately lead to the prolongation of APTT. The anticoagulant effect was due to the Metallo/serine proteolysis activity of the extract.

Sorghum bicolor was reported by to possess anticoagulant activity and prolong the APTT and PT. The plant also possesses anti-thrombotic properties and since thrombin is required for the formation of blood clot, it is not surprising that blood clotting is being prolonged. However this effect was observed only in the APTT values of this present study, thus, the research done by is not in total agreement with this present study.

In the anemic groups, it was observe that the concentration (dose) of APHM used had an impact on all parameters (Table 4). From the results, it was observed that the PT and APTT were prolonged when anemia was induced into the rabbits and after the administration of anti-sickling polyhedral mixture, a reduction in these values was observed. The difference was statistically significant ($p<0.05$). This implies that APHM alleviates the increase in PT and APTT values caused by induced anemia.

The result of this study shows that after the administration of APHM, a general increase in platelet count in apparently healthy rabbits was observed and the 500 mg/kg concentration is statistically significant ($p<0.05$). Some plants of the mixture like Dualism Guineans and Securidaca longependuculata are reported to promote the production and function of platelets. The increase in blood platelet count of apparently rabbits after the administration of the APHM could be due to direct action of D Guineans and S. longependuculata to cause an increase in platelet count or increased production of thrombocyte.

Following the induction of anemia, a reduction in platelet count was observed (Table 4) and upon the administration of anti-sickling polyhedral mixture, the platelet count reduces further, this implies that the administration of anti-sickling polyhedral mixture does not alleviate the reduction of platelet count caused by the induction of anemia. There was significant change from the baseline values ($p<0.05$).

Erythrocyte sedimentation rate has been reported to be of clinical significance in sickle cell disease. In this study, the ESR values were generally higher compared to control groups in apparently healthy groups (Table 3). The induction of sickle cell anemia caused an increase in the ESR values; this is not surprising as it has been reported by Eastman, (1984) that sickle cell disease condition leads to lowered hematocrit levels and viscosity which in turn increases the ESR. The treatment of the rabbits with the anti-sickling polyhedral mixture caused a further increase in the ESR and the difference is statistically significant ($p<0.05$).

D-dimers are products obtained from degradation of cross-linked fibrin. Its assay is commonly used to rule out thromboembolism. In this study, there is a general increase in the level of D-dimers in rabbits administered the APHM compared to the control group in apparently healthy rabbits (Table 3). Some of the plants in this research like have been reported to have fibrinolysis properties.

The induction of anemia caused an increase in D-dimer level. Sickle cell anemia is usually accompanied with haemostatic complications in which thromboembolism are one of them. Since D-dimers are products of fibrin degradation, it is not surprising that the D-dimer levels are increased upon induction of anemia. Elevated levels of D-dimers in the blood indicate that thrombi have been formed and degraded in the body. However, after the administration of Anti-Sickling Polyhedral Mixture (APHM), it was observed that the D-dimer level was decreased and the difference is statistically significant ($p<0.05$). This could mean that the phytochemicals of the mixture has anti-sickling properties, thus stopping haemostatic complications that may have accompanied SCD.

CONCLUSION

The findings of this study revealed that there is a deviation from baseline values of all haemostatic parameters after the administration of the anti-sickling polyhedral mixture, indicating that the mixture does in fact has an effect on hemostasis in both apparently healthy and anemic rabbits. The result also revealed that the effects observed are dose dependent.

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