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EVALUATION OF THE RELATIONSHIP BETWEEN FIBRINOGEN, PLATELET AND DIFFERENTIAL WHITE CELL COUNT IN FEMALE RATS CO-ADMINISTERED CRUDE ETHANOL LEAF EXTRACT OF *Ageratum conyzoides* AND NIGERIAN BONNYLIGHT CRUDE OIL

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The present study was focused on the possibility of using ethanol leaf extract of *A. conyzoides* to ameliorate the consequences of the synergistic interaction between platelets, fibrinogen and some fractions of white blood cell with the goal of protecting the cardiovascular system against the consequences of exposure to the crude oil in rats. Twenty female Wistar rats (120-150g body weight) were divided into four groups of five rats each. The rats in group I served as the control group and were oral gavaged 3ml/kg of normal saline; group II gavaged 748.33mg/kg body weight of the extract of *A. conyzoides*. Group III animals were gavaged3ml/kg body weight of Nigerian Bonnylight crude oil (NBLCO).Group IV animals were gavaged 748.33mg/kg body weight of the extract of *A. conyzoides*, and 3ml/kg body weight of NBLCO. The results showed that NBLCO ingestion significantly increased monocyte, neutrophil and eosinophil counts while it significantly reduced total white blood cells, lymphocyte counts and platelets with respect to groups I and II (the control and *A. conyzoides* groups respectively) ($p<0.05$). The co-administration of *A. conyzoides* with NBLCO to group IV animals significantly reversed the value of the aforementioned parameters with respect to NBLCO-treated group (group III) ($p<0.05$). The co-administration of *A. conyzoides* with NBLCO to animals in group IV significantly reduced platelet count with respect to groups I and II (control and *A. conyzoides* groups respectively) ($p<0.05$). Similarly, the NBLCO significantly increased serum fibrinogen concentration with respect to groups I and II (the control and *A. conyzoides* groups respectively) ($p<0.05$). The co-administration of *A. conyzoides* with NBLCO to animals in group IV significantly reduced serum fibrinogen concentration with respect to NBLCO-treated group III ($p<0.05$) but was significantly higher than groups I and II (control and *A. conyzoides* groups respectively) ($p<0.05$). It is concluded that the NBLCO by significantly increasing serum fibrinogen concentration, neutrophil, eosinophil and monocyte on the one hand and significantly reducing platelets count on the other hand, can aggravate and escalate the synergistic interaction between fibrinogen and the aforementioned cells to cause cellular injury in which the co-administration of ethanol leaf extract of *Ageratum conyzoides* can ameliorate.

KEYWORDS: Nigerian Bonnylight crude oil, Erythrocyte Membrane, Erythrocyte Haemolysis, Osmotic Fragility, *Ageratum conyzoides*

INTRODUCTION

Blood flow characteristics which is influenced by various factors including plasma proteins play an important role in the pathogenesis of thrombotic events leading to cardiovascular-related disorders. The major plasma proteins include albumin, globulin and fibrinogen. In addition, platelets, fibrinogen and some polymorphonuclear and mononuclear leukocytes play significant role in determining blood flow characteristics and in pathogenesis of thrombotic events associated with cardiovascular disorder. Lacombe *et al.,* (1988) observed that an elevated fibrinogen concentration in particular facilitates aggregation in red blood cell suspension.

Polymorphonuclear and mononuclear leukocytes may be activated through the release of cytokines such as tumor

necrosis factor- α (TNF-α) (Guha *et al*., 2000; Shanmugam *et al*., 2003), transforming growth factor-β1 (Korpinen, 2001), superoxide (Kedziora-Kornatowska, 1999), monocyte chemo-attractant protein-1 to participate in the pathogenesis of microvascular and macrovascular complications. In coronary artery disease, high level of neutrophil, eosinophil and monocyte counts predict future occurrence (Madjid *et al*., 2004)

Platelets through chemokines secretion can possibly induce pathology in heart failure (Aukrust *et al*., 1998; Damas *et al*., 2000).This can occur when activated platelets stimulate macrophage chemo-attractant protein-1 (MCP-1) coupled direct platelet-monocyte contact (Aukrust *et al*., 1998) to escalate pathologic activities. Macrophage chemo-attractant protein-1 and macrophage proinflammatory protein-1a (MCP-1a) are potent chemo-attractants of monocytes and lymphocytes that can in turn modulate other cell functions.

Fibrinogen is a coagulation factor that is associated with subclinical atherosclerosis (Fowkes *et al*., 1993; Rosengren and Wilhelmsen, 1996; Paramo *et al*., 2004). Up regulation of adhesion molecules by blood leukocytes and inflammatory cytokines in endothelial cells is associated with the recruitment and accumulation of leukocytes and platelets on the endothelial cells lining the vasculature. This in turn promotes neutrophils, monocytes, lymphocyte, platelets and endothelial cell interactions (Lavie, 2008). Such cellular interactions between blood cells and endothelial cells may result in injury to the endothelium (Lavie, 2008).Exposure to environmental insults such as crude oil can trigger off this cascade of events (Ita *et al*., 2016) which could result in disease conditions such as atherosclerosis. Atherosclerosis is associated with deranged lipid metabolism (Wang and Briggs, 2004) and micro-inflammation, where free radical agents can oxidize low density lipoprotein to promote atherosclerosis (Morrow and Ridker, 1999; Wang and Briggs, 2004).This process involves the attraction of other cells such as circulating lymphocyte and macrophages (Powell, 1988) with potentials to produce inflammatory cytokines. The events leading to the production of inflammatory cytokines are coupled with the capacity of macrophages to take up LDL and transform such into foam cells (Morrow and Ridker, 1999). Furthermore, inflammatory cytokines are known to promote adhesion of platelets to the vascular endothelium, attracting leukocytes and indeed enhancing progression of plaque growth.

The mechanism underlying the interactions between fibrinogen, leukocytes and platelets is in one way or the other associated with inflammation, and is implicated in the pathogenesis of various cardiovascular diseases, neurodegenerative disorder, autoimmune disease, diabetes and cancer (Mates *et al*., 1999; Kayode *et al*., 2009; Vaghasiya and Chanda, 2010).

The search for substance(s) with potential to mitigate inflammatory events and reduce the risk of cardiovascular disorder will definitely open a window of therapeutic advantage in this direction.

In view of the important role played by medicinal plants in providing valuable therapeutic agents in traditional and modern medicine practices, *Ageratum conyzoides,* an herbal plant with enormous beneficial effects becomes promising. *A. conyzoides* is an annual herbaceous weed with a history of traditional medicinal benefits in many countries including Nigeria. The extracts of this herbal plant are widely used in folk medicine as purgative, analgesic (Tandon *et al*., 1994), to treat wounds and burns (Okunade, 2002), treatment of high blood pressure, fever, diabetes, pneumonia and numerous infectious diseases (Xuan *et al*., 2004), potential to increase blood cell formation as a blood booster (Ita, *et al*., 2007) and is hepatoprotective (Ita, *et al*., 2009). The present study was therefore focused on the possibility of using ethanol leaf extract of *A. conyzoides* to ameliorate the consequences of the synergistic interaction between platelets, fibrinogen and some fractions of white blood cell with the ultimate goal of protecting the cardiovascular system against the consequences of exposure to crude oil in rats.

MATERIALS AND METHODS

The crude petroleum used in this study was obtained from the Exxon Mobil laboratory, Ibeno, Nigeria.

Collection of plant material

The whole plant was obtained from the Botanical farm of the Department of Pharmacognosy and Natural Medicine, University of Uyo, Uyo, Nigeria. Specimen of the leaves was authenticated by a staff of the Department of Botany and Ecological Studies, University of Uyo, Uyo. A voucher specimen (UUH 3517) was deposited at the Herbarium.

Preparation of leaf extract

The leaves of *A. conyzoides* were rinsed with distilled water and shade dried for two weeks. The dried leaves were ground into powder with an electric blender. Four hundred grams (400 g) of the powdered leaf sample was macerated in 700ml 70% ethanol, and left overnight in a refrigerator at 4^{o}C. The mixture was filtered with a cheese cloth and the filtrate obtained was concentrated under reduced pressure using a rotary evaporator (at 37^{o}C) to about 10% of its original volume. The concentrate was then kept in a water bath at 37^{o}C for complete evaporation to dryness yielding 40.64g (10.15%) of the extract.

Acute toxicity test

Acute toxicity study (LD_{50}) of *Ageratum conyzoides* was estimated using Lorke's method (Lorke, 1993). A total of 25 mice weighing between 15-22g were divided into five groups with five mice per group. Mice in the five groups were administered 3000mg/kg, 3500mg/kg, 4000mg/kg, 4500mg/kg and 5000mg/kg of body weight respectively (intraperitoneally). All experimental animals were observed for physical signs of toxicity such as gasping, writhing, decreased respiratory rate, and death after 24 hours.

The median lethal dose of *Ageratum conyzoides* was calculated as geometrical means of the maximum (most tolerable) dose producing 0% mortality (a) and the minimum (least tolerable) dose producing 100% mortality (b) using the formula:

$LD_{50} = \sqrt{ab}$
$LD_{50} = \sqrt{3500 \times 4000}$
$= 3741.66mg/kg$

The acute toxicity test for the NBLCO also involved 25 mice weighing between 15-22g which were divided into five groups with five mice per group. Mice in the five groups were administered intraperitoneally 10ml/kg, 15ml/kg, 20ml/kg, 25ml/kg and 30ml/kg of body weight respectively.

$LD_{50} = \sqrt{10 \times 20}$
$= 14.14ml/kg$

Experimental animals

Female Albino Wistar rats weighing between 150-180g were obtained from the Animal House Facility of the Faculty of Basic Medical Sciences University of Uyo, Uyo, Nigeria and were kept in a well-ventilated section of the Animal House. While the experiment progress the rats were allowed free access to feed (vital feeds, Grand Cereals Ltd, Jos) and water *ad libitum*.The animals were kept in separate experimental room and allowed to acclimatize for a period of one week before commencement of studies.

Experimental design and treatment of animals

A total of twenty (20) adult female Albino Wistar rats were randomly divided into four groups (group I, II, III and IV) of five (5) rats each. Group I served as the control and was oral gavaged with 3 ml/kg body weight of normal saline. Group II was oral gavagedwith748.33mg/kg body weight of ethanolic leaf extract of *Ageratum conyzoides*, this dose was calculated as 20% of the lethal dose (LD_{50}) of 3741.66 mg/kg. Group III was oral gavaged with 3 ml/kg body weight of NBLCO, which was calculated as 20% of the lethal dose (LD_{50}) of 14.14 ml/kg. The group IV animals in addition to 3 ml/kg body weight of NBLCO were supplemented with 748.33 mg/kg body weight of ethanolic leaf extract of *Ageratum conyzoides*. In all cases, the doses were based on the rat's most recently recorded body weight. The calculated volume in milliliter (ml) was applied daily for thirty one (31) days. The experimental procedures involving the animals and their care were conducted in conformity with the approved guidelines by the Research and Ethical Committee of the Faculty of Basic Medical Sciences, University of Uyo, Uyo. Nigeria.

Collection of blood sample for analysis

After thirty one (31) days of administration, the rats were anaesthetized with chloroform soaked in swap of cotton wool in a killing chamber. Blood was collected by cardiac puncture with a 5 ml sterile syringe and needle. The total volume of blood collected was 4 ml, which was divided into two portions. One portion was transferred into plain sample bottles. This was allowed to stand for 2 hours to clot after which the serum was separated by centrifugation (RM-12 micro centrifuge, REMI, England) at 4000 rpm for 10 minutes. The serum obtained was stored at $-20^{o}C$ until required for analysis. The second portion was used for total white blood cell, differential white cell and platelets counts using an automatic haematology analyzer (Mindray Hematology analyzer, BC-2300).

Statistical analysis

Data were expressed as the mean + standard error of the mean. Statistical analysis was carried out using window SPSS package (SPSS 22.00 version). Data were analyzed using one way analysis of variance (ANOVA), results obtained were further subjected to test for least significant difference (LSD). Values of $P<0.05$ were considered significant.

RESULTS

White blood cell and the differential cell count

As could be observed in figure 1, NBLCO ingestion significantly increased monocyte, neutrophil and eosinophil counts while it significantly reduced total white blood cells and lymphocyte counts with respect to groups I and II (the control and *A. conyzoides* groups respectively) ($p<0.05$). The co-administration of *A. conyzoides* with NBLCO to group IV animals significantly reversed the aforementioned parameters with respect to NBLCO-treated group (group III) ($p<0.05$).

Fibrinogen

The mean serum fibrinogen concentrations obtained in this study are shown on figure 1. Ingestion of NBLCO significantly increased serum fibrinogen concentration with respect to groups I and II (the control and *A. conyzoides* treated groups respectively) ($p<0.05$). Co-administration of *A. conyzoides* and NBLCO to animals in group IV significantly reduced serum fibrinogen concentration with respect to NBLCO-treated group III ($p<0.05$) but was significantly higher than groups I and II (control and *A. conyzoides* groups respectively) ($p<0.05$).

Platelets

Ingestion of NBLCO significantly lowered the platelet count with respect to groups I and II (the control and *A. conyzoides* groups respectively) ($p<0.05$) as illustrated in figure 2. Co-administration of *A. conyzoides* with NBLCO to animals in group IV significantly increased platelet count with respect to NBLCO-treated group III ($p<0.05$) but was significantly lower than groups I and II (control and *A. conyzoides* treated groups respectively) ($p<0.05$).

Table 1: Comparison of total white blood cell count and the differential white cell count in rats following exposure to NBLCO and ethanol leaf extract of *Ageratum conyzoides* .

Groups	WBC ($x10^3$µL)	Lymphocyte (%)	Monocyte (%)	Neutrophil (%)	Eosinophil (%)
I (Control)	2.90 ± 0.33	47.50 ± 2.43	4.17 ± 0.48	49.83 ± 1.49	2.33 ± 0.33
II (748.33 mg/kg body weight of extract)	3.23 ± 0.35	47.67 ± 1.87	3.50 ± 0.62	54.17 ± 3.40	1.50 ± 0.22
III (3 ml/kg body weight of NBLCO)	1.22 ± 0.19a,b	26.50 ± 2.58a,b	8.00 ± 0.58a,b	71.83 ± 3.02a,b	11.83 ± 1.11a,b
IV (3 ml/kg body weight of NBLCO plus 748.33 mg/kg body weight of extract	2.97 ± 0.17c	47.33 ± 2.68c	5.83 ± 0.31a,b,c	48.83 ± 2.91b,c	7.17 ± 0.91a,b,c

Legend:
a = significantly different from group I ($p<0.05$)
b = significantly different from group II ($p<0.05$)
c = significantly different from group III ($p<0.05$)

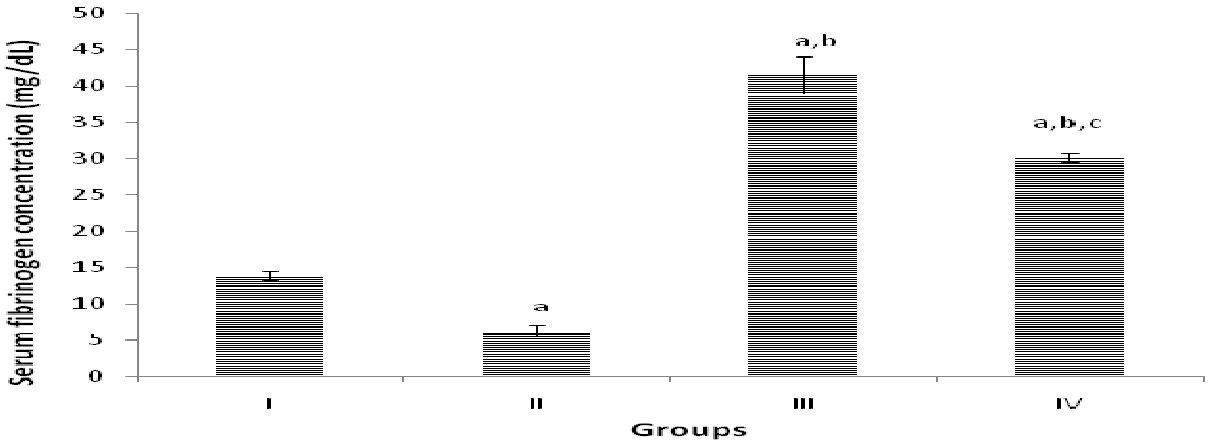

Figure 1: Serum fibrinogen levels in the various animal groups. Values are mean + SEM. a= $p<0.05$ vs group I, b = $p<0.05$ vs group II, c= $p<0.05$ vs group III

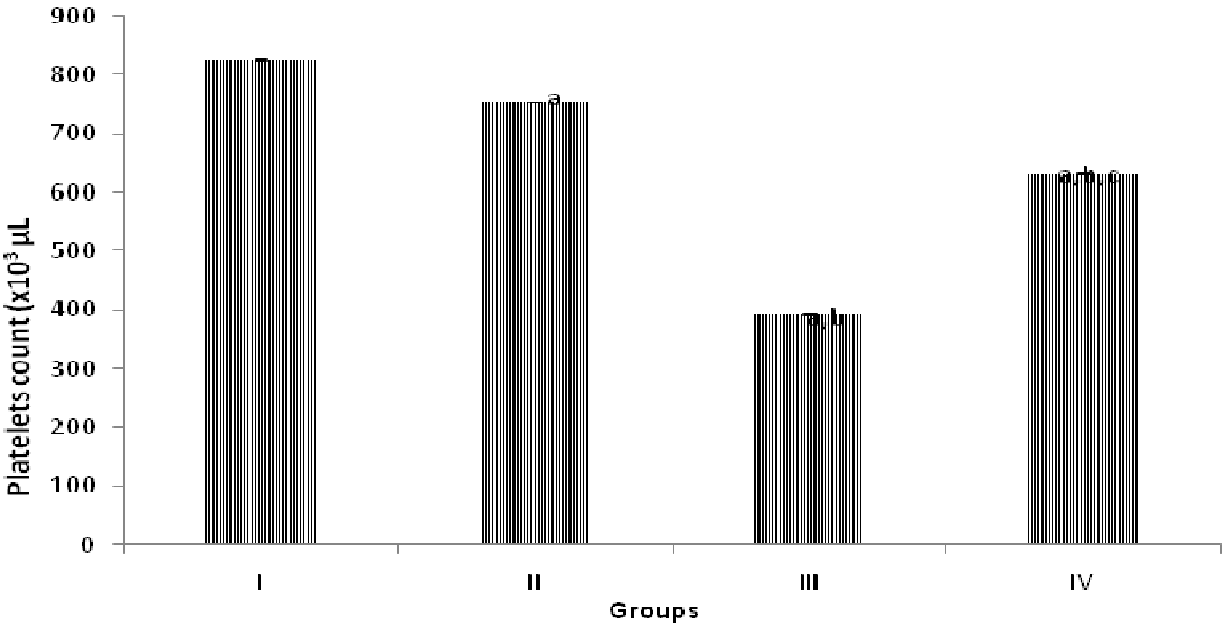

Figure 2: Platelets count in the various animal groups. Values are mean + SEM. a= p<0.05 vs group I, b = p<0.05 vs group II, c= p<0.05 vs group III.

DISCUSSION

The potential of NBLCO to aggravate and escalate the synergistic interaction between fibrinogen, platelets, polymorphonuclear and mononuclear leukocytes culminating in inflammatory disorder and the ameliorative potentials of ethanol leaf extract of *Ageratum conyzoides* was the focus of this study. Ingestion of NBLCO significantly elevated serum fibrinogen concentration, neutrophils, eosinophils and monocytes, while it significantly reduced platelet count. NBLCO has recently been reported to induce the development and progression of inflammation in rats (Ita *et al*., 2016). As observed in the present study, the significant elevation of neutrophils and monocytes count by NBLCO could instigate transendothelial migration which is associated with leukocyte trafficking to sites of inflammation or injury involving co-operative interaction between these cells and endothelial cell adhesion molecules such as integrins. Integrin present in platelets can bind fibrinogen and become activated by binding collagen or thrombin to escalate clot formation. This probably accounts for the significantly reduced circulating platelet observed in this study, particularly in the presence of anti-haemopoietic agent like NBLCO. This activation causes conformational changes in the integrin which is associated with cytoskeleton remodeling. This could trigger intravascular coagulation involving other coagulating factors and plays a central role in thrombus formation in health and disease. Activation of coagulation activities and deposition of fibrin is instrumental to vascular thromboembolism. Venous thromboembolism for instance is triggered by intravascular activation of coagulation and thrombin-mediated intraluminal fibrin deposition (von Bruhl *et al*., 2012; Darbousser *et al*., 2012), where the fibrin network provides a scaffold for the binding of endothelial cells, leukocytes, platelets and plasma proteins to the already initiated clot formation. Therefore abnormalities in fibrinogen level such as hyperfibrinogemia as observed in this study is a risk factor for venous thrombus formation because elevated plasma fibrinogen level increases risk of the venous thromboembolism. Interestingly, fibrin network structure reflects the fibrinogen concentration and the presence of elevated fibrinogen increases fibrin network density, clot stiffness, and the resistance of clots to fibrinolysis (Machlus *et al*., 2011). Fibrinogen has the potential to mobilize leukocyte and to upgrade the signaling process thereby providing positive feedback for the thrombin-mediated regulation of fibrin network structure. Thus, adhesion of neutrophils and monocyte to activated endothelium has been proposed to be a necessary initial step for venous thrombus formation (von Bruhl *et al*., 2012; Darbousser *et al*., 2012). The potential of fibrinogen to mobilize leukocytes and sustain the aforementioned process might be responsible for the high percentage of neutrophils and monocytes induced by NBLCO in this study.

When tissues become inflamed, leukocytes including lymphocytes are recruited to the sites of inflammation by mechanisms involving molecules expressed on the leukocytes and on the endothelium. There appears to be unrestricted emigration of neutrophils, eosinophils and monocytes from the blood stream in response to molecular changes on the surface of blood vessels that signal NBLCO injury.

It has been reported that platelets express fibrinogen receptor on their surfaces which facilitates platelet-fibrinogen interaction; this then activates the platelets to enhance platelet aggregation (Spangenberg *et al*., 1993). Such

micro-aggregates are also reported to enhance platelet-leukocyte interactions (De-Gaetano *et al*., 1990)and to escalate inflammatory events. Indeed, platelets play a pivotal role involving their massive trafficking of platelets into the process of inflammation and resulting in the significantly lower platelets in circulation as well as total white blood count following NBLCO administration to rats.

Co-administration of ethanol leaf extract of *Ageratum conyzoides* reversed the effects of NBLCO and significantly raised the total white blood cell, lymphocytes and platelets while significantly reducing monocytes, neutrophils, eosinophils and fibrinogen concentration. This is not surprising as extract of *Ageratum conyzoides* has been reported to stimulate haematopoiesis probably through its direct effect on haematopoietic systems (Ita *et al*., 2007). Furthermore, *Ageratum conyzoides* is reported to be a natural reservoir of antioxidants (Jagetia, 2007), it is possible that this antioxidant potentials played a prominent role in reversing the effect of NBLCO as most of the crude oil effects are directly related to generation of oxidant radicals.

CONCLUSION

It is concluded that the NBLCO by significantly increasing serum fibrinogen concentration, neutrophil, eosinophils and monocyte on the one hand and significantly reducing platelets count on the other hand, can aggravate and escalate the synergistic interaction between fibrinogen and the aforementioned cells to cause cellular injury in the body which was reversed by the co-administration of ethanol leaf extract of *Ageratum conyzoides*.

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