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ANTI-INFLAMMATORY EFFECTS OF AQUEOUS EXTRACT OF *AZADIRACHTA INDICA* A. JUSS (MELICEAE) ON HISTAMINE AND EGG ALBUMIN-INDUCED PAW EDEMA IN WISTAR RATS

MAHDI, M.A.* AND GAIUS, G.
Department of Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences, Gombe State University, Gombe, Nigeria

ABSTRACT

Inflammation is a response to infections, injury, stress or trauma. Several medicinal plants were reported to be useful in the management of inflammation in traditional medicine. This study was aimed at investigating the effect of aqueous extract of *Azadirachta indica* on two models of acute inflammation; Histamine-induced inflammation and fresh egg albumin-induced inflammation in rats. Five (5) groups of rats containing five (5) animals per group were used for the study. Rats in group 1 were administered 1 mL/kg normal saline intraperitoneally that served as control for comparison with the test group. Groups 2, 3 and 4 rats were administered with aqueous extract of *Azadirachta indica* at a dose of 500, 1000 and 1500 mg/kg respectively while group 5 was administered diclofenac 20 mg/kg all of which were easily administered via intraperitoneal route. In the egg-albumin induced inflammation study, five (5) groups of five (5) rats each were administered different doses of drugs. Group 1 received 1 mL/kg normal saline, while groups 2-4 were given 20, 40 and 60 mg/kg of extract respectively. The fifth group received 20 mg/kg diclofenac. All drugs for this study were administered intraperitoneally. Data was analyzed using one-way analysis of variance ANOVA and presented as Mean ± Standard Deviation. The median lethal dose (LD_{50}) was found to be greater than 5000 mg/kg. The histamine-induced rat paw edema significantly decreased at 3 hours in groups 3-4 given 20, 40 and 60 mg/kg of extract $p < 0.01$, $p < 0.001$, $p < 0.001$) respectively compared to normal saline control group. Both the aqueous extract of *Azadirachta indica* at 20, 40 and 60 mg/kg and diclofenac at 20 mg/kg were not able to significantly alter rat paw size. Therefore, we conclude that aqueous extract of *Azadirachta indica* produce dose dependent anti-inflammatory effect in histamine-induced paw edema model of Wistar rats.

Keywords: *Azadirachta indica*, Acute inflammation, edema, extract, diclofenac, paw size
***Correspondence:** mahdi.mohammed30@yahoo.com; 2348103617554, 2349014208402

INTRODUCTION

Inflammation is part of the body's defense mechanism. It is the process by which the immune system recognizes and removes harmful and foreign stimuli and begins the healing process [1]. "Inflammation, a basic way in which the body reacts to infections, irritation or other injury, the key feature being redness, warmth, swelling and pain. Inflammation is now recognized as a type of nonspecific immune response." [2]. Despite available information about the signaling pathways that link energy accumulation [adiposity] to chronic inflammation, little is known about the real biological significance of inflammation [3].

Given that inflammation is a response triggered by damage to living tissues, the response is a defense mechanism that evolved in higher organisms to protect them from infections and injury. Its purpose is to localize and eliminate the injurious agent and to remove damaged tissue components so that the body can begin to heal. The response consists of changes in blood flow, an increase in permeability of blood vessels, and the migration of fluid, proteins, and white blood cells (leukocytes) from the circulation to the site of tissue damage. An inflammatory response that lasts only a few days is called acute inflammation, while a response of longer duration is referred to as chronic inflammation [3].

Inflammation is an imperative and fundamental response of the immune system against any harmful stimuli, microbial invasion, infected cells, toxic substances and injury, and acts by removal of injurious stimulus thereby initiating the healing process. Basically, it is the defense system of the body. When inflammation goes significant, it develops serious complications including neurodegenerative disorders, autoimmune diseases, neoplastic diseases, and cardiac complications. Initially, inflammation commences as an acute condition and if failed to regulate the pro-inflammatory stimulus for prolonged period, it gets transformed into chronic form. Acute inflammation is regulated and chronic inflammation is deregulated form. But it's a challenge to differentiate between acute and chronic inflammation based on the molecular

pharmacological basis, [4]. As a fundamental biological process which was regarded as the most frequent sign of disease, inflammation is an expression of the body's local and protective response against microbial invasion, entrance of antigen and damage of its cells and tissues. Successful and controlled inflammatory response is a useful process, which leads to the clearance of injurious stimuli and restore normal physiology. Resolution of inflammation and diffusion of inflammatory stimulus is controlled by a number of soluble mediators [5].

Although acute inflammation is usually beneficial, it often causes unpleasant sensations, such as the pain of a sore throat or the itching of an insect bite. Discomfort is usually temporary and disappears when the inflammatory response has done its job. But in some instances, inflammation can cause harm. Tissue destruction can occur when the regulatory mechanisms of the inflammatory response are defective or the ability to clear damaged tissue and foreign substances is impaired. In other cases, an inappropriate immune response may give rise to a prolonged and damaging inflammatory response. Examples include allergic, or hypersensitivity reactions, in which an environmental agent such as pollen, which normally poses no threat to the individual, stimulates inflammation, and autoimmune reactions, in which chronic inflammation is triggered by the body's immune response against its own tissues [6].

The responses may be frightful in Rheumatoid Arthritis, Crohn's disease, inflammatory bowel disease and metabolic disorders. Recently, management of Arthritis was a major challenge for the clinicians, the reason being delayed recognition, lack of knowledge about the physiological and molecular aspects arthritis. There are a limited number of drugs available. For example, Nonsteroidal anti-inflammatory drugs (NSAIDs), which gives symptomatic relief from pain and inflammation but also show some side-effects, and lack propensity for targeted therapy. In the past few years, advances of new drugs like Interleukin (IL-6) inhibitor, JAK inhibitor, anti TNF and currently available disease modifying anti rheumatic drugs (DMARDs) have provided relief in this context. Molecular Pharmacological understanding and efforts towards the signal transduction pathways of anti-inflammatory targets and their expression pattern is the major pathway to have an insight which serves as a tool for designing oral drug therapy pertaining to the enhancement of clinical use of natural and synthetic medicines [7].

The factors that can stimulate inflammation include microorganisms, physical agents, chemicals, inappropriate immunological responses, and tissue death. Infectious agents such as viruses and bacteria are some of the most common stimuli of inflammation. Viruses give rise to inflammation by entering and destroying cells of the body; bacteria release substances called endotoxins that can initiate inflammation. Physical trauma, burns, radiation, injury, and frostbite can damage tissues and also bring about inflammation, as can corrosive chemicals such as acids, alkalis, and oxidizing agents. As mentioned above, malfunctioning immunological responses can incite an inappropriate and damaging inflammatory response. Inflammation can also result when tissues die from a lack of oxygen or nutrients, a situation that often is caused by loss of blood flow to the area. [5, 7].

The four cardinal signs of inflammation: redness (Latin *rubor*), heat (*calor*), swelling (*tumor*), and pain (*dolor*) were described in the 1st century AD by the Roman medical writer Aulus Cornelius Celsus. Redness is caused by the dilation of small blood vessels in the area of injury. A heat result from increased blood flow through the area and is experienced only in peripheral parts of the body such as the skin. Fever is brought about by chemical mediators of inflammation and contributes to the rise in temperature at the injury. Swelling, called edema, is caused primarily by the accumulation of fluid outside the blood vessels. The pain associated with inflammation results in part from the distortion of tissues caused by edema, and it also is induced by certain chemical mediators of inflammation, such as bradykinin, serotonin, and the prostaglandins [1].

According to the National Institute of Environmental Sciences, inflammation and pain are associated to virtually all diseases which are caused by infections or injury – they are the body's mechanism for healing. Such diseases include autoimmune diseases like rheumatoid arthritis, cardiovascular diseases such as high blood pressure and heart diseases. Gastrointestinal disorders for instance inflammatory bowel disease, ulcerative colitis or Crohn's disease. Others are lung diseases like chronic obstructive pulmonary diseases (COPD), type 2 diabetes, and cancer. The anti-inflammatory activity of *Azadirachta indica* has been earlier reported by Alzohairy and other workers [8]. The aim of this study was to evaluate the anti-inflammatory effect of the aqueous stem bark extract of *Azadirachta indica* in Wister rats.

MATERIALS

Drugs and chemicals

The following drugs/chemicals were obtained and used for the studies:

Fresh egg albumin, Histamine – analytical grade, BDH Poole, Diclofenac potassium 75 mg (Jawa Pharmaceutical); Batch number 201108; NAFDAC numberA4-8761; Expiry date 11/2023.

Animals

Thirty-seven (37) adult Wister of both sexes weighing 20-35 g were obtained from Animal House Facility, University of Jos, Jos-Nigeria. The animals were maintained on standard diet and water *ad libitum*. They were housed in propylene cages at room temperature throughout the period of the study. All animals exhibiting ill-health were excluded from the study.

Plant materials

Stem bark extract of *Azadirachta indica* were collected from Zambuk town of Yamaltu/Deba LGA Gombe State in the month of November, 2022. The plant material was deposited at Herbarium unit of the Department of Biological Sciences, Faculty of Science, Gombe State University for identification.

METHODS

Preparation of plant material

The dried plant materials were reduced into powdered form and 2.7 kg of the powder macerated in 1 L of distilled water at ambient temperature for a period of seven (7) days with intermittent agitation. The extract was then filtered with the aid of Whatsman No. 1 filter paper. The filtrate was then evaporated to dryness to a dark brown residue which was referred to as crude aqueous stem bark extract of *Azadirachta indica* and kept in a desiccator until required for use.

Drug preparations and administration

Acute toxicity study: Stock concentrations of 100 mg/mL was prepared, from which serial dilutions were made to obtain concentrations of aqueous stem bark extract of *Azadirachta indica* at 10 mg/mL and 1 mg/mL for oral administration to groups 1, 2 and 3 of phase I respectively. Similarly, for phase II stock concentrations of 500 mg/mL, was diluted serially to 160 mg/mL, 290 mg/mL, in distilled water which were given orally to groups I-III mice respectively.

Anti-inflammatory study: A stock concentration of 200 mg/mL of the extract was prepared and serial dilutions were done to achieve working concentrations of 50 mg/mL, 100 mg/mL and 150 mg/mL, for graded dosing to groups 2-4 mice in the histamine-induced anti-inflammatory study. However, 10 mg/mL, stock of *Azadirachta indica* was prepared for fresh egg albumin-induced inflammatory study. Working concentrations were 2, 4, and 6 mg/mL, respectively. The route of administration for this model was intraperitoneal.

Standard drugs: The standard drug used in both models of inflammation was 75 mg Diclofenac potassium manufactured by Jawa Pharmaceuticals Limited. A stock of 1 mg/mL was prepared and a working concentration of 0.2 mg/mL was administered orally to each mouse according to body weight

Acute toxicity study

Lorke's [9] method was used for the determination of oral median lethal dose (LD_{50}) of aqueous stem bark extract of *A. Indica* in mice. Thirteen (13) Wister rats were used in the study. The test involved two stages. In the first Phase, nine (9) rats were divided into three (3) groups of three (3) rats each and were administered with aqueous stem bark extract of *Azadirachta indica* at doses of 10 mg/kg, 100 mg/kg and 1000 mg/kg body weight orally respectively. The rats were monitored for signs and symptoms of toxicity such as sniffing, restlessness, hyperactivity, leaking of paws, defecation, stretching and protrusion of the eye balls for the first 4 h and for mortality after 24 hours. In the second Phase, three (3) fresh rats were divided into three (3) groups of one (1) rat each and were administered with extract at doses of x_1, x_2, and x_3 mg/kg respectively, based on result of the first phase. The rats were also observed for signs and symptoms of toxicity for the first 4 hour and death over a period of 24 hour. The LD_{50} value was calculated as geometric mean of the lowest lethal dose and highest non-lethal dose for which the animal survived. LD_{50} was calculated as thus;

$LD_{50} = \sqrt{(D_0 \times D_{100})}$

D_0 = highest dose that did not produce mortality

D_{100} = lowest dose that produced mortality [10].

Anti-inflammatory study

Histamine-induced inflammation model [11]: The following procedure was adopted: All rats were weighed and their paw sizes measured and recorded (baseline data). They were grouped and administered the following according to body weight: Normal saline 1 mL/kg to group 1 rats. Extract at graded doses of 500 mg/kg, 1000 mg/kg, 1500 mg/kg to groups 2-4 rats respectively and Diclofenac 20 mg/kg to group 5 rats. After 1 hour, 1% histamine i.e. 0.1 mL was administered to all groups of rats. The paw sizes were measured at 0 hr before histamine was administered, then observe and measure paw size at 1, 2, 3 and 4 hours respectively.

Egg albumin-induced inflammation model in rats: The method of Barung [12] was adopted in which fresh egg albumin was extracted from locally purchased egg. The mice were grouped into five (5) groups. Group 1 received 1 mL/kg normal saline. The extract was administered at graded doses of 1000 mg/kg, 1500 mg/kg and 5000 mg/kg to groups 2-4 rats respectively. Diclofenac 20 mg/kg was administered to group five (5) rats. Following administration of the extract, after 30 minutes, paw oedema was induced by administration injection of 1 mL fresh egg albumin via the hind paw. Paw sizes were measured at 0, 1, 2, 3 and 4 hours respectively using venire caliper.

Statistical analysis

Data obtained were expressed as mean ± standard deviation (SD). Levels of statistical significance between groups were analyzed using statistical computer software - One Way Analysis of Variance calculator for Social Science. P values < 0'05

RESULTS

Percentage yield of aqueous extract of *Azadirachta indica*

The maceration of 2.7 kg stem bark extract of *Azadirachta indica* resulted in an oily dark brown colored extract residue weighing 16.67 g which is equivalent to 0.62% $^{w}/_{w}$ yield.

Median lethal dose (LD_{50}) of aqueous stem bark extract of *Azadirachta indica*

The oral median lethal dose (LD_{50}) of the aqueous extract of *Azadirachta indica* in Wistar rats was found to be > 5000 mg/kg hence classified as practically non-toxic.

Histamine-induced inflammation model in rats

The result indicated a statistically significant decrease ($p < 0.01$, $p < 0.001$) of paw size at 3 hours after administration of 500 and 1000 mg/kg aqueous extract of *Azadirachta indica* compared to normal saline group. The effect of the extract at 1000 mg/kg was similar to that of standard group given 20 mg/kg Diclofenac

Fresh egg albumin-induced model of inflammation in rats

The result indicated that both the extract at graded doses of 20, 40 and 60 mg/kg were not capable of producing significant change ($p < 0.01$) in paw size from 0 through 4 hours after induction of inflammation with 0.1% fresh egg albumin.

DISCUSSION

The plant *Azadiracta indica* (Neem) was known to have brought ''good health'' to those who administer them [13, 14]. Therefore, it is a relevant material for screening of anti-inflammatory activity. Considering safety, our result of the acute toxicity study with the aqueous stem bark extract of *Azadirachta indica* indicated that the median lethal dose (LD_{50}) was practically non-toxic for oral administration in rats. In a similar study with the aqueous leaf extract of *Azadirachta indica* given intraperitoneally, the LD_{50} was 4800 mg/kg [15]. Thus, aqueous stem bark and leaf of *Azadirachta indica* may be safe for research and medicinal use. The aqueous extract of *Azadirachta indica* significantly and dose-dependently decreased the paw size of rats after histamine-induced paw edema. Histamine is not the only mediator of acute inflammation and immediate hypersensitivity responses. It is as well involved in chronic inflammation and other events in immune responses. Histamine stimulates macrophages, dendritic cells, T lymphocytes, B lymphocytes and endothelial cells. It regulates antigen-specific Th1 and Th2 cells, and related antibody isotype responses [16]. By inhibition of cyclooxygenase peripherally, the extract might have stopped the production of proinflammatory substances prostaglandins, thromboxanes and leukotriene. Alternatively, the aqueous stem bark extract of *Azadirachta indica* may act centrally by inhibiting pain nociception similar to opioid analgesics such as morphine or could have a histamine antagonist action. Egg albumin is an inducer of acute inflammation and has the following constituents: ovalbumin (54%), ovotransferrin (12%), ovomucoid (11%), ovomucin (3.5%), and lysozyme (3.5%) [17]. These proteins are the major allergens constituents of egg white of albumin which triggers inflammation. Anurachalam [18] and other workers [19] used fresh egg white to induce rat paw inflammation. In addition, maximum anti-inflammatory effect was seen at 3 hours after challenge with fresh egg albumin in rats [20] administered polyherbal formula. In our study, we observed that the aqueous extract of *Azadirachta indica* had no effect on fresh egg albumin-induced paw edema. It implies that the aqueous extract of *Azadirachta indica* have no effect on fresh egg albumin-induced inflammation in rats. However, it significantly reduced histamine-induced paw size at doses of 500 and 1000 mg/kg in rats at 3 hours of challenge.

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Table 1: Anti-inflammatory effects of *Azadirachta indica* in histamine-induced paw edema of Wister rats

Group	Treatment (mg/kg)	0 hour	1 hour	2 hours	3 hours	4 hours
1	Normal saline	0.24 ± 0.232	0.37 ± 0.189	0.38 ± 0.241	0.40 ± 0.15	0.33 ± 0.241
2	AI 500	0.24 ± 0.232	0.36 ± 0.232	0.35 ± 0.150	0.30 ± 0.15*	0.27 ± 0.189
3	AI 1000	0.22 ± 0.189	0.34 ± 0.177	0.37 ± 0.189	0.28 ± 0.241**	0.27 ± 0.189
4	AI 1500	0.30 ± 1.910	0.36 ± 0.276	0.41 ± 0.276	0.34 ± 0.095	0.28 ± 0.116
5	Diclofenac 20	0.22 ± 0.189	0.40 ± 0.000	0.34 ± 0.177	0.28 ± 0.241**	0.284 ± 0.209
		p = 0.060066	p = 0.088593	p = 0.093132	p = 0.00122	p = 0.236807

Data are presented as Mean ± Standard deviation and analyzed using one-way ANOVA followed by Turkey HSD post hoc test; *p* values less than 0.01 are considered statistically significant; * = p < 0.01, ** = p < 0.001; AI = *Azadirachta indica*

Table 2: Effects of aqueous extract of *Azadirachta indica* on egg albumin-induced paw edema of Wister rats

Group	Treatment	Time of Measurement of Paw Size				
	(mg/kg)	0 hour	1 hour	2 hours	3 hours	4 hours
NS	1 mL/kg	0.29 ± 0.0361	0.3 ± 0.05	1.32 ± 1.6327	0.29 ± 0.0656	0.32 ± 0.0289
AI	20	0.26 ± 0.0231	0.25 ± 0.0643	0.36 ± 0.0404	0.29 ± 0.0115	0.28 ± 0.0208
AI	40	0.17 ± 0.0115	0.29 ± 0.0551	0.3 ± 0.000	0.25 ± 0.0379	0.22 ± 0.0265
AI	60	0.19 ± 0.0208	0.29 ± 0.0451	0.3 ± 0.0917	0.27 ± 0.0416	0.24 ± 0.0361
DCF	20	0.17 ± 0.0115	0.3 ± 0.0153	0.34 ± 0.0361	0.28 ± 0.0200	0.25 ± 0.0153
P Value		0.00016	0.7703	0.40521	0.67979	0.01046

Data is presented as Mean ± Standard Deviation and analyzed using repeated measure analysis of variance; p value less than 0.01 is considered statistically significant; NS = normal