Original Article

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Anti-thromboinflammation of Triherbal Extract in Diabetic Rats

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Abstract:

Objective: To evaluate the effects of a blended aqueous extract of Moringa oleifera, Ocimum gratissimum, and Vernonia amygdalina on some markers of thromboinflammation in diabetic rats.

Material and Methods: We administered 150 mg/kg body weight of the individual extracts and the blended extract (1:1:1) to diabetic induced rats for 28 days. Acute toxicity of the extracts were determined by the Lorke technique while the markers of thromboinflammation, involving platelet count, mean platelet volume, platelet distribution width, plateletcrit, platelet large cell ratio, absolute white blood cell, lymphocytes, neutrophils, monocytes, basophils and eosinophils, were determined as part of the complete blood count using an automated hematology analyzer.

Results: The LD50 of both the individual and blended extracts were observed to be above 5,000 mg/kg body weight. There was a significant increase in the serum glucose and markers of thromboinflammation involving the total white blood cell count, neutrophil count, platelet count, plateletcrit, mean platelet volume, platelet distribution width and the platelet large cell ratio, but a decrease in the lymphocyte and eosinophil count for the diabetic non-extract treated rats. The diabetic rats treated with the blended extract showed a significant restoration of the parameters.

Conclusion: This study revealed that the blended aqueous extracts of Moringa oleifera, Ocimum gratissimum, and Vernonia amygdalina are non-toxic and may have synergistic effects in limiting thromboinflammation in a diabetic state.

Keywords: blended extracts, diabetes mellitus, inflammation, subclinical, thrombosis, triherbal

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Introduction

Thromboinflammation refers to the intricate crosstalk between subclinical chronic inflammation and thrombosis (development of thrombosis due to an inflammatory state)[1,2]. It results in loss of the normal antithrombotic and anti-inflammatory functions of endothelial cells, leading to the dysregulation of coagulation, complement activation, platelet activation, and leukocyte trafficking[3,4]. It has been reported as a major pathological process underlying complications in diabetes mellitus; and, it provides a good therapeutic target for the management of patients[1,4].

The availability of synthetic drugs for the treatment of diabetes mellitus may be common, but because of the high costs and side effects, attention is focused on the use of medicinal herbs[5]. The blended aqueous extracts of Moringa oleifera, Ocimum gratissimum, and Vernonia amygdalina are a common decoction used for the management of diabetes mellitus in Nigeria[6]. The anti-diabetic activities of the individual aqueous extracts of Moringa oleifera, Ocimum gratissimum, and Vernonia amygdalina have been reported by different studies; however, there is a paucity of scientific data on the effects of the blended aqueous extracts on the markers of thromboinflammation[7–13]. The pharmacological effects of these plants including anti-diabetic, anti-inflammatory, antithrombotic and anti-oxidative effects have been attributed to the presence of different secondary metabolites, such as flavonoids, alkaloids, tannins, saponnin, terpenoids, and phenols. The anti-oxidative effects of Moringa oleifera have been reported by some studies and attributed to some secondary metabolites[8,9]. Anti-diabetic and anti-inflammatory effects have been reported for various parts of Ocimum gratsimum due to its secondary metabolite content[10,11]. The pharmacological effects of the aqueous extracts of vernonia amygdalina, including the anti-diabetic, antithrombotic and antiflammatory effects, have been reported by other studies[12,13].

Moringa oleifera, commonly known as the miracle tree, belongs to the family Moringaceae; it is a short, slender, deciduous, perennial tree that grows to about 10m tall with drooping branches and feathery pale green leaves[7–9]. The species are native to Africa, Southeast Asia, South America, and the Caribbean and grow in a variety of soils, including semi-dry, desert, and tropical rainfall[9].

Ocimum gratissimum, commonly known as scent herb, belongs to the family Lamiaceae and grows about 1–3 cm tall with an erect stem, slender branches, and marginalized leaves. It is common in tropical rainforest zones[10,11].

Vernonia amygdalina, commonly known as bitter leaf, is a perennial herb belonging to the family Asteraceae; it is indigenous to Africa and grows to 701 cm tall with a flaky rough stem and leaves which are medium to dark green[12,13].

The present study was designed to evaluate the effect of the blended triherbal aqueous extracts of moringa oleifera, Ocimum gratissimum, and Vernonia amygdalina on the platelet count, mean platelet volume, platelet distribution with, plateletcrit, platelet large cell ratio, absolute white blood cell and differential white blood cells involving lymphocytes, neutrophils, monocytes, basophils, and eosinophils in diabetic rats.

Material and Methods

Ethical clearance

Ethical clearance for the study was obtained from the Animal Research Ethics Committee of the Faculty of Allied Sciences, Enugu State University of Science and Technology, Enugu, Nigeria, with assigned number: FAHS/EC/2024/002.

Plant authentication

The leaves were identified by Dr. C. N Ugwu, a botanist, with assigned herbarium numbers MP197, MP198 and MP199 for Moringa oliefera, Ocimum gratissimum, and Vernonia amagadlina, respectively.

Preparation of leaf extracts

The extracts were prepared using a previously reported procedure[14]. Fifty grams of each was soaked separately in 100 ml of boiled distilled water for 24 hours. They were then filtered using a N0. 1 Whatman filter paper to obtain a fine filtrate for each extract. The filtrates were allowed to dry in an oven (Techmel and Techmel, 420, USA) at 50 °c to obtain the crude extract. The crude extracts were stored in an air-tight container and were later reconstituted in distilled water to give the required concentration of 150 mg/kg body weight for the individual extracts, while the blending of individual extracts, was achieved by mixing them in the ratio 1:1:1 to give the required concentration of 150 mg/kg body weight.

Phytochemical screening

The presence of different secondary metabolites in each of the extracts and the blended extract was detected by chemical reactions for alkaloids (Mayer's and Dragendorff test), tannins (ferric chloride test), steroids (Lieshermann-Burchard test), Saponins (froth test), terpenoids (salxowski test), flavonoids (Ammonia and sulphuric acid test), glycosides (killer-kiliani test) and phenols (ferric chloride test), as previously reported[15]. The quantitative values of the constituents were determined by measurement of the color development for the different tests using a spectrophotometer (Multiskan FC; Thermo Fischer, Scientific, USA)[16].

Animal handling

Wistar rats weighing 160-200 g were used for the study. The animals were allowed an acclimatization period of 2 weeks. They were housed in cages at a room temperature of 25-28 °C with moisture control under a naturally illuminated environment of 12:12 hour's dark/light. They were fed standard rat pellets and water ad libitium in line with the rules of the National Institute of Health Guide for the Care and Use of Laboratory Animals[17].

Acute toxicity testing

Lorke's technique was used to determine the LD50 of the extracts[18]. This was conducted in 2 phases using 36 rats (12 rats for each extract). In the first phase, 3 groups of 3 rats in each cage were administered 100, 500, and 1,000 mg/kg of the aqueous extracts orally. Rats were observed for signs of toxicity and mortality within 24 hours, with particular attention during the first 4 hours of the experiment. The second phase was followed in similar conditions by the administration of 2,000, 3,000 and 5,000 mg/kg to the next 3 groups of one rat in each cage to detect the signs of toxicity and mortality during 24 and 72 hours, respectively.

Induction of diabetes

Diabetes was induced using Alloxan (AZN) monohydrate (Sigma Chemical Co., St. Louis, MO, USA), according to previous reports[19]. Alloxan was injected intraperitoneal for 2 days at a concentration of 20mg/kg each dissolved in 0.9% saline. Diabetes was confirmed 3 days later in induced rats showing random blood glucose level ≥200 mg/dL by using a glucometer (Accu-Chek, India) to test blood samples from the tail vein.

Experimental design

Animals were randomly distributed into 7 groups, each consisting of 5 rats, and were treated daily for 28 days by oral gavage, as shown in Table 1. Three milliliters of blood was collected from the inferior vena cava under chloroform anesthesia into ethylenediamine-tetracetic acid (EDTA) for the estimation of thromboinflammation markers.

Determination of thromboinflammation markers

Markers of thrombosis involving platelet count (PLT), mean platelet volume (MPV), platelet distribution width (PDW) plateleterit (PCT) and platelet large cell ratio (P-LCR), as well as the markers of inflammation including white blood cell count (WBC) and differential counts

(lymphocytes, neutrophils, monocytes, basophils and eosinophils) were determined as part of a complete blood count assay using the automated hematology analyzer (Mindray, 530 BC, China).

Data analysis

Data were analyzed using the statistical package for social sciences (SPSS) version 25 (IBM Crop, Armok, NY, USA) software. Quantitative data (markers of thromboinflammation) were presented as mean and S.D. for various groups. One-way analysis of variance (ANOVA) was used to compare all the groups, followed by the Turkey post-hoc test. p-value<0.05 was considered significant.

Results

Acute toxicity effect of the extracts

Acute toxicity tests carried out on both the individual extracts (MOAE, VAAE, and OGAE) and the blended extract (BAE) did not yield any signs of toxicity in the rats up to a dose of 5,000 mg/kg after 24 hours (Tables 2 and 3). We, therefore, adopted an LD50 of more than 5,000 mg/kg body weight.

Table 1 The treatment regimen for various groups (Experimental design)

Group	Treatment
1	Non-diabetic nor extract treated (normal control)
2	Diabetic non-extract treated (negative control)
3	Diabetic + treated with 40 mg/kg metformin (positive control)
4	Diabetic + treated with 150 mg/kg VAAE.
5	Diabetic + treated with 150 mg/kg MOAE
6	Diabetic + treated with 150 mg/kg OGAE
7	Diabetic + treated with 150 mg/kg BAE

VAAE=vernonia amygdalina aqueous extract, MOAE=moringa oleifera aqueous extract, OGAE=ocimum gratisimum aqueous extract, BAE=blended aqueous extract

Table 2 The 24-hour acute toxicity (LD50) test of low doses of the extracts (phase 1)

Extracts	Concentration (mg/kg body weight)	Mortality/No of rats
VAAE	100	0/3
	500	0/3
	1000	0/3
MOAE	100	0/3
	500	0/3
	1000	0/3
OGAE	100	0/3
	500	0/3
	1000	0/3
BAE	100	0/3
	500	0/3
	1000	0/3

Number of deaths per group=0, Number of rats per group=3, VAAE=vernonia amygdalina aqueous extract, MOAE=moringa oliefera aqueous extract, OGAE=ocimum gratisimum aqueous extract, BAE=blended aqueous extract

Table 3 The 24-hour acute toxicity (LD50) test of high doses of the extracts (phase 2)

Extracts	Concentration (mg/kg body weight)	Mortality/No of rats
VAAE	2000	0/1
	3000	0/1
	5000	0/1
MOAE	2000	0/1
	3000	0/1
	5000	0/1
OGAE	2000	0/1
	3000	0/1
	5000	0/1
BAE	2000	0/1
	3000	0/1
	5000	0/1

Number of deaths per group=0; Number of rats per group=1, VAAE=vernonia amygdalina aqueous extract, MOAE=moringa oliefera aqueous extract, OGAE=ocimum gratisimum aqueous extract, BAE=blended aqueous extract

Qualitative and quantitative phytochemical composition of the extracts

The extracts revealed the presence of flavonoids,

alkaloids, saponins, tannins, steroids, terpenoids, and phenols (Table 4). The mean values of the secondary metabolites for the blended extract were significantly higher compared to the individual extracts (Table 5).

Pharmacological effect of the extracts

Serum glucose concentration for the negative control was significantly higher compared to those of the normal control, positive control, and the rats treated with the individual extracts, as well as those treated with the blended extract.

There was a significant increase in the total white blood cell and neutrophil counts of the negative control group compared to the normal control, positive control, the diabetic rats treated with the individual extracts and the blended extract, with the rats treated with the blended extract showing similar values for the parameters compared to the normal and positive control groups. The lymphocyte, monocyte, basophil, and eosinophil count for the negative control group was significantly lower compared to the normal control, positive control, the group treated with the individual extracts and the blended extract, with the rats treated with the blended extract showing similar values for the parameters compared to the normal and positive controls groups (Table 6).

For the markers of thrombosis, there was a significant increase in the platelet count, plateletcrit, platelet distribution width, mean platelet volume, and the platelet large cell ratio for the negative control group compared to the normal control, positive control, the groups treated with the individual extracts and those treated with the blended extract, with those treated with the blended extract having similar values for the parameters compared to the normal and positive control groups (Table 7).

Mean body weight of the animals

The diabetic rats showed a non-significant increase in body weight compared to their initial body weights prior to treatment with the extracts. The diabetic rats that were not treated with the extract (negative control group) revealed significant reductions in body weight while the normal control rats revealed a significant increase in body weight after the experimental period compared to their weights prior to the experiment (Table 8).

Table 4 The phytochemical composition of the extracts (qualitative test)

Constituent	MOAE	OGAE	VAAE	BAE
Flavonoids	++	+	++	+++
Alkaloids	++	+	+++	+++
Saponins	+	+	++	++
Tannins	+	+	+	++
Steroids	++	+	+	++
Terpenoids	+	+	+	++
Phenols	++	–	+	++

Key: (–) Absent, (+) Present, (++) Moderately present, (+++) Abundantly present, MOAE=moringa oliefera aqueous extract, OGAE=ocimum gratisimum aqueous extract, VAAE=vernonia amygdalina aqueous extract, BAE=blended aqueous extract

Table 5 The phytochemical compositions of the extracts (quantitative test)

Constituent	MOAE	OGAE	VAAE	BAE
Flavonoids	5.9 ± 0.7^{a}	2.1 ± 0.4^{b}	9.0 ± 0.3^{c}	12.6 ± 0.5^{d}
Alkaloids	5.4 ± 0.4^{a}	1.6 ± 0.4^{b}	11.1 ± 0.3^{c}	15.1 ± 0.3^{d}
Saponins	1.5 ± 0.4^{a}	1.3 ± 0.3^{a}	7.7 ± 0.3^{b}	10.3 ± 0.8^{c}
Tannins	4.0 ± 0.4^{a}	2.2 ± 0.6^{b}	4.5 ± 0.4^{a}	8.8 ± 0.2^{c}
Steroids	8.4 ± 0.4^{a}	2.7 ± 0.6^{b}	2.5 ± 0.2^{b}	11.2 ± 0.4^{c}
Phenols	0.4 ± 0.2^{a}	–	0.4 ± 0.2^{a}	6.5 ± 0.3^{b}
Terpenoids	1.4 ± 0.4^{a}	1.1 ± 0.3^{a}	1.0 ± 0.3^{a}	2.8 ± 0.4^{b}

Values are mean±S.D. of 3 independent measurements, S.D.=standard deviation, mean values having different lowercase letters of the alphabet as superscripts are considered significantly different (p-value<0.05) within the rows, MOAE=moringa oliefera aqueous extract, OGAE=ocimum gratisimum aqueous extract, VAAE=vernonia amygdalina aqueous extract, BAE=blended aqueous extract

Table 6 Some markers of subclinical inflammation among the study groups

Treatment	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
WBC (10^9/L)	5.3±0.9[a]	16.9±1.4[b]	5.8±0.7[a]	10.8±0.4[c]	13.0±0.8[d]	13.5±0.8[d]	6.2±0.7[a]
Neutrophil (%)	53.6±2.6[a]	77.6±4.4[b]	59.8±3.0[a]	67.2±2.6[c]	72.6±2.9[b]	72.4±6.2[b]	56.2±2.9[a]
Lymphocyte (%)	39.8±3.0[a]	19.4±3.9[b]	37.6±2.9[a]	26.8±1.1[c]	24.8±1.9[b,c]	23.7±3.5[b,c]	36.8±2.3[a]
Eosinophil (%)	3.2±0.8[a,b]	2.0±0.7[a]	2.0±0.7[a]	3.1±0.9[a,b]	1.8±0.9[a]	2.4±1.0[a]	3.8±0.4[a,b]
Monocyte (%)	1.6±1.1[a,b,c]	0.6±0.5[a,b,c]	0.2±0.4[a,b]	2.0±0.7[a,b]	0.6±0.6[a,b,c]	1.2±1.2[a,b,c]	1.8±0.4[a,c]
Basophil (%)	1.8±1.1[a,b]	0.6±0.5[a]	0.4±0.4[a]	1.0±1.0[a,b]	0.4±0.4[a]	0.8±0.8[a,b]	1.8±0.4[a,b]
RBS (mg/dL)	84.2±5.9[a]	260.6±7.8[b]	86.8±4.4[a]	215.0±6.9[c]	228.8±2.7[c]	221.9±3.3[c]	136.8±12.1[d]

Data are presented as mean±S.D.; mean values having different lowercase letters of the alphabet as superscripts are considered significantly different (p-value<0.05) within the rows, S.D.=standard deviation, WBC=white blood cell, RBS=random blood sugar

Table 7 Some markers of subclinical thrombosis among the study groups

Treatment	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
PLT (10^9/L)	236.8±16.2[a]	408.0±39.0[b]	287.9±20.6[c]	293.8±19.0[c]	305.6±9.9[c]	311.1±10.7[c]	233.1±5.7[a]
MPV (fl)	9.9±1.5[a]	18.2±2.2[b]	14.0±1.2[c]	13.7±1.8[c]	15.3±0.8[b,c]	14.3±0.7[c]	8.7±1.0[a]
PDW (fl)	8.8±1.2[a]	16.8±1.7[b]	11.6±0.8[c]	13.3±1.2[c]	12.5±1.1[c]	13.5±0.7[c]	8.7±1.5[a]
PCT (%)	0.3±0.1[a]	0.9±0.3[b]	0.2±0.1[a]	0.3±0.2[a]	0.4±0.2[a]	0.3±0.2[a]	0.3±0.1[a]
P-LCR (%)	21.0±2.0[a]	41.1±3.8[b]	27.0±1.4[c]	25.2±1.5[c]	35.3±1.8[d]	35.0±1.3[d]	21.0±2.0[a]
RBS (mg/dL)	84.2±5.9[a]	260.6±7.8[b]	86.8±4.4[a]	215.0±6.9[c]	228.8±2.7[c]	221.9±3.3[c]	136.8±12.1[d]

Data are presented as mean±S.D.; mean values having different lowercase letters of the alphabet as superscripts are considered significantly different (p-value<0.05) within the rows, S.D.=standard deviation, PLT=platelet, MPV=mean platelet volume, PDW=platelet distribution width, PCT=plateletcrit, P-LCR=platelet-large cell ratio, RBS=random blood sugar

Table 8 Mean body weight of the rats before and after experimental protocol

Group treatment	Initial weight (g)	Final weight	t-value	p-value
1 Non-diabetic nor extract treated (normal control)	160.40±8.10	171.12±2.41	4.082	0.003*
2 Diabetic non-extract treated (negative control)	160.52±2.34	119.20±3.90	0.127	0.001*
3 Diabetic + treated with 40 mg/kg metformin (positive control)	161.08±5.73	165.19±6.32	0.384	0.155
4 Diabetic + treated with 150 mg/kg VAAE	160.17±4.48	163.83±9.36	0.698	0.242
5 Diabetic + treated with 150 mg/kg MOAE	160.84±6.92	164.59±7.50	1.992	0.317
6 Diabetic + treated with 150 mg/kg OGAE	160.60±11.65	162.75±2.87	2.513	0.529
7 Diabetic + treated with 150 mg/kg BAE	161.94±1.88	166.82±4.77	1.360	0.104

S.E=standard error, significant* at p-value<0.05, VAAE=vernonia amygdalina aqueous extract, MOAE=moringa oliefera aqueous extract, OGAE=ocimum gratisimum aqueous extra, BAE=blended aqueous extra

Discussion

The present study investigated the synergistic effects of the blended aqueous extracts of Moringa oleifera, Ocimum gratissimum, and Vernonia amygdalina on some markers of thromboinflamation in diabetic rats. The absence of mortality or clinical signs of toxicity at doses up to 5,000 mg/kg in both the rats treated with the individual extracts and the blended extract shows that the extracts are safe. Similar to the values reported by other studies for LD50 evaluation of triherbal formulations, values above 500 mg/kg body weight are considered non-toxic and relatively safe[20,21]. The observed significant increase in the concentrations of the secondary metabolites for the blended extract suggests that the extracts may be synergistic in their pharmacological activity. Indeed, the traditional practice of blending different medicinal plant extracts is an approach aimed at achieving the desired therapeutic effectiveness based on the concept that a combination of herbal extracts may be advantageous over the use of the individual extracts, as it may provide an increased and good mixture of the secondary metabolites required to optimize therapy[21]. Polyherbal extracts applied for the management of the coronavirus disease (COVID-19) were reported to show no significant changes in the total white blood cell counts and neutrophils of the treated rats compared to the controls[21]. This is not in agreement with the findings of the present study, which found significant increases in the parameters for the diabetic rats treated with the blended extract compared to the controls, though we did not use the same combination of extracts.

The significant increase in the serum glucose for the diabetes induced rats suggests the presence of hyperglycemic-induced thromboinflammation. Hyperglycemia usually occurs following the administration of diabetogenic compounds such as alloxan monohydrate[19]. Alloxan monohydrate is capable of generating free radicals, which destroy the beta cells of the islets of Langerhans, with the resultant reduction in insulin production, which leads to an increase in serum glucose and thromboinflammation[22]. The significant reduction in the serum glucose concentration of the rats treated with the blended extracts is in consonance with the findings of a previous study which reported the hypoglycemic effect of the blended aqueous extracts of Moringa oleifera, Ocimum gratissimum, and Vernonia amygdalina in alloxan-induced diabetic rats[6].

Similar values of the markers of thromboinflammation recorded for the groups treated with the blended extract, positive control group, and the normal control could be attributed to the increased values of the secondary metabolites recorded for the blended extract. Terpenoids and flavonoids are known to possess anti-inflammatory activities, while tannins have been reported to exert anti-thrombotic activities and equally active hypoglycemic activities[23,24]. This finding is similar to the findings of a previous study that reported an increase in these parameters in alloxan-induced diabetic rats treated with the blended extracts of Moringa oleifera, Ocimum gratissimum, and Vernonia amygdalina[6]. Increased platelet count, platelet indices involving the mean platelet volume, plateletcrit, platelet distribution width, and the platelet large cell ratio observed in the present study have been described by many studies as reliable markers of subclinical thrombosis, while increased white blood cell count and subtypes have also been described as reliable markers for subclinical inflammation in diabetic patients[25-30]. Any non-significant differences observed for both the serum glucose and the various markers of thromboinflammation for the diabetic rats treated with the blended extract and those treated with metformin suggest that the blended extract may be as effective as the standard drug (metformin) used in clinical settings for the management of diabetes.

Conclusion

The present study demonstrates that the blended aqueous extracts of Moringa oleifera, Ocimum gratissimum, and Vernonia amygdalina are non-toxic and synergistic in reducing thrombinflammation in diabetic rats.

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Conflict of interest

The authors declare no conflict of interest.

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