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EVALUATION OF INVITRO ANTI-INFLAMMATORY POTENTIAL OF AQUEOUS *Acanthus montanus* (AHON-EKUN) LEAVES EXTRACT

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Abstract: Inflammation is a normal response to any noxious stimulus that threatens the host and may vary from localized response to a generalized one This study is aimed at evaluating the invitro anti-inflammatory potential of aqueous leaves extract of Acanthus montanus (Family:Acanthaceae). In vitro anti-inflammatory potentials were evaluated using standard experimental protocols such as Inhibition of albumin denaturation, Anti-lipoxygenase activity, Membrane stabilization and Proteinase inhibitory action at different concentrations of the extract with Diclofenac sodium used as the standard drug. Aqueous extract of A.montanus leaves were tested for the presence of alkaloid, flavonoid ,tannin, glycoside, steroids, phenol and phlobatannins.The total phenol and flavonoid content were evaluated as well. The total phenol and flavonoid content were found to be (64.99±0.6 mg of Gallic acid/g of equivalent) and (26.97± 0.5mg of Quarcetin/g of equivalent).The IC_{50} values of the extract and diclofenac were 224.53µg/ml and 183.1µg/ml for inhibition of albumin denaturation, 137.03µg/ml and 159.62µg/ml for Anti-lipoxygenase activity, 250.2µg/ml and 195.44µg/ml for membrane stabilization and 198µg/ml and 191.4µg/ml for Proteinase Inhibitory Action. However, the extract exhibited a significant ($P<0.05$) inhibitory activity in protein denaturation with an IC_{50}value of(198µg/ml) compared to the standard drug(191.4µg/ml).The Inhibition of Albumin Denaturation might be the possible mechanism by which the extract elicit its anti-inflammatory effect. The results obtained in the present study indicate that the aqueous extract of Acanthus montanus is a potent source of anti-inflammatory agents and this justified its uses in the treatment of various infections.

Keywords: *Acanthus montanus, Inflammation, membrane stabilization, Anti-lipoxygenase activities.*

1.0 Introduction

Inflammation comes from the Latin word ‘inflammare,' which means ‘to burn.' Inflammation is the body's protective response to a variety of harmful stimuli such as diseases and damage (Vijayalakshmi, Ravichandiran, Velraj, Helmatha&Sudharani, 2011). Pain, redness, swelling, and dysfunction of the affected bodily part are all common symptoms of inflammation (AmiraDad, Schinell&Jose-luis,2012). The increased migration of plasma and leukocytes from the blood into the wounded tissues causes acute inflammation, which is the body's initial response to damaging stimuli. Chronic inflammation causes a gradual shift in the types of cells present at the site of inflammation, and is characterized by tissue damage and healing at the same time due to the inflammatory process (Kumar & Collins, 1998). Rheumatoid arthritis, asthma, chronic peptic ulcer disease, TB, Pelvic inflammatory disease, and Crohn's disease are examples of inflammatory disorders.

Non-steroidal anti-inflammatory medicines (NSAIDS) like ibuprofen, aspirin, and diclofenac are the most often used drugs to treat inflammation-related discomfort. These medications work by blocking or lowering the pain caused by an enzyme that contributes to inflammation. The usage of NSAIDS over an extended period of time has a cost. The usage of nonsteroidal anti-inflammatory drugs (NSAIDs) can result in substantial gastrointestinal side effects. Even worse, certain NSAIDS have been associated to high blood pressure, a higher risk of congestive heart failure, stroke, and myocardial infarction.

For century, medicinal plants have been a source of a wide range of biologically active substances, which have been employed widely as crude material or as purified compounds to treat a variety of diseases (Arif et al,

2009). Medicinal plants are commonly utilized in traditional medicine to cure a variety of diseases (Njoku&Ezeibe, 2007; Ogukwe, Oguzie, Unaegbu&Okolue, 2004).*Acanthus montanus*belongs to the family of Acanthaceae, it is also known as "Bear's breeches", "Mountain thistle" or "Alligator plant". It is a striking small shrub with sparse branches and soft stem. It grows wild in grasslands, woods, scrub and rocky hills of the Balkans, Romania, Greece, Eastern Mediterranean and Africa.(Huxley,1992).*A.montanus h*as a dark-green leaves with spikes of pale pink flowers which prefers shady conditions, dry situations too (Huxley, 1992).

A. montanus has long been used in folk medicine to treat a variety of ailments, including respiratory, gastrointestinal, circulatory, and urinary problems (Igoli, Ogaji, Tor-Anyin&Igoli, 2005). According to Asongalem, Foyet, Ekobo, Dimo, and Kamtchouing (2004), the aqueous extract of A.montanus exhibits strong anti-inflammatory action. However, the anti-inflammatory properties of this herb have not been scientifically proven in vitro. This sparked more research into the possibility that the plant had anti-inflammatory properties.

2.0 Materials and Methods

2.1 Plant Material

Fresh leaves of *A.montanus* plant were collected from a local farm in Ikire, Apomu, Osun State, Nigeria(7.35º North Latitude,4.18º East Longitude) in February, 2019. Botanical identification and authentication were performed by Mr. T.K. Odewo, a Senior Superintendent of the Taxonomy Department, Forestry Research Institute of Nigeria (FRIN), Ibadan, Nigeria. A voucher specimen with authentication number (FHI, 112236) was deposited.

2.1.2 Preparation of Plant Extracts

Fresh *A.montanus* leaves were thoroughly rinsed and air-dried at room temperature for 15 days. The dried samples was grinded into fine powder using a Master chef electric blender Model MC-BL 1644 and stored in an air-tight container at room temperature until use.

2.1.3 Extraction of the plant material

Aqueous extract of the plant was prepared by macerating 100g of the dry powdered plant material in 1 litre of distilled water in a clean 5L round bottom flask and the closure was sealed with aluminium foil, then kept at room temperature for 72hours (for thorough extraction). At the end of the 72hours, the extract were filtered first through a Whatmann's filter paper No. 1 (125mm) and funnel and then through into a clean beaker. The aqueous extract(filtrate) obtained after filtration was kept in a sterile bottle and freeze-dried.

2.2 Qualitative Phytochemical Screening

The Phytochemical screening was carried out in order to determine the active ingredients of the plant which are responsible for the anti-inflammatory effect. The Phytochemical analysis was carried out using the method described by (Odebiyi&Sofowora, 1978) for the detection of phenolics,alkaloids,steroid,tannins,terpenoids,phlobatannins,glycosides and flavonoids.

2.2.1 Steroid Test

To 1cm^3 of the extract solution, 5 drops of concentrated H_2SO_4 was added. Red colouration indicates the presence of steroids.

2.2.2 Glycoside Test

To 1cm^3 of the extractsolution, 10cm^3 of 50% $H_2SO_4$was added in a test tube. The mixture was heated in boiling water for 15 minutes. 10cm^3 of Fehling's solution was added and the mixture boiled. A brick red precipitate indicates the presence of glycosides.

2.2.3 Flavonoid Test

The plant extracts(3cm^3) was added to 1$cm^3$of 10% Hcl in a test tube. A yellow colouration indicates the presence of flavonoids.

2.2.4 Alkaloid Test

To 3cm^3 of the plant extracts,1cm^3 of 1% HCl was added in a test tube. The mixture was heated for 20 minutes, cooled and filtered. The filtrate was used in the following tests: 2 drops of Wagner's reagent was added to 1cm^3 of the extracts. A reddish brown precipitate indicates the presence of alkaloids.

2.2.5 Phenolic Test

To 1cm^3 of the plant extracts in a test tube, 2 drops of 5% $FeCl_3$ was added. A greenish precipitate indicates the presence ofphenolics.

2.2.6 Tannin Test

To 1cm3 of the extracts, 1cm^3 of freshly prepared 10% KOH was added in a test tube. A dirty white precipitate indicates the presence of tannins.

2.2.7 Phlobatannin Test

The extracts (1cm^3)was added to 1% HCl in a test tube. A red precipitate indicates the presence of phlobatannins**.**

2.3 Quantitative Phytochemical Analysis

2.3.1 Estimation of Total Phenolic Content

The total phenolic content of sample was estimated according to the method of (Makkar,Norsambuu,Lkhavatsere& Becker, 1997). The aliquots of the extract was taken in a test tube and made up to the volume of 1ml with distilled water. Then 0.5ml of Folin-Ciocalteu reagent (1:1) with water) and 2.5ml of sodium carbonate solution (20%) were added sequentially to the test tube. Soon after vortexing the reaction mixture, the tubes were placed in the dark for 40 min. and the absorbance was measured at 725 nm by UV-spectrophotometer against the reagent blank. Using Gallic acid monohydrate, a standard curve was prepared. The linearity obtained was in the range of 1-10 µg/ml. Using the standard curve, the total phenolic content was calculated and expressed as Gallic acid equivalent in mg/g of extract.

2.3.2 Determination of Total flavonoid

Total flavonoid content was measured by aluminium chloride colorimetric assay. 1ml of extracts or standard solution of Quarcetin (500µg/ml) was added to 10ml volumetric flask containing 4ml of distilled water. To the above mixture, 0.3ml of 5% $NaNO_2$ was added. After 5 minutes, 0.3ml of 10% $AlCl_3$was added. After 6 minutes, 2ml of 1M NaOH was added and the total volume was made up to 10ml with distilled water. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510 nm. Total flavonoid content of the plant was expressed as percentage of Quarcetin equivalent per 100 g of fresh mass.

2.4 INVITRO ANTI-INFLAMMATORY ASSESSMENT

The leaf extract of *A.montanus* was subjected to preliminary assessments using standard procedures to determine its anti-inflammatory activities.

2.4.1 Extract Preparation

5mg of crude aqueous extracts of *A.montanu*s was dissolved in 5ml of distilled water to produce a solution of 5mg/ml.

2.4.2 Inhibition of Albumin Denaturation

The Inhibition of Albumin Denaturation was assayed according to (Sakat, Juveka&Gambhire, 2010). The 5ml of reaction mixture was comprised of 0.2ml of eggs albumin, 2.8ml of phosphate buffered saline (PBS, pH 6.4) and 2ml of varying concentration of extracts. Similar volume of double distilled water served a control. Then the

mixture was incubated at 37 °C in incubator for about 15mins and then heated at 70 °C for 5mins. After cooling, their absorbance was measured at 660nm by using pure blank. Diclofenac sodium (standard drug) was used as reference drug and treated as such for determination of absorbance. The percentage inhibition of protein denaturation was calculated by the formula below;

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control- Absorbance of Sample}}{\text{Absorbance of Control}} X\ 100$$

3.4.3 Anti-lipoxygenase Activity

The lipoxygenase activity was assayed according to the method of (Tappel, 1962) with slight modifications (Wallace& Wheeler, 1975). A total volume of 200 µl assay mixture contained, 160 µl sodium phosphate buffer (100 mM, pH 8.0), 10µl test extract (50 to 250 µg) extracted material in 100 mMTris buffer pH 7.4) and 20 µl lipoxygenase enzyme. The contents were pre-incubated for 10 min at 25°C. The reaction was initiated by the addition of 10 µl linoleic acid solution as substrate. The change in absorbance was observed after 6 min at 234 nm. All reactions were performed in triplicates in 96-well Microplate Reader Spectra Max 190 (Molecular Devices, USA). The positive and negative controls were included in the assay. The percentage inhibition (%) was calculated by the formula below:

$$\text{Inhibition (\%)} = \frac{\text{(Absorbance of control – Absorbance of test sample)}}{\text{Absorbance of control}} x100$$

3.4.4 Membrane Stabilization

3.4.4.1 Preparation of Red Blood Cells (RBC's)

Fresh whole mammalian blood (10ml) was collected and transferred to heparinzed centrifuge tubes. The tubes were centrifuged at 3500rpm for 10min and were washed three times with equal volume of normal saline. The volume of the blood was measured and reconstituted as 10% v/v suspension with normal saline.

2.4.5 Proteinase Inhibitory Action

The test was performed according to the modified method of (Oyedepo&Femurewa, 1995) and (Sakat, Juvekar&Gambhire,2010). The reaction mixture (2 ml) was containing 0.06 mg trypsin, 1ml of 20 mMTrisHCl buffer (pH 7.4) and 1 ml test sample of different concentrations. The reaction mixture was incubated at 37°C for 5 min and then 1ml of 0.8% (W/V) casein was added. The mixture was inhibited for an additional 20 min, 2ml of 70% perchloric acid was added to terminate the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank. The experiment was performed in triplicate. The percentage of inhibition of proteinase inhibitoryactivity was calculated with the formula below:

$$\text{Percentage inhibition} = \frac{\text{(Absorbance of control – Absorbance of sample)}}{\text{Absorbance of control}} X\ 100$$

3.5 STATISTICAL ANALYSIS

The experimental data obtained were expressed as Mean ± S.E.M .The difference between

the extract and control were compared using One way Analysis of Variance (ANOVA) followed by Duncan's Test (Control vs Test) using the SPSS Software Version 20 . $P<0.05$ was considered statistically significant. The IC_{50} values were calculated using Microsoft Excel version 2016.

3.0 RESULT

3.1 PRELIMINARY PHYTOCHEMICAL SCREENING

Phytochemical screening was conducted on the leaf sample to test for the presence of certain phytochemicals and the following result was obtained.

Table 1: Qualitative Phytochemical Screening of Aqueous Extract of *A.montanus*

Phytochemical test	*A.montanus* Leaves extract
Steroid test	+
Flavonoid test	+
Alkaloid test	+
Phenolic test	+
Tannins test	+
Phlobatannin test	-
Glycoside test	+

Keys: + = Test Substance Present, - = Test Substance Absent

Table 2: Quantitative Phytochemical Analysis of Aqueous *A.montanus* Leaves Extract

Plant part	Total Phenolic content	Total Flavonoid content
A.montanus leaves extract	64.99± 0.6	26.97± 0.5

The total phenolic and total flavonoid content were expressed as 64.99 0.6 mg of Gallic acid/g of equivalent and 26.97 0.5mg of Quarcetin/g of equivalent.

3.3 INVITRO ANTIINFLAMMATORY ACTIVITY

Table 3:Effect of Aqueous *A.montanu* leaves extract on Inhibition of Albumin denaturation.

Concentration(mg/ml)	Mean±SEM	
	AMA	Standard (diclofenac)
50	0.12^{a}±0.01	0.10^{a}±0.01
100	0.09^{a}±0.01	0.09^{a}±0.00
150	0.08^{a}±0.00	0.07^{a}±0.00
200	0.06^{a}±0.00	0.05^{a}±0.00
250	0.06^{a}±0.00	0.05^{a}±0.00
IC_{50}	224.53	183.1

Values are represented as mean ± SEM (in triplicates).$p< 0.05$ considered as IC_{50} significant when compared to the Standard drug (Diclofenac). The result of Inhibition of Albumin Denaturation indicated that the IC_{50} values of the extract were significantly higher than that of the Diclofenac which were 224.53 µg/ml and 183.1µg/ml respectively. The Standard drug, Diclofenac significantly inhibited the activity of Albumin Denaturation. However, it was discovered that at a concentration of 250µg/ml, the standard drug, Diclofenac exhibited the highest percentage Inhibition of Albumin Denaturation at 58.6± 0.03%.

3.3.2 Anti-lipoxygenase Activity

Table 4:Effect of aqueous *A.montanus* leaves extract on Anti-lipoxygenase activity

	Mean±SEM	
Concentration	AMA	Standard (diclofenac)
50	0.58^{a}±0.02	0.62^{a}±0.04
100	0.58^{a}±0.02	0.78^{b}±0.02
150	0.55^{a}±0.02	0.87^{b}±0.04
200	0.59^{a}±0.01	1.02^{b}±0.02
250	0.66^{a}±0.05	1.07^{b}±0.04
IC_{50}	137.03	159.62

Values are represented as mean ± SEM (in triplicates).p< 0.05 considered as IC_{50} significant Anti-lipoxygenase activity of the aqueous leaf extract *of A.montanus* at a concentration of 250µg/ml demonstrated a stronger percentage Lipoxygenase Inhibition of 81.02 ± 0.05% with an IC_{50} value of 137.03µg/ml. The leaf extract also demonstrated the highest Lipoxygenase Inhibition with an IC_{50} value of 137.03µg/ml compared to that of the standard drug, Diclofenac.

3.3.4 Membrane Stabilization Activity

Table 5:Effect of Aqueous *A. montanus* leaves extract on Membrane Stabilization

	Mean±SEM	
Concentration (mg/ml)	AMA	Standard (Diclofenac)
50	0.11^{a}±0.01	0.12^{a}±0.01
100	0.10^{a}±0.00	0.07^{a}±0.01
150	0.09^{b}±0.00	0.07^{a}±0.00
200	0.07^{ab}±0.00	0.06^{a}±0.00
250	0.06^{ab}±0.00	0.05^{a}±0.00
IC_{50}	250.2	195.44

Values are represented as mean S.E.M (in triplicates). P<0.05 considered as IC_{50} significant when compared with the standard drug.

The result of Membrane Stabilization study indicated that the highest percentage erythrocyte stabilization of 51.I9% and 56.68% at a concentration of 250µg/ml with IC_{50} Values of 250.2µg/ml and 195.44µg/ml

respectively. However, the standard drug (Diclofenac) exhibited the highest erythrocyte membrane stabilizing activity with an IC_{50} value of 195.44µg/ml.

4.3.5 Proteinase Inhibitory Action

Table 6: Effect of Aqueous *Acanthus montanus* leaves extract on Proteinase Inhibitory Action

	Mean± SEM	
Concentration(mg/ml)	AMA	Standard (control)
50	0.92^{a}±0.04	0.93^{a}±0.03
100	0.72^{a}±0.04	0.71^{a}±0.05
150	0.63^{a}±0.02	0.61^{a}±0.01
200	0.52^{a}±0.02	0.55^{a}±0.03
250	0.50^{a}±0.0	0.45^{a}±0.03
IC_{50}	198	191.4

From the result of Proteinase Inhibitory Action, the standard drug(Diclofenac) exhibited a higher percentage Inhibition of 59.75 ± 0.03% at a concentration of 250µg/ml with an IC_{50} value of 191.4µg/ml compared to that of the extract at 198µg/ml. It was also observed that the Diclofenac showed a higher Proteinase inhibition with an IC_{50} value of 191.4µg/ml.

4.4 DISCUSSION

Inflammation is a common reaction to any unpleasant stimuli that poses a hazard to the host, and it can range from a localized to a widespread response (Medzhitov, 2008).

The leaves of A.montanus can be used to make a crude aqueous extract that can be used to treat a variety of human ailments. The existence of significant phytochemical elements such as alkaloid, steroids, flavonoid, tannin, glycosides, terpenoid, and phenolics was discovered as a consequence of phytochemical screening. These findings are consistent with Ebana et al., (2016) and Eze&Amadi's research (2014). As a result, the existence of these phytochemicals could explain the plant's anti-inflammatory properties.

When biological proteins are denatured, they lose their biological function. Protein denaturation is the breakdown of a protein's structure.Because of the inflammation, it's well-documented. The term "denaturation" refers to a change in the structure of a protein.Bonding types include electrostatic, hydrogen, hydrophobic, and disulphide (Bagad, Umarkar, Tatiya&Surana, 2011). The effect of various doses of A.montanus leaf extract on albumin denaturation is depicted in Figure 1. (Table 2). In a dose-dependent way, the leaf extract inhibited albumin denaturation significantly.From the result, it was observed that *A.montanus* showed a significant activity with an IC_{50} value of (224.53µg/ml) when compared to the standard drug Diclofenac with an IC_{50} value of (183.1µg/ml). The result obtained is in accordance with (Fatma, Sokindra& Shah, 2013) who investigated the anti-oxidant and anti-inflammatory activities of ethanolic extract of *Moringaoleifera.*

Lipoxygenases (LOXs) are a group of non-heme iron-containing enzymes involved in the conversion of arachidonic, linoleic, and other polyunsaturated fatty acids into physiologically active metabolites that are known inflammatory and immune response mediators (Azila& Don, 2012). Lipoxygenase inhibitors are used to treat a variety of inflammatory illnesses and boost the immune system's response to viral and bacterial infections.
Lipoxygenase inhibition has been demonstrated in a variety of plant extracts.. The result of this study revealed that Inhibition of Lipoxygenase enzyme was concentration dependant. According to the result obtained from this study, *A.montanus* inhibit the action of lipoxygenase enzyme with an IC_{50} value (137.03µg/ml) when compared to the standard drug Diclofenac with an IC_{50} value (159.62µg/ml). This result is in concord with the findings of Mohammad et al, (2014) who reported the Lipoxygenase inhibitory activities *of Carallumaarabica*with an IC_{50}

values of 30.77µg/ml. The study verified that the ethanol extract and ethyl acetate fraction from *Carallumaarabica* showed appreciable anti-inflammatory via Lipoxygenase inhibitory activity with an IC_{50} value of 30.77µg/ml. The membrane stabilization assay is a frequently used technique for determining the anti-inflammatory properties of test chemicals and plant extracts. Membrane stabilization has been utilized as a technique in the past.. This method can be utilized to test anti-inflammatory activity in vitro since the erythrocyte membrane is similar to the lysosomal membrane. This present study uncovers that the standard drug diclofenac exhibited the highest erythrocyte membrane stabilization activity with IC_{50}value of 56.68 ± 0.0034% (Table 4).The result revealed that a significant dose-dependent inhibitory activity and the extract showed a significant Inhibitory activity when compared with the standard drug with an IC_{50} values of (250.2µg/ml and 195.44µg/ml). Fawole et al, (2010) reported that flavonoids are known to possess anti-inflammatory activity. The result obtained in this study is in agreement with earlier submissions of (Anosike, Obidoa&Ezeanyika,2012) who reported a significant membrane stabilizing activities of *Solanum aethiopicum* against heat induced lysis of Red Blood cells. Proteinases, such as trypsin, are necessary enzymes for the hydrolysis of proteins, Proteins have peptide bonds. The functions of these enzymes in inflammation, cardiovascular disease, tissue remodeling, matrix degradation, auto-immune arthritis, and skin allergies have all been widely researched. As a result, inhibiting these enzymes therapeutically will be a novel contribution. From the result, it was observed that inhibition was concentration dependent and the standard drug Diclofenac when compared with the extract demonstrated the highest Proteinase inhibitory activity with an IC_{50} values of (191.4 µg/ml and 198µg/ml).(Table 5). Several plants and natural products have been recorded to demonstrate considerable proteinase inhibition. This result is in correlation with Gulnaz, Wethroe&Jyoti, (2014) who conducted an experiment on the ethanolic extract of *Randiauliginosa* exhibited significant proteinse inhibition. Proteinase activity was significantly inhibited by the Ethanolic extract of *Randi auliginosa*, it exhibited significant anti-proteinase activity. It showed maximum inhibition of 66% at 600 g/ml and that of standard was found to be 73% at the same concentration.
Previous phytochemical studies on Acanthus montanus leaves extract indicated the existence of bioactive chemicals in large quantities.

Many alkaloids and flavonoids-containing plants have been shown to have diuretic, antispasmodic, anti-inflammatory, and analgesic properties (Mahato,Gangul&Sahu,1982).

The presence of all of these bioactive chemicals may have been responsible for A.montanus's strong anti-inflammatory activity.The in-vitro anti-inflammatory activity of *A.montanus* extract as it was observed in this study justified its use in ethno-medicine. This plant can be used as an alternative source of treatment of inflammatory diseases.

5.0 CONCLUSION

It could be concluded that the leaf extract contains phytochemicals such as alkaloid, steroids, flavonoid, tannin, glycosides and phenolics. The results of In-vitro anti-inflammatory tests also suggested that the aqueous leaf extract of *Acanthus montanus* possesses potent anti-inflammatory activities which could be attributed to the presence of the bioactive compounds.

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