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In-vitro evaluation of anti-inflammatory properties of ethanol extract of ten locally obtained plants in Uyo, Nigeria

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ABSTRACT

Background: Many diseases are associated with inflammatory processes. To prevent or manage these diseases, anti-inflammatory therapies are required. The current anti-inflammatory drugs often result in therapy failure or precipitate intolerable adverse effects; hence, there is a need for new and more effective anti-inflammatory agents. The anti-inflammatory activity of methanol extracts of ten locally obtained plants namely: *A. conyzoides* ; *A. indica*, *M. oleifera*, *A. vera*, *T. fruticosum*, *Z. officinale*, *O. gratissimum*, *V. amygdalina*, *C. papaya*, and *A. sativum*, were studied.

Methods: The *in-vitro* anti-inflammatory activity of the plants' extracts was studied using albumin denaturation assay and membrane stabilization assay against aspirin and diclofenac sodium as standard drugs.

Results: The results showed a dose dependent increase in anti-inflammatory activity in both models. In protein denaturation inhibition, aspirin, showed maximum inhibition of 67% at a concentration of 100 µg/mL, while the extracts' maximum inhibition was observed at a concentration of 400 µg/ml, with *A. indica*, and *M. oleifera having the highest activity at* 61% and 59% respectively. For heat induced haemolysis, the maximum inhibition was observed at the concentration of 400 µg/ml, with *A. indica*, *M. oleifera* having the highest inhibition at 61% and 64% compared to aspirin, which showed 70% protection. For hypotonicity induced haemolysis, the maximum inhibition was observed at the concentration of 400 µg/ml, with *A. indica* and; *M. oleifera*, having 70% respectively compared to diclofenac sodium, which showed 53% protection at 100 µg/mL.

Conclusion: These results suggest that these plants are potential sources of leads for the development of better and more effective anti-inflammatory agents.

Keywords: *Allium sativum, Azadirachta indica,* Anti-inflammatory activity, Protein denaturation inhibition, hypotonicity, haemolysis

1.0 INTRODUCTION

The process of inflammation includes the reaction of the tissues of the body to trauma, toxins, heat, microbes, and other elements that can cause injury to body tissues and it is a complex set of interactions among soluble factors and cells that can arise in any tissue in response to traumatic, infectious, post-ischaemic, toxic, or autoimmune injury [1]. Inflammatory response involves several phases which include local activation of inflammatory factors when tissue damage occurs, breakdown of blood vessel barriers, recruitment of circulating inflammatory cells, and activation of tissue repairs to restore tissue function [2]. Inflammation is an adaptive response and is usually for the benefit of the body. A successful inflammatory response results in the elimination of the offending agents, followed by repairs of the affected tissues, which is mediated by recruited and tissue macrophages [3]. However, when there are genetic deficiencies or disease conditions that can cause a malfunction in the inflammatory processes, the inflammatory response may cause more damage than the offending pathogen [1]. Inflammation plays a critical role in the development of many complex diseases and disorders, including cardiovascular diseases, autoimmune diseases, neurodegenerative diseases, metabolic syndromes, and cancers [4]. The vascular and cellular reactions of inflammation are triggered by soluble factors that are produced by various cells or derived from plasma proteins and

are generated or activated in response to the inflammatory stimulus [5]. According to Kumar *et al.* [5] the four cardinal signs of inflammation are rubor (redness), tumor (swelling), calor (heat), and dolor (pain). Pain is one of the major presentations of inflammation, and it is crucial that it is appropriately treated or managed [6]. Pain is managed with analgesics, and non-steroidal anti-inflammatory drugs (NSAIDs) are a key component of the pharmacological management of both acute and chronic pain [7]. Anti-inflammatory drugs exert their analgesic activities by inhibiting cyclooxygenase (COX) isozymes, interfering in the conversion of arachidonic acid to prostaglandins E2, prostacyclins, and thromboxanes [7]. Anti-inflammatory drugs can be obtained from numerous sources, but nature remains the best source [8]. Today, there is a strong demand by the public for less expensive preventive or curative anti-inflammatory treatments from natural sources [9]. The study and discovery of natural products with anti-inflammatory properties will expand the available options of anti-inflammatory agents, which can be used to prevent and manage pains and various chronic inflammatory conditions. Natural source offers a means of providing healthcare in both developed and developing countries of the world [10]. In recent years, there has been an increase in the effort to survey plants as a source of new drugs, and this has resulted in the discovery of many new bioactive compounds from plants [11]. Plants, terrestrial microorganisms such as fungi and bacteria, and marine animals have produced the majority of natural compounds utilized for medical purposes due to their anti-inflammatory action [12]. Some natural products employed as anti-inflammatory agents include alpha-lipoic acid, curcumin, fish oil containing omega-3 fatty acid, etc. This study was designed to investigate the phytochemical constituents and the anti-inflammatory activities of ten (10) randomly selected but commonly used plants in Uyo, the capital of Akwa Ibom State, Nigeria. These plants are commonly found growing luxuriantly without any pastoral care and as such abundantly available in this locality. They are majorly deployed as medicinal agents in many conditions but rarely as anti-inflammatory agents.

2.0 MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipement and Apparatus

The followings were the equipment and apparatus used for the work: Water bath (Selecta), Centrifuge (800-1 Centifugal machine, Vokus Madi Ventures, China), weighing balance (S. Mettler), UV-visible spectrophotometer (LaboMed Inc.), test tubes, beakers, stirring rods, spatula, volumetric flask, measuring cylinder, maceration tanks, and glass funnel.

2.1.2 Solvents, reagents, and chemicals
Ethanol, bovine serum albumin, 1N HCl, fresh whole human blood, normal saline, phosphate buffer saline, Alsever's solution, dextrose, sodium citrate, citric acid, sodium chloride, hypo saline, distilled water, Acetic acid, Acetic anhydride, Concentrated sulphuric acid, Ferric chloride, Acetone, Mayer's reagent, Dragendoff's reagent, sodium hydroxide, chloroform, and magnesium metal.

2.1.3 Standard anti-inflammatory drugs
Aspirin (Anacin), Diclofenac sodium (From Drugfield Pharmaceutical Limited)

2.2 Methods

2.2.1 Plant Collection and Identification
The plants were collected fresh from farmlands in Ibup Street in Uyo LGA of Akwa Ibom state on 21st February 2021. Taxonomical identification and authentication were carried out by the Department of Botany, Faculty of Science, University of Uyo, and taxonomic serial numbers were assigned to the individual plants.

2.2.2 Extraction
The collected plants were washed, sliced into pieces, and shade dried. On drying, they were pulverized with a mortar and pestle. The powders were weighed (50 g each) into extraction tanks and ethanol (200mL, 70%) was added until they were completely submerged. After four days, the mixtures were filtered and the filtrates were concentrated in a water bath at 50ºC to obtain the extract. The extracts were then appropriately labelled.

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2.2.3 Phytochemical Screening of the Extracts

The plant extracts were subjected to phytochemical analysis using the methods of Sofowora, (1993) [13], and Trease and Evans (1996) [14].

2.2.4 Determination of in vitro Anti-inflammatory Properties

2.2.4.1 Inhibition of albumin denaturation [15]

1% aqueous solution of bovine albumin fraction was prepared by weighing Bovine serum albumin (1g) into a little quantity of distilled in a water beaker and the mixture was transferred into a 100 mL volumetric flask. Distilled water was then added to the mark. 0.5 ml of different concentrations of the extract (100 µg, 200 µg, 300 µg, and 400 µg) was placed in separate test tubes. 2.5 ml of 1% aqueous solution of bovine albumin fraction was added to the test tubes, followed by 0.3 ml of 1N HCl to adjust the pH of the reaction mixture. The reaction mixtures were incubated in a water bath at 37ºC for 20 minutes and then heated to 51ºC for 20 minutes. The mixtures were cooled under a running tap, and the turbidity of the mixtures was measured using a UV-Visible spectrophotometer at 660nm. In the control, 1 ml of distilled water was added in place of the sample extract. Aspirin (Acetylsalicylic acid, 100 µg/ml) was used as the reference drug. This was repeated for all the samples. The experiment was performed in triplicate. The percentage inhibition of protein denaturation was calculated with the formula:

Percentage inhibition = $(Abs_{control} - Abs_{sample}) / Abs_{control} \times 100$

Where $Abs_{control}$ is the absorbance of control not containing the extract or drug, and Abs_{sample} is the absorbance of the test samples.

2.2.4.2 Inhibition of heat induced haemolysis [15]

Preparation of 10% red blood cell suspension: 10 mL of fresh whole blood was obtained from healthy human volunteers who had not taken NSAIDs for two weeks prior to the experiment into EDTA bottles. The blood was transferred into a test tube and centrifuged at 3000 rpm for 10 minutes. The plasma layer was separated from packed red blood cells layer. The packed red blood cells layer was washed thrice with an equal volume of normal saline (0.9% w/v) by introducing the saline into it, shaking to mix, centrifuging at 1500 rpm for 2 minutes, and collecting the packed red blood cells layer. Thereafter, 5 mL of the blood was measured into a 50 mL volumetric flask and normal saline was added to the 50 mL mark, making a 10% v/v suspension.

Determination of inhibition of heat induced haemolysis: The reaction mixtures contained 2 mL of different concentrations (100 µg, 200 µg, 300 µg, and 400 µg) of each of the plant sample extract and 2 mL of the 10% v/v red blood cells suspension in a test tube. The test tubes were incubated in a water bath at 56ºC for 30 minutes. Afterwards, the test tubes were cooled under a running tap and then centrifuged at 2500 rpm for 5 minutes. The absorbance of the supernatant of each test tube was determined at 560nm. In the control, 2 mL of normal saline was added in place of the sample extract. Acetyl salicylic acid (100 µg/ml) was used as the reference drug. The experiment was performed in triplicate.
The percentage inhibition of protein denaturation was calculated with the formula:

Percentage inhibition = $(Abs_{control} - Abs_{sample}) / Abs_{control} \times 100$

Where $Abs_{control}$ is the absorbance of control not containing the extract or drug, and Abs_{sample} is the absorbance of the test samples.

2.2.4.3 Determination of hypotonicity induced haemolysis [16, 17]

Preparation of red blood cells suspension: Blood was collected—into an EDTA bottle—from a healthy human volunteer who had not taken any NSAIDS for the previous two weeks before the experiment. The blood was transferred into two test tubes, 5 mL each, and then mixed with an equal volume of Alsever's solution (2% dextrose, 0.7% sodium citrate, 0.5% citric acid and 0.4% sodium chloride in water). The mixture was centrifuged at 3000 rpm for 10 minutes, and the plasma layer was separated from the packed red blood cells layer. The packed red blood cells layer was washed thrice with an equal volume of isosaline saline (0.85% w/v) by introducing the saline into it, shaking to mix, centrifuging at 1500 rpm for 2 minutes, and collecting the packed red blood cells layer. Thereafter, 5 mL of the packed cells was measured into a 50 mL volumetric flask and isosaline was added to the 50 mL mark, making a 10% v/v suspension.

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Determination of inhibition hypotonicity induced haemolysis: The prepared assay mixture containing 1 mL phosphate buffer (0.15M), 2 mL of hypo saline (0.36% w/v), 0.5 ml of the 10% v/v red blood cells suspension and 1mL of various concentrations of each plant extract (100µg/mL, 200µg/mL, 300ug/mL, and 400µg/mL) was incubated at 37ºC for 30 min and centrifuged. Thereafter, absorbances of the supernatant solutions obtained were read at 560 nm. In the control, 2 mL of distilled water was added in place of the extract. Diclofenac sodium (100 µg/mL) was used as the reference drug. The experiment was performed in triplicate.

The percentage inhibition of protein denaturation was calculated with the formula:

Percentage inhibition = $(Abs_{control} - Abs_{sample})/Abs_{control} \times 100$

Where $Abs_{control}$ is the absorbance of control not containing the extract or drug, and Abs_{sample} is the absorbance of the test samples.

2.3 Statistical Analysis

Results are expressed using multiple comparisons of Mean ± SEM by one-way analysis of variance (ANOVA), followed by the *Tukey-Kramer* multiple comparisons test. Results were considered statistically significant at $p<.05$

3.0 RESULTS

3.1 Phytochemical Screening

Table 1: Phytochemical Screening of the extracts of the plants

Plants	Alkaloids	Saponins	Tannins	Flavonoids	Phenols	Terpenoids
Allium sativum	+	+	+	+	+	+
Aloe vera	+	+	+	+	+	+
Ageratum conyzoides	+	+	+	+	+	+
Azadirachta indica	+	+	+	+	+	+
Carica papaya	+	+	+	+	+	+
Moringa oleifera	+	+	+	+	+	+
Ocimum gratissimum	+	+	+	+	+	+
Talinum fruticosum	+	+	+	+	+	+
Vernonia amygdalina	+	+	+	+	+	+
Zingiber officinale	+	+	+	+	+	+

3.2 Evaluation of anti-inflammatory activity

Table 2: Percentage inhibition of ethanol extract of the plants on albumin denaturation

Conc. (µg/mL)	*A. conyzoides*	*A. indica*	*M. oleifera*	A. *vera*	*T. fruticosum*	*Z. officinale*	*O. gratissimum*	*V. amygdalina*	*C. papaya*	*A. sativum*	*Aspirin*
0	0	0	0	0	0	0	0	0	0	0	
100	18±0.01	37±0.29	31±0.11	33±0.23	28±0.91	36±0.01	12±0.08	3±0.11	9±1.11	6±0.01	67±0.00
200	23±0.03	44±0.12	37±0.13	36±0.45	39±0.29	41±0.03	27±0.02	7±0.02	23±0.09	11±0.09	67±0.00
300	36±0.09	46±0.10	43±0.03	41±0.02	43±0.55	45±0.03	35±0.02	15±0.02	25±0.79	24±0.05	67±0.00
400	54±0.01	61±0.33	59±0.09	48±0.22	45±0.25	49±0.07	45±0.09	30±0.12	29±0.88	36±0.11	67±0.00

Data are given as Mean±SEM ($n=3$; $p<0.05$)

Table 3: Percentage inhibition of ethanol extract of the plants on heat induced haemolysis of erythrocyte

Conc. (µg/mL)	*A. conyzoides*	*A. indica*	*M. oleifera*	A. *vera*	*T. fruticosum*	*Z. officinale*	*O. gratissimum*	*V. amygdalina*	*C. papaya*	*A. sativum*	*Aspirin*
0.	0	0	0	0	0	0	0	0	0	0	0
100.	51±0.02	20±0.03	56±0.11	10±0.21	55±1.01	54±0.41	10±0.01	10±0.31	5±0.05	11±0.05	70±0.02
200.	55±0.03	54±0.02	58±0.21	54±0.31	56±1.01	55±0.61	20±0.11	23±0.11	11±0.06	19±0.32	70±0.00
300.	58±0.01	60±0.02	63±0.41	55±0.01	58±0.08	60±0.32	41±0.02	24±0.31	25±0.01	40±0.09	70±0.01
400.	58±0.02	61±0.01	64±0.11	55±0.31	58±0.05	61±0.62	55±0.02	43±0.04	46±0.62	48±0.04	70±0.02

Data are given as Mean±SEM ($n=3$; $p<0.05$)

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Table 4: Percentage inhibition of ethanol extract of the plants on hypotonicity induced haemolysis of erythrocyte

Concentration (µg/mL)	*A. conyzoides*	*A. indica*	*M. oleifera*	A. *vera*	*T. fruticosum*	*Z. officinale*	*O. gratissimum*	*V. amygdalina*	*C. papaya*	*A. sativum*	*Diclofenac*
0	0	0	0	0	0	0	0	0	0	0	0
100	24±1.20	31±2.20	22±1.30	35±0.20	19±2.90	11±0.20	8±0.20	34±5.90	22±0.60	8±0.2	53±0.20
200	43±1.0	45±0.20	59±1.40	37±0.60	24±3.10	13±1.60	17±2.50	43±0.20	26±2.20	16±1.9	53±1.00
300	52±1.1	64±0.50	66±1.30	46±0.80	30±1.50	19±0.70	31±0.40	44±1.90	49±1.30	31±1.4	53±1.20
400	59±0.9	70±2.20	70±1.40	55±03.20	37±2.20	23±2.20	51±1.20	46±2.00	52±3.20	51±2.2	53±1.00

Data are given as Mean±SEM (n=3; $p<0.05$)

4.0 DISCUSSION

The result of the phytochemical screening of the plants extracts showed the strong presence of bioactive/polyphenolic compounds such as alkaloids, flavonoids, tannins, steroids, and phenols (Table 1), which may be responsible for the observed anti-inflammatory activity of the plant extracts.

Protein denaturation is a process in which proteins lose their tertiary and secondary structures as a result of exposure to an external stress or substance, such as a strong acid or base, a concentrated inorganic salt, an organic solvent, or heat) [15]. Protein denaturation is a well-known cause of inflammation and rheumatoid arthritis [18]. The ability of plant extract to reduce protein denaturation was investigated as part of the research into the mechanism of anti-inflammatory effect [15].

In this research work, aspirin, a standard anti-inflammatory drug, showed maximum inhibition of 67% at a concentration of 100 µg. For the extracts, the maximum inhibition was observed at a concentration of 400µg/ml, and they are as follows: *A. conyzoides*, 54%; *A. indica*, 61%; *M. oleifera*, 59; *A. vera*, 48%; *T. fruticosum*, 45%; *Z. officinale*, 49%; *O. gratissimum*, 45%; *V. amygdalina*, 30%; *C. papaya*, 29%; and *A. sativum*, 36% (Table 2).

In the study of membrane stabilization activity, the ability of the plant extract to inhibit the denaturation of the membrane of erythrocytes was determined. Numerous studies show that lysosomes influence glucocorticoid signalling and the inflammatory response; these studies suggest that lysosomal activity is negatively linked with glucocorticoid anti-inflammatory effects [19]. The membrane of an erythrocyte is similar to that of a lysosome [16], and the ability of an agent to promote its stability implies that the agent may also be able to stabilize the lysosomal membrane [15].

The studied extract exhibited a membrane stabilization effect by inhibiting heat and hypotonicity induced haemolysis of the erythrocyte membrane. For heat induced haemolysis, the maximum inhibition was observed at the concentration of 400µg/ml, and they are as follows: *A. conyzoides*, 58%; *A. indica*, 61%; *M. oleifera*, 64; *A. vera*, 55%; *T. fruticosum*, 58%; *Z. officinale*, 61%; *O. gratissimum*, 55%; *V. amygdalina*, 43%; *C. papaya*, 46%; and *A. sativum*, 48%. All the results were compared with a standard drug, aspirin, which showed 70% protection (Table 3). For hypotonicity induced haemolysis, the maximum inhibition was observed at the concentration of 400µg/ml, and they are as follows: *A. conyzoides*, 59%; *A. indica*, 70%; *M. oleifera*, 70; *A. vera*, 55%; *T. fruticosum*, 37%; *Z. officinale*, 23%; *O. gratissimum*, 51%; *V. amygdalina*, 46%; *C. papaya*, 52%; and *A. sativum*, 51%. All the results were compared with a standard drug, diclofenac sodium, which showed 53% protection at 100µg/ml (Table 4).

5.0 Conclusion

The results indicate that the ethanol extracts of these local plants possess anti-inflammatory properties which may be due to the strong occurrence of bioactive/polyphenolic compounds such as alkaloids, flavonoids, tannins, steroids, and phenols in the extracts. The extracts serve as free radical inhibitors or scavengers or acting possibly as primary oxidants inhibited the heat induced albumin denaturation, proteinase activity and stabilized the red blood cells membrane. The extracts also reduced the activity of lipoxygenase. Purification of these bioactive compounds is necessary and may lead to the discovery of 'lead' compounds with better activity compared to already existing drugs in the market which can be used for designing potent anti-inflammatory drugs which for the treatment of various diseases such as cancer, neurological disorder, aging and inflammation. This study further brings to the fore the fact that these plants which are abundantly found in this locality have anti-inflammatory potentials.

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Significance of the Research
From the experiment, it is observed that all the samples analyzed possessed some level of anti-inflammatory activity, and the extent of inhibition of inflammation increased with an increase in concentration. This research has highlighted the anti-inflammatory potentials of these locally obtained plants which they were not identified with before this research work was undertaken. This is useful in orthodox medicine as it can serve as a source of potential Leads in the synthesis of new, more effective anti-inflammatory agents. Also, this is useful in herbal medicine as it could proffer remedies for some inflammatory conditions.

Authors' Contributions
Conceptualization, ECJ. Methodology, IVJ; Software, ECJ; Formal analysis, IVJ and ECJ. Investigation, ECJ and IVJ; Resources, ECJ and IVJ; Writing—original draft preparation, IVJ; writing—review and editing, ECJ; Supervision, ECJ; Project administration, ECJ.

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Conflicts of Interest: The authors declare no conflict of interest.

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