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Screening extracts of *Asystasia vogeliana* Benth. for anti-inflammatory potentials

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Abstract

This study screened different parts of *Asystasia vogeliana* for anti-inflammatory potentials. Fresh *A. vogeliana* leaf, stem, and aerial parts were separately air-dried, pulverised, and extracted in 80% methanol (MeOH) for 72 hrs. Filtrates were separately concentrated to produce leaf, stem, and aerial extracts, respectively. Acute oral toxicity was determined on the leaf and stem extracts using OECD method. Then, extracts were screened for in vivo anti-inflammatory potentials via egg albumin-induced paw oedema model. The powdered leaf part was extracted with dichloromethane (DCM) and ethyl acetate (EtOAc) for 72 hrs and processed to produce DCM and EtOAc leaf extracts. Then, MeOH, DCM, and EtOAc leaf extracts were screened for in vitro anti-inflammatory activities via bovine erythrocyte membrane stabilisation, anti-denaturant, anti-tryptic, and nitric oxide scavenging assays. No mortality or sign of toxicity was observed at 2000 mg/kg. Compared with the untreated negative control, the various parts of *A. vogeliana* demonstrated ¬in vivo anti-inflammatory activities by reducing the paw oedema size at different time intervals. Most significant anti-inflammatory activity was obtained with the aerial extract. DCM leaf extract demonstrated the highest erythrocyte membrane stabilization (85.46±0.01%), anti-denaturant (89.52±0.01%), and NO scavenging (IC50 6.77±0.94 mg/ml) activities. MeOH leaf extract showed least in vitro anti-inflammatory activity. The study concludes that the stem and aerial parts of *A. vogeliana* are also a good source of anti-inflammatory compounds, in addition to the leaf part. No toxic effect was observed at 2000 mg/kg. Therefore, our findings substantiate the conventional use of *A. vogeliana* leaves as remedy for inflammatory-related conditions.

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Introduction

Inflammation is a biological reaction of the immune system to harmful stimuli, which could be pathogens or non-pathogens (Chen *et al*., 2018). The pathogenic stimuli may be bacteria, fungi, viruses, or parasites. The non-pathogenic stimuli may be chemicals, drugs, UV exposure, and free radical stress (Achoui *et al*., 2010). Therefore, an inflammatory response could be pathogen-associated or non-pathogen-associated. The pathogen-associated inflammatory response (PAIR) is activated when a pathogen (bacterium, virus, or fungus) invades the body tissue and triggers an immune response. The non-pathogen-associated inflammatory response (NPAIR) is triggered in response to harmful processes like tissue injury, cell death, cancer, free radicals, and degeneration. These factors may trigger acute or chronic inflammatory responses in vital organs (heart, liver, kidney, gastro-intestinal tract, lung, etc.), leading to diverse tissue injuries (Chen *et al*., 2018). Following damage, the organs express inflammatory mediators to initiate cascades that expel the harmful agent(s) (Abdulkhaleq *et al*., 2018). The inflammatory mediators may be pro-inflammatory or anti-inflammatory, depending on the prevailing homeostatic conditions (Vignali and Kuchroo, 2012). Following release, the pro-inflammatory mediators and chemokines initiate the recruitment of neutrophils and monocytes to the site of tissue damage (Bertram, 2004). On arrival at the injury site, these inflammatory cells secrete reactive oxygen species (ROS), reactive nitrogen species (RNS), proteases (trypsin and chymotrypsin), and cytokines (tumour necrosis factor alpha) to neutralize the pathogens and clean up the debris afterward (Boora *et al*., 2014; Praveena *et al*., 2010).

The free radicals act through a cascade leading to the downstream activation of kinases. Activated kinases phosphorylate the inhibitor kappa β (Iκβ), which is a cytoplasmic protein that regulates the activity of the nuclear factor kappa β protein (NFκβ) (Abdulkhaleq *et al*., 2018). Phosphorylation causes the Iκβ to dissociate from NF-κβ complex and is subsequently degraded via the ubiquitin-proteasome system.

Free NFκβ is translocated into the nucleus, where it binds to a gene response element for transcriptional activation of pro-inflammatory genes for inflammatory proteins including cytokines, chemokines, cyclooxygenase-II, and inducible nitric oxide synthase (Hume *et al*., 2001). The release of inflammatory proteins intensifies the inflammatory reactions until the culprit is arrested and expelled (Gebhardt *et al*., 2009). In chronic inflammation, the neutrophils (primary cell type in acute inflammation) are steadily replaced by more aggressive macrophages which essentially secrete pro-inflammatory cytokines (tumour necrosis factor-alpha). Prolonged secretion of cytokines is responsible for most forms of chronic conditions, including arthritis, asthma, neurodegenerative diseases, Alzheimer's, etc. (Iwalewa *et al*., 2007).

Over the years, inflammatory disorders have been controlled by the use of steroidal and nonsteroidal anti-inflammatory drugs (Bertram, 2004; Stankov, 2012). However, both drug types have been shown to cause unwanted side effects (Harirforoosh *et al*., 2013; Greaves, 1976). Therefore, there is need for new drug alternatives (Achoui *et al*., 2010; Chen *et al*., 2018). Fortunately, many medicinal plants have been employed in several ethnomedicine for treatment of disease conditions including inflammatory disorders (Furman *et al*., 2019; Popoola *et al*., 2017) with fewer or no side effects (Zhou *et al*., 2011).

During the screening of some medicinal plants traditionally used in Nigeria for management of chronic inflammatory conditions, *Asystasia vogeliana* was selected based on the local information. The study plant, *Asystasia vogeliana* (Family: Acanthaceae), is a straggling herb with several ethnomedicinal benefits in Nigeria. The leaves of *A. vogeliana*, or the whole plant, are squeezed in clean water, and the extract solution is drunk for treatment of malaria, gastric disorders, and gonorrhea (Popoola *et al*., 2017). The whole plant of *A. vogeliana* is boiled with *Cassia alata* leaves, *Citrus aurantifolia* (lime) fruits, and *Cymbopogon citratus* leaves.

The combined extract or infusion is drunk as a remedy for malaria, fever, hypertension, gonorrhea, gastric disorders, cancer, tumours, epilepsy, and diabetes (Popoola *et al.*, 2017). The leaf extract of *A. vogeliana* is also drunk as a remedy for hepatitis in Nsukka, a metropolis in Enugu, Nigeria (Ugwuanyi *et al.*, 2020). In south-west Nigeria, the leaves of *A. vogeliana* are boiled in clean water, and the infusion is drunk as a tonic for boosting the blood volume in anemic conditions. Also in Cameroun, the leaf decoction of *A. vogeliana* is drunk as a remedy for the treatment of reproductive diseases and management of HIV (Gildas *et al.*, 2017). Many of the health conditions treated with the various extracts of *A. vogeliana* were found to share similar physio-pathological mechanisms as inflammation. Therefore, the objective of this study was to assess the acute oral toxicity and anti-inflammatory properties of various solvent extracts of *Asystasia vogeliana*. This study aims to contribute crucial insights into the safety profile and potential therapeutic value of *A. vogeliana* extracts, thus expanding our understanding of their viability for possible pharmaceutical applications. In Nigerian ethnomedicine, the leaves of *Asystasia vogeliana*, or the whole plant is consumed for different medicinal purposes (Popoola *et al.*, 2017). Hence, different parts of the plant such as the leaves, stem, and aerial (the combined stem and leaf parts) parts were screened for anti-inflammatory potentials.

Materials and methods

Collection of plant sample

Fresh aerial samples of *A. vogeliana* were collected at Ile-Ife from the geographic coordinates Lat: N07°29.728' Long: E004°33.008' in Osun State, Nigeria. The samples were identified and authenticated with specimen number IFE-17776 at the IFE HERBARIUM, Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria.

Wistar rat

The study obtained ethical approval (IPH/OAU/12/1627) from the Institute of Public Health (IPH) at Obafemi Awolowo University, Ile-Ife, to use laboratory animals. Subsequently, forty-eight Wistar rats of both sexes, weighing between 100-150 g, were purchased from the Animal Breeding House within the Faculty of Health Sciences at OAU, Ile-Ife, Nigeria. The rats were given a two-week period to acclimatize in the animal housing unit at the Department of Biochemistry and Molecular Biology, OAU, Ile-Ife. Throughout the acclimatization, the rats were provided with unrestricted access to standard chow and water.

Preparation of A. vogeliana crude extracts

The leaves, stems, and aerial parts were air-dried and separately pulverized using a grinding machine. Then 200 g of each part were separately extracted in 80% (v/v) methanol for 72 hours under controlled laboratory conditions. Each mixture was filtered and then concentrated using a vacuum-assisted rotary evaporator to produce methanol leaf (LE), stem (SE), and aerial (SLE) extracts.

Evaluation of the acute toxicity of A. vogeliana leaf and stem extracts

The OECD limit test method (OECD, 2008) was adopted for oral acute toxicity test. Adult female rats (100–150 g) were fasted overnight but allowed free access to water. After fasting the animals overnight, an oral administration of the extract (2000 mg/kg body weight), not exceeding 1 ml/100 g bwt, was carried out using an intubation cannula. The initial animal was administered with *A. vogeliana* leaf extract (2000 mg/kg) or *A. vogeliana* stem extract (2000 mg/kg) and observed for toxicity signs or mortality over 24 hours. Following observation, four additional animals were dosed to make a total of five animals per group. Continuous observation occurred at 30 minutes, intermittently within the first 24 hours (with emphasis on the initial 4 hours), and subsequently daily for two weeks. The calculation of the median lethal dose (LD_{50}) was performed at the end of the study.

Induction of paw edema in wistar rats using egg albumin

In vivo anti-inflammatory potentials of the leaf, stem, and aerial extracts were assessed using the egg

albumin-induced paw edema model in Wistar rats (Miya *et al.*, 2016). Forty-eight Wistar rats (100-150 g) of both sexes were divided into eight groups (n = 6) and treated as follows: Group 1 received 5% DMSO (2 ml/kg) as the normal control, while Group 2 received diclofenac sodium (10 mg/kg) as the positive control. Groups 3 and 4 were orally pre-treated with leaf extract (LE) at 250 and 500 mg/kg, respectively. Similarly, Groups 5 and 6 were pre-treated with stem extract (SE) at 250 and 500 mg/kg, and Groups 7 and 8 were pre-treated with aerial extract (SLE) at 250 and 500 mg/kg. All pre-treatments were administered an hour before injecting freshly prepared egg albumin (0.1 ml) into the left hind paw of each rat. The initial paw size (mm) was measured using a digital Vernier Caliper at time zero (0) hour. Subsequently, measurements were taken at 1, 2, 3, and 4 hours after egg albumin induction to evaluate the inflammation. Differences in paw size were statistically analyzed using the STAT 3.0 package.

Preparation of dichloromethane (DCM) and ethyl acetate (EtOAc) extracts from A. vogeliana leaves

Having validated *in vivo* anti-inflammatory potentials of the various parts of *A. vogeliana* in edema model, the powdered leaf sample (193 g) of *A. vogeliana* was further macerated in DCM and EtOAc for 72 hours. The filtrate solutions were subjected to vacuum-assisted concentration to produce DCM and EtOAc leaf extracts, respectively.

In vitro anti-inflammatory assays

Preparation of 2% (v/v) red blood cells

Fresh whole blood sample of bovine was collected from an abattoir located in Ile-Ife, Nigeria. The blood was collected into an anticoagulant solution containing 3.8% trisodium citrate and gently agitated to prevent hemolysis. The sample was centrifuged at 3000 rpm (704 x g) for 10 minutes. The supernatant was removed and the packed red cells were washed repeatedly with normal saline solution (0.85% NaCl) until a clear supernatant was achieved. After removal of the supernatant, 2 ml of the packed red cells were made up to 100 ml using normal saline, making 2% (v/v) bovine erythrocytes (Oyedapo *et al.*, 2010).

Membrane stabilization assay

The membrane stabilization activity of DCM, EtOAc, and MeOH leaf extracts of *A. vogeliana* was assessed using a modified method of Oyedapo *et al.* (2010). The reaction mixture consisted of 1.0 ml of hyposaline (0.25% w/v NaCl), 0.5 ml of phosphate buffer (0.15 M, pH 7.4), varying concentrations of extract/drug (0-350 µg/ml) adjusted to 3.0 ml using normal saline (0.85% w/v NaCl), and 0.5 ml of 2% (v/v) red blood cells. The reaction mixture was incubated at 56°C for 30 minutes, followed by centrifugation at 3000 rpm (704 x g) for 10 minutes. After centrifugation, the supernatant was collected and absorbance of the released hemoglobin was measured at 560 nm against a reagent blank. The experiment was performed in triplicates. The positive control was performed using aspirin (NSAID) and prednisolone (SAID). The percentage of membrane stability was calculated as follows:

% stability= [100–{(Absorbance of test drug)-(Absorbance of drug control)/Absorbance of blood control}] × 100

The absence of the drug in the blood control resulted in 100% lysis.

Tryptic inhibitory assay

The combined methods of Oyedapo and Famurewa (1995) and Shivraj and Park (2013) were adopted to assess the inhibitory activities of *A. vogeliana* leaf extracts on trypsin enzyme. The bovine serum albumin (BSA) was used as the substrate, while aspirin served as the positive control. The reaction mixture consisted of 0.03 ml of trypsin (0.06 mg), Tris HCl buffer (20 mM, pH 7.4; 0.5 ml), and the extract/drug (0.5 ml; 10-50 µg/ml) to a total volume of 1.0 ml. The reaction mixture was pre-incubated at 37°C for 5 minutes. Then, BSA (0.8%, 0.5 ml) was added, and incubated again at 37°C for 20 minutes. The reaction was stopped by adding 5% trichloroacetic acid (1 ml). The mixture was centrifuged to clarify the cloudy suspension. The supernatant was collected and the absorbance was measured at 280 nm against a buffer blank. The anti-tryptic activity was quantified as follows:

Percentage inhibition = {(Abs of control - Abs of test)/ (Abs of control)} × 100

Where, Abs of control represents 100% hydrolysis of BSA.

Protein denaturation inhibitory assay

The anti-denaturant activity of *A. vogeliana* leaf extracts was determined using BSA as the reference protein, and diclofenac sodium as the positive drug (Mizushima and Kobayashi, 1968; Akinpelu *et al.*, 2018). The reaction mixture consisted of 0.5 ml BSA (0.25 mg/ml) and varying concentrations of the extracts (0 - 400 µg/ml) to a total volume of 3.0 ml. The reaction mixture was pre-incubated at 37°C for 20 minutes, and then, heated at 57°C for 3 minutes. After allowing cooling, 2.5 ml of phosphate buffer (0.5 M, pH 6.3) was added and mixed thoroughly. Then, 1.0 ml was pipetted into clean test tubes followed by the addition of alkaline copper reagent (1.0 ml) and Folin-Ciocateu's reagent (1.0 ml). The reaction mixture was incubated at 55°C for 10 minutes. After cooling, the absorbance was measured at 650 nm against the reagent blank.

Quantity of protein left = (Abs of Test –Abs of Control)/(Abs of Test)

Percentage of Inhibition = (Quantity of protein left/ Total protein) × 100

Where, Absorbance of control represents 100% denaturation.

Nitric oxide radical scavenging assay

The nitric oxide radical scavenging activity of the *A. vogeliana* leaf extracts was evaluated (Green *et al.*, 1982; Marcocci *et al.*, 1994). The reaction mixture consisted of 0.1 ml extract (0.3125, 0.625, 1.25, 2.5, 5, and 10 mg/ml) and 0.9 ml of sodium nitroprusside (2.5 mM) prepared in phosphate buffer saline. The mixture was incubated under light for 150 minutes. Then, 0.5 ml of 1% sulfanilic acid prepared in 5% phosphoric acid was added to the reaction mixture, and incubated in the dark for an additional 10 minutes. Subsequently, 0.5 ml of 0.1% NED (N-1-napthylethylenediamine dihydrochloride) was added, and the absorbance of the formed chromophore was measured at 546 nm. The nitric oxide scavenging activity was calculated as:

% Inhibition = [(Ablank-Asample)/Ablank] × 100

Ablank = absorbance of control

Asample = absorbance of test compound

Sample concentration providing 50% inhibition (IC_{50}) was extrapolated from the graph by plotting the percentage inhibition of scavenging activity against the extract concentration.

Phytochemical screening of different leaf extracts of A. vogeliana

The various leaf extracts of *A. vogeliana* were subjected to phytochemical screening based on the procedures described by Harborne (1984) and Trease and Evans (2002).

Data analyses

The data from the study was analysed using the GraphPad Prism 5.0 package. The values were presented as Mean ± SEM (n=3). The differences between the control and treated groups were analysed via ANOVA and values were considered statistically significant at $p \leq 0.05$.

Results

Percentage yields of A. vogeliana crude extracts

The percentage yields of the various crude extracts of *A. vogeliana* are presented in Table 1. The methanol leaf extract had the highest yield of 15.69%. This was closely followed by the methanol aerial extract which account for 10.73%. The ethyl acetate leaf extract, on the other hand, gave the least yield 4.29%. The yield percent highlights how each solvent affected the quantities of the components extracted from the various parts of *A. vogeliana*. This information can help us or other researchers to optimise the extraction methods and cut cost.

Table 1. Percentage yield crude extracts obtained from *A. vogeliana*.

A. vogeliana extract	Percentage yield (%)
Methanol leaf extract	15.69
Methanol stem extract	10.00
Methanol aerial extract	10.73
Dichloromethane leaf extract	7.64
Ethyl acetate leaf extract	4.29

Acute toxicity effect of methanol leaf and stem extracts of A. vogeliana

Oral administration of 2000 mg/kg to the test animals did not produce any death or sign of toxicity. Suggesting that the extracts are relatively nontoxic at 2000 mg/kg bwt. Based on this result, two lower doses 250 and 500 mg/kg were selected for *in vivo* acute anti-inflammatory study in Wistar rats induced with paw oedema.

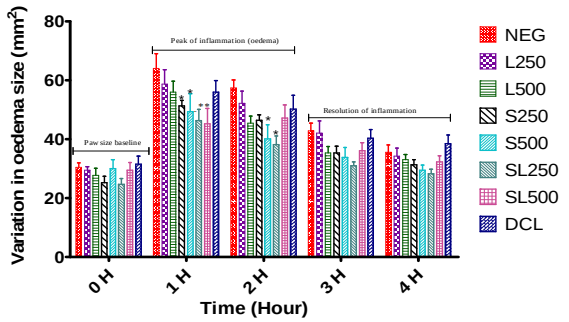

Fig. 1. Anti-inflammatory effect of methanol leaf, stem, and aerial extracts of A. vogeliana on egg albumin-induced paw oedema in rats.

LE: leaf extract; SE: stem extract; SLE: aerial extract. Mean values with * was significantly lower than the negative control group (NEG). The lower the values, the higher the potency level. NEG: Negative control that received 5% DMSO (2 ml/kg); L250: Group treated with 250 mg/kg leaf extract; L500: Group treated with 500 mg/kg leaf extract; S250: Group treated with 250 mg/kg stem extract; S500: Group treated with 500 mg/kg leaf extract; SL250: Group treated with 250 mg/kg aerial extract; SL500: Group treated with 250 mg/kg aerial extract; DCL: Diclofenac (10 mg/ml).

Anti-inflammatory effects of methanol leaf, stem, and aerial extracts in wistar rats with paw oedema

The methanol extracts obtained from the leaves, stems, and aerial parts of *A. vogeliana* showed different anti-inflammatory effects. When compared with the negative control group, the extracts attenuated the paw edema sizes significantly in the first two hours of assessment (Fig. 1). Compared with the negative control group, the inhibitory effect of the leaf extract at 250 (L250) and 500 mg/kg (L500) was not statistically significant across the assessment period. The stem extract at 250 mg/kg significantly reduced the paw oedema size after the first one hour (1 h) of induction. At 500 mg/kg, the stem extract exerted a protracted anti-inflammatory effect by producing a significant reduction in the oedema size for the first 2 hours when compared with the negative control. As the time progressed to the third and fourth hours, no significant inhibition was observed. Likewise, the aerial extract at 250 mg/kg (SL250) produced a significant reduction oedema size in the first 2 hours, while the 500 mg/kg (SL500) aerial extract showed reduction in oedema for the first 1 hour of induction when compared with the negative control. Individually, when the effect of the leaf extracts and stem extract was compared using a two-way analysis of variance, no significant difference was observed. However, the effects of the combined extract (aerial) at 250 and 500 mg/kg were significantly superior to the leaf extract at 250 and 500 mg/kg alone.

Membrane stabilization activity of A. vogeliana leaf extracts

The membrane stabilizing activity of the MeOH, DCM, and EtOAc leaf extracts of *A. vogeliana* on bovine erythrocytes exposed to both heat and hypotonic induced lyses is shown in Fig. 2.

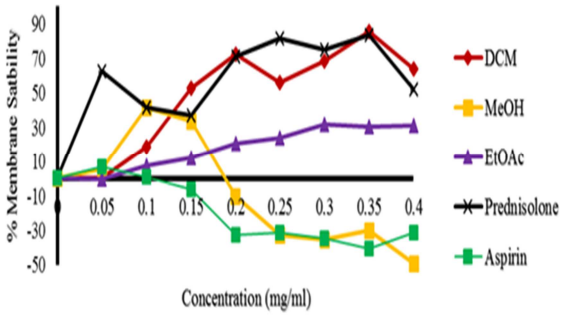

Fig. 2. Membrane stabilization activity of *A. vogeliana* leaf extracts.

Value represents Mean ± SEM (n= 3). DCM: Dichloromethane extract; MeOH: Methanol extract; EtOAc: Ethyl acetate extract

The results obtained were compared with nonsteroidal (aspirin) and steroidal (prednisolone) anti-inflammatory drugs as controls. The DCM leaf extract was found to significantly ($p \leq 0.05$) inhibit the lysis of the stressed erythrocyte when compared with aspirin (6.74±0.02%) but not prednisolone (83.49±0%) reference drug. DCM leaf extract had the

highest percentage stabilization activity (85.46±0.01%) at 0.35 mg/ml. The MeOH and EtOAc leaf extracts had percentage inhibition of 41.27±0.01 and 31.66±0.03% at 0.10 and 0.30 mg/ml, respectively. Furthermore, the EtOAc leaf extract demonstrated a monophasic mode of protection against the induced lysis, while the DCM leaf extract and prednisolone showed a biphasic mode of protection. MeOH leaf extract and aspirin were entirely biphasic; and protected the RBCS only at very low concentrations. The concentration-dependent effects of the leaf extracts can provide an insight into the potential formulation when applied therapeutically.

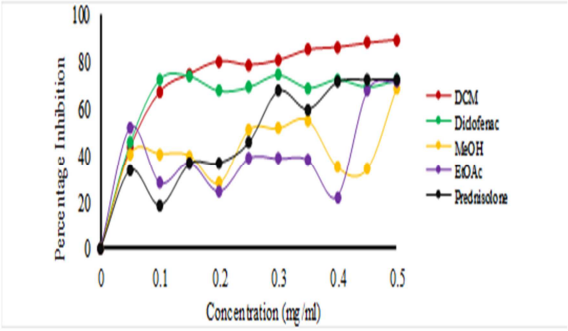

Fig. 3. Percentage inhibition of BSA denaturation by crude extract.

Inhibition of protein denaturation

The percentage inhibition of heat-induced albumin denaturation of *A. vogeliana* leaf extracts is shown in Fig. 3. The maximum percentage inhibitions for DCM, EtOAc, and MeOH extracts were 89.52±0.00, 71.58±0.03, and 68.94±0.0, respectively, at 0.50 mg/ml. Prednisolone and diclofenac had percentage inhibitions of 72.64±0.0 and 74.34±0.02%, at 0.50 and 0.30 mg/ml, respectively. There was no significant difference between the leaf extracts and the control drugs. The DCM leaf extract showed a gradual and steady increase in the inhibition percent in a concentration-dependent manner. The anti-denaturant effects of the DCM leaf extract correlated well with diclofenac sodium, and was significantly higher than MeOH and EtOAc extracts. The EtOAc leaf extract showed the least percentage inhibition across the concentrations tested. The results suggest that *A. vogeliana* leaf extracts exhibit notable potential as inhibitors of protein denaturation. The elevated anti-denaturation effect of DCM leaf extract underscores the promising application in the management of chronic inflammatory conditions such as rheumatoid arthritis.

Inhibitory effects of A. vogeliana leaf extracts on tryptic activity

Table 2. Tryptic inhibitory activity of A. vogeliana leaf extracts.

Sample	IC_{50} (µg/ml)
EtOAc extract	13.27±2.42
DCM extract	19.47±4.81
MeOH extract	25.46±2.56
Aspirin	64.83±17.49

Each value represents a Mean ± SEM (n= 3). DCM: Dichloromethane extract; MeOH: Methanol extract; EtOAc: Ethyl acetate extract

The inhibitory effects of the leaf extracts of *A. vogeliana* on trypsin activity are shown in Table 2. The EtOAc leaf extract exhibited a slightly stronger inhibitory effect (IC_{50} = 13.27±2.42 µg/ml) than the DCM (IC_{50} = 19.47±4.81 µg/ml) and MeOH (IC_{50} = 25.46±2.56 µg/ml) leaf extracts. All *A. vogeliana* leaf extracts demonstrated greater potency in inhibiting trypsin activity than aspirin (IC_{50} = 64.83±17.49 µg/ml). Trypsin plays a pivotal role in the modulation of inflammatory responses. Therefore, in the quest for new anti-inflammatory drug discovery, the inhibition of trypsin holds significance as it offers new avenues for designing effective treatments for various inflammatory conditions.

Table 3. Nitric oxide radical scavenging activities of DCM and EtOAc leaf extracts.

Sample	IC_{50} (µg/ml)
Ascorbic acid	0.06±2.52
DCM Extract	6.77±0.94
EtOAc Extract	8.28±0.99

Value represents Mean ± SEM (n= 3). DCM: Dichloromethane extract; EtOAc: Ethyl acetate extract; IC_{50}: Median inhibition concentration

Nitric oxide (NO) radical scavenging activity of A. vogeliana leaf extracts

The DCM and EtOAc leaf extracts that exhibited more pronounced anti-inflammatory effects were evaluated for NO scavenging activity. The NO radical scavenging assay was chosen above other antioxidant assays because it is directly linked to the anti-inflammatory focus of our study. NO is required for optimal immune function, excessive generation can

result in chronic inflammation and tissue damage. The DCM leaf extract showed more potency at scavenging NO radicals (IC_{50} = 6.77±0.94 mg/ml) than the EtOAc extract (IC_{50} = 8.28±0.99 mg/ml). The ascorbic acid control showed considerably higher NO radical scavenging activity (IC_{50} = 0.06±2.52 mg/ml) than the leaf extracts (Table 3).

Table 4. Phytochemical constituents of different extracts of *A. vogeliana* leaves.

Phytochemical	DCM extract	EtoAc extract	MeOH extract
Alkaloids	+++	+++	+++
Carbohydrates	+	+	+
Flavonoids	++	++	-
Cardiac Glycosides	++	-	-
Phenols	-	-	-
Phlobatannins	-	-	-
Resins	++	+	-
Saponins	-	-	-
Sterols	-	-	-
Tannins	++	++	-
Terpenoids	+	+	-

Key: (+): Weak positive; (++): Positive test; (+++): Strongly positive); (-): Negative test

Phytochemical constituents of A. vogeliana leaves

The phytochemical constituents of methanol, dichloromethane, and ethyl acetate extracts of *A. vogeliana* are presented in Table 4. Alkaloids, carbohydrates, flavonoids, cardiac glycosides, resins, tannins, and terpenoids were present in DCM leaf extract. Also, alkaloids, carbohydrates, flavonoids, resins and tannins were present in the ethyl acetate extract. While the methanol leaf extract showed positive tests for alkaloids and carbohydrates. The presence of alkaloids, flavonoids, and tannins in the DCM and ethyl acetate leaf extracts of *Asystasia vogeliana* suggests a potential for bioactive compounds with medicinal properties. These compounds are often associated with various biological activities, including anti-inflammatory, antioxidant, and antimicrobial effects. The presence of alkaloids and carbohydrates in the methanol leaf extract further implies the possibility of bioactive constituents that could contribute to the plant's pharmacological properties.

Discussion

This study screened different parts of *A. vogeliana* Benth for potential anti-inflammatory properties and also determined the acute oral toxicity. Toxicity and inflammation are inter-related: if a drug is toxic, it can trigger inflammatory reactions. This explains why an acute toxicity test was conducted to have an insight into the safety profile of *A. vogeliana*. Fortunately, administration of the leaf and stem extracts at a dose of 2000 mg/kg body weight was without a visible toxic effect. Suggesting that the consumption of *A. vogeliana* at 2000 mg/kg bwt is not associated with adverse inflammatory reactions. Consequently, the LD_{50} was estimated to be >2000 mg/kg body weight threshold.

The highest yield of the plant extract was produced from the MeOH leaf. Methanol is a polar solvent and when mixed with water, further increases the strength to thoroughly extract the phytoconstituents. Aqueous-methanol have been shown to extract polyphenols and flavonoids (Komolafe, 2014; Medini *et al.*, 2014). All the pharmacological activities of medicinal plants are attributable to the presence of phytoconstituents they contain. When humans consume medicinal plants, these therapeutic constituents in them combat diseases and ailments. Curcumin, from *Curcuma longa* root, was shown to have multiple anti-inflammatory targets, including prostaglandins, lipoxygenase, free radical, TNFα, and IL-1 (Farhood *et al.*, 2018).

Members of Acanthaceae have been reported to contain alkaloids, cardiac glycosides, carbohydrates, flavonoids, phenols, proteins, quinones, saponins, terpenoids, and tannins (Khan *et al.*, 2017). Some of these metabolites were also confirmed in this present study with *A. vogeliana* and could be responsible for the biological activities of the plant.

Egg albumin-induced paw oedema is a globally recognized *in vivo* technique for quick screening of compounds with anti-inflammatory potentials (Oyemitan *et al.*, 2008; Miya *et al.*, 2016). In this study, the stem, and aerial (combined leaf and stem)

parts were able to significantly attenuate the paw oedema size within the first 2 hours of induction when compared with the negative control. At a low dose, the stem significantly reduced the paw oedema size after the first one hour (1 h) of induction. However, at a higher dose, the stem extract was able to significantly reduce the oedema size for the first 2 hours at a stretch. Similarly, the aerial extract at both doses was able to significantly reverse the paw oedema size. In some part of the country (Nigeria), only the leaf part of *A. vogeliana* is employed for disease management. This present study has shown very clearly that significant anti-inflammatory potency also resides in the stem and aerial parts of *A. vogeliana*.

Our finding corroborates Adeyemi *et al.* (2011) who observed that *Asystasia gangetica* (co-generic species) exhibited anti-inflammatory and analgesic properties. The *in vivo* anti-inflammatory activities of *A. vogeliana* stem and aerial extracts were significantly higher than diclofenac sodium at 10 mg/kg. The observed effect could be attributed to the selected dose (low dose). The observed effect may show a different profile at a higher dose of diclofenac sodium.

Egg albumin-induced paw oedema model is known to interfere with the early phase of inflammation characterised by neutrophil infiltration, free radical release, and prostaglandin synthesis. These inflammatory mediators are released in two phases, namely the neurogenic phase (analgesic phase) and inflammatory phase (Oyemitan *et al.*, 2008). The neurogenic phase involves the release of acute phase reactants (neutrophils, histamines, serotonins, reactive proteins) leading to the acute induction of pains. The inflammatory phase involves the release of prostanoids by cyclooxygenase 2 leading to inflammation (Miya *et al.*, 2016). The *A. vogeliana* extracts probably combated these processes by reversing the neurogenic reactions and cyclooxygenase-mediated phase (Miya *et al.*, 2016).

Furthermore, both the polar (MeOH) and moderately polar extracts (DCM and EtOAc) protected the stressed bovine erythrocytes significantly ($p \leq 0.05$) better than aspirin (NSAID). However, the DCM extract competed significantly with prednisolone (SAID) and performed better than MeOH and EtOAc extracts. The *A. vogeliana constituents* may have interacted with erythrocyte membrane and alter their surface charges to shield it from lysing effects of heat and hyposaline (Anosike *et al.*, 2019). Phytochemicals such as flavonoids, saponins, and polyphenols elicit significant membrane stabilizing effect (Oyedapo *et al.,* 2010; Akinpelu *et al.,* 2018). Therefore, the protection of the stressed erythrocytes by *A. vogeliana* leaf extracts could be attributed to presence of these secondary metabolites.

Furthermore, *A. vogeliana* leaf extracts also inhibited albumin protein denaturation *in vitro*. The anti-denaturant effects of DCM leaf extract correlated well with diclofenac sodium. One of the cardinal symptoms of inflammation is heat and loss of tissue function which is attributed to tissue protein denaturation (Mizushima and Kobayashi, 1968). NSAIDs used in the management of inflammatory conditions have been reported in the protection of proteins against denaturation (Akinpelu *et al.*, 2018). Inhibitors of protein denaturation have been applied in the treatment of rheumatoid arthritis.

Also the DCM leaf extracts of *A. vogeliana* inhibited the activity of trypsin even significantly higher than the aspirin drug used in the positive control. During inflammatory reactions, the proteinases residing in the lysosomal granules of neutrophils are released to degrade the inflammatory stimuli and clear the damaged cell debris. Once the stimulus is completely degraded, the hepatocytes secrete alpha-1 proteinase (α_1) inhibitors (Puente and Lopez-Otin, 2004) to deactivate the proteinases. Depending on the inflammatory signal, the level of proteinases may overwhelm the presence of the endogenous inhibitors, thus, allowing tissue protein digestion to run unabated. Medicinal plants have been shown to

possess anti-inflammatory property through the inhibition of trypsin activity (Shalini *et al.*, 2015).

Furthermore, oxidative stress is a trigger of inflammation and prolonged inflammation is known to induce oxidative stress (Marrassini *et al.*, 2018). Agents capable of scavenging NO radicals may be considered anti-inflammatory. The DCM leaf extract elicited significant NO radical scavenging activity than EtOAc leaf extract. The results corroborates Marrassini *et al.* (2018) who observed that the aerial extracts of *Urera aurantiaca* reduced NO production in LPS-stimulated macrophages at higher concentrations. Subathraa and Poonguzhali (2012) also reported that the aqueous extract of *Acorus calamus* contained compounds that inhibited NO production. *Combretum racemosum* extract and fractions were reported to scavenge NO radicals better than ascorbic acid (Bamidele *et al.*, 2011). Also, Boora *et al.* (2014) specified phenolic constituents of the plant as strong inhibitors of NO.

Having demonstrated significant anti-inflammatory activity, this study strongly recommends the isolation and characterisation of the bioactive constituents of *A. vogeliana* Benth to understand their chemical nature(s). The study also recommend further studies on the stem and aerial extracts of *A. vogeliana*.

Conclusion

The study shows that in addition to the *A. vogeliana* leaves being used in Nigerian traditional medicine as remedy for inflammatory conditions, the stem and aerial parts are also a good source of anti-inflammatory compounds. Administration of *A. vogeliana* orally at 2000 mg/kg did not produce mortality. *In vivo* anti-inflammatory screening of the various extracts progressively attenuated the paw edema size. Similarly, *in vitro* anti-inflammatory evaluation of the leaf extracts showed that DCM leaf extract is the most potent extract. Therefore, our findings validate the conventional use of *A. vogeliana* leaves as remedy for inflammatory conditions. In addition, our finding also shows that the stem and the aerial parts even harbour stronger anti-inflammatory benefits.

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