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Antihypertensive Potential of *Azadirachta indica* Methanolic Leaf Extract in Salt-induced Hypertensive Rat Model

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ABSTRACT

Hypertension is a condition arising from persistently elevated blood pressure, leading to severe complications, including death. Factors such as high salt consumption and family history predispose individuals to hypertension. *Azadirachta indica* (AI) has shown potential in reducing high blood pressure, irregular heart rhythms, and abnormal heartbeats. This study aimed to assess the antihypertensive effect of *Azadirachta indica* methanolic leaf extract (AIMLE) on salt-induced hypertension in albino rats. The study involved 25 healthy male albino rats (150 - 250g), divided into five groups (A-E, n=5). Group A received normal salt feed and water (vehicle), group B received high-salt feed (HSF), group C received HSF and 500 mg/kg AIMLE, group D received HSF and 1000 mg/kg AIMLE, and group E received HSF and 10 mg/kg Nifedipine. Systolic (SBP) and diastolic (DBP) blood pressures were measured using the tail cuff method. Antidiuretic hormone (ADH) and Angiotensin-Converting Enzyme (ACE) levels were analyzed using ELISA methods. Nitric Oxide, Potassium, Bicarbonate, and Sodium were assayed using the colorimetric method. Administration of HSF caused a significant ($p < 0.05$) progressive increase in SBP and DBP after 8 weeks when compared with the normal control. However, treatment with the serial doses of AIMLE resulted in a significant ($p < 0.05$) dose dependent decrease in SBP, DBP and serum nitric oxide concentrations as well as decrease in serum levels of ACE, ADH, potassium, bicarbonate, and sodium ions when compared with untreated HSF group, after 8 weeks. These findings suggest that *Azadirachta indica* leaf extract may offer a natural remedy for managing hypertension.

Keywords: *Azadirachta indica*, ADH, Salt-induced hypertension, Antidiuretic, ACE

Introduction

Hypertension is one of the common health problems worldwide, with an estimated rate of 1.28 billion adults within the age range of 30 to 79 globally[1]. It is an elevation in the blood pressure beyond the normal range with two consecutive measurements ≥ 140/90 mmHg[2]. Approximately 30% of men and 50% of women aged 65–75 years present with hypertension. About 1.56 billion people are hypertensive worldwide[3]. This value indicates a 60% increase in the prevalence of hypertension with the majority, roughly two-thirds, residing in regions characterized by low and middle income[2]. It is particularly concerning that a substantial 46% of individuals with hypertension are unaware of their condition, and merely 42% have the opportunity to undergo formal diagnosis and receive subsequent treatment[2]. In Nigeria, hypertension stands out as the most frequently diagnosed cardiovascular disease risk equivalent, contributing to nearly one-fourth of emergency admissions in urban healthcare facilities[4]. This persistent non-communicable ailment has a worldwide impact, across socio-economic strata, gender, and age categories[5]. Hypertension is associated with an increased susceptibility to severe health complications, such as myocardial infarction, stroke, cardiac death, heart failure, vascular disease, and renal insufficiency[6]. Notably, the average blood pressure within the Nigerian population surpasses that of European and American populations[7]. In Nigeria, the prevalence of hypertension falls within the range of 12% to 36.8%[8,9]. Although several factors may predispose an individual to the development of the hypertensive state and other cardiovascular diseases, Numerous studies have pointed various risk factors associated with hypertension, encompassing factors such as aging, obesity, sedentary lifestyle, familial history, African heritage, smoking, contraceptive pill usage, and excessive alcohol and salt consumption[10].

Despite progress in the realms of preventing, detecting, managing, and regulating elevated blood pressure, hypertension persists as a substantial public health predicament[11]. Some studies have investigated the prospective utility of complementary and alternative medicine in the treatment of hypertension[11]. The recognition and adoption of complementary and alternative medicine have experienced substantial growth within the medical community and among patients over the past two decades[12]. Herbal medicine is increasingly popular as an alternative treatment globally. Herbal products have been instrumental in research and the development of new drugs over the years[13,14]. The plant kingdom is a vast and largely untapped reservoir of potential therapeutic agents, offering a wealth of opportunities for drug development and discovery[12]. *Azadirachta indica*, commonly known as neem, is a member of the *Meliaceae* family and is renowned for its therapeutic properties. It is predominantly found in tropical and semi-tropical regions across the world. Various parts of the neem tree, including the seeds, leaves, flowers, and bark, are widely employed for diverse purposes[15]. The medicinal plant *Azadirachta indica*, which belongs to the family *Meliaceae*, reportedly has diverse medicinal efficacies in the prevention or treatment of several diseases. It has been reported to be effective in experimental hypertension associated with increased water retention and fluid overload[16]. *Azadirachta indica* contains numerous constituents, such as nimbolide, limonoids, nimbidin, and nimbin, which modulate various genetic pathways and other biological activities, contributing to disease management[17]. Among the initial polyphenolic flavonoids isolated from fresh neem leaves were quercetin and ß-sitosterol, renowned for their antifungal and antibacterial properties[17]. This plant showcases a broad spectrum of biological and pharmacological activities, including its antibacterial effects[17], antifungal properties[18], and anti-inflammatory attribute[19]. Previous research has substantiated its involvement in various activities, including anti-inflammatory, antiarthritic, antipyretic, hypoglycemic, antigastric ulcer, antifungal, antibacterial, and antitumor actions[19]. The management of hypertension involves the use of several antihypertensive drugs, including diuretics,

sympatholytic drugs, renin inhibitors, ACE inhibitors, calcium channel blockers, β-adrenergic and α1/β-adrenergic antagonists, and vasodilators[19]. Some of these drugs can lead to a variety of adverse effects, such as edema, excessive fatigue, headaches, vomiting, skin rashes, irregular heart rate, blurred vision, and muscular cramps[19]. The current study investigates the antihypertensive effects of a methanolic extract of *Azadirachta indica* in a model of hypertension induced by a high-salt diet in Albino rats.

Material and Methods

Plant Collection

Fresh leaves of *Azadirachta indica* were obtained in March to June 2024 from the Kwara State University garden in Malete, Kwara State, Nigeria. The plant was identified and authenticated at the Department of Plant Biology, University of Ilorin, Nigeria, with voucher number UILH|001|860|2023 deposited in the herbarium.

Preparation of the Extract

Azadirachta indica leaves were harvested, washed, air-dried, and ground into powder. Subsequently, 1000 g of *Azadirachta indica* powder was soaked in 3000 ml of methanol for three days with intermittent stirring. The mixture was sieved through Whatman number one Filter Paper. The filtrate was then subjected to drying using a rotary evaporator (40°C) to obtain a dried residue. This extract was stored in a refrigerator prior to when it was used for the preparation of test solutions.

Ethical approval

The approval for the experimental routine was authorized by the Center for Research and Innovation, Kwara State University, Malete (Ref: KWASU/CR&D/REA/2023/0020).

Experimental animal

All twenty-five male albino rats weighed between 150g and 250g were kept in the laboratory animal house of Kwara State University, Malete. The animals were treated following the guidelines outlined in the 'Guide for the Care and Use of Laboratory Animals' (National Academic Press, Washington DC, USA, 1996). They were housed in polypropylene cages (22.5 cm^2 x 37.5 cm) under standard conditions of room temperature (24-27°C) with a 12-hour light-dark cycle. The rats had unrestricted access to a standard pellet diet and water.

Study design

Hypertension was induced by high salt loading rats containing 8% sodium chloride (NaCl) and normal salt feed containing 5% NaCl. Twenty-five albino rats were randomly assigned into five groups A to E, each group has 5 rats. Group A (normal control) received 0.5% normal-salt feed (NSF), Group B (negative control) received 8% high-salt diet (HSF), Groups C received 8 % HSF + 500 mg/kg body weight (bwt) *Azadirachta indica* methanolic leaf extract (AIMLE) and group D received 8% HSF + 1000 mg/kg bwt AMLE and Group E (positive control) was given 8% HSF +10 mg/kg bwt Nifedipine.

Hemodynamic assessment

The baseline blood pressure (systolic blood pressure {SBP} and diastolic blood pressure {DBP} in mmHg) was measured in conscious albino rats and then monthly for two consecutive readings using a tail cuff pressure plethysmograph (Kent Scientific CODA).

Biochemical analysis

All the rats were anaesthetized and held safe at the end of the treatment period. Three millilitres of blood was collected using heparinized capillary tube from the medial canthus of the eye under the nictitating membrane. The blood was dispensed into the plain bottle, allowed to clot and then centrifuged at 3000 revolution per minute for 10 minutes. Thereafter, the serum was

aliquoted into cryovial tube for storage at - 20° C prior to the biochemical analysis.

The competitive inhibition enzyme immunoassay technique was employed for the assay of anti-diuretic hormone (ADH) and angiotensin I-converting enzyme (ACE). A monoclonal antibody specific for ADH, ACE, and NO has been pre-coated onto a micro titre plate. A competitive inhibition reaction is launched between biotin-labeled ADH/ACE and unlabeled ADH/ACE, respectively (Calibrators or samples) with the pre-coated antibody specific for ADH, ACE, and NO. After incubation, the unbound conjugate is washed off. Next, avidin conjugated to Horseradish Peroxidase (HRP) is added to each micro titer plate well and incubated. The amount of bound HRP conjugate is inversely proportional to the concentration of ADH/ACE in the sample. After the addition of the substrate solution, the intensity of color developed is inversely proportional to the concentration of ADH/ACE in the sample [20]. Nitric oxide (NO) concentration was determined by the spectrophotometric method of Ridnour *et al.* [21]. Electrolyte levels (sodium, potassium, and bicarbonate) were measured in millimole per liter using an Ion Selective Electrode machine. This is based on a thin selective membrane across which only the specific ion can be transported. The transport of ions from a high concentration to a low concentration through selective binding with some sites within the membrane creates a potential difference. Serum nitric oxide levels were determined using a colorimetric assay kit (Promega Corporation, USA) based on the Griess reaction [22].

Statistical Analysis

The data were presented as mean ± standard error of mean (SEM). Multiple group comparison was carried out using one-way analysis of variance (ANOVA) and Tukey's post hoc test. GraphPad Prism version 9.0 was the software employed for graph plotting. Data were considered statistically significant at $p < 0.05$.

Results

At baseline, there were no significant differences ($P<0.05$) for systolic blood pressure (SBP) across the groups. However, administration of high salt feed (HSF) resulted in a significant ($P<0.05$) and progressive increase in systolic blood pressure with percentage increase of 43.8 and 125.5% at 4 and 8 weeks, respectively, when compared with the normal control (Figure 1). Meanwhile, groups treated with AIMLE at 500 and 1000 mg/kg body weight doses showed a significant ($P<0.05$) dose-dependent decrease in SBP. The diastolic blood pressure (DBP) at baseline revealed no significant difference across the groups; treatment with HSF led to a significantly ($P<0.05$) elevated DBP with percentage increase of 108.3% and 177.3% at 4 and 8 weeks, respectively, compared with the normal control (Figure 2). Significantly ($P<0.05$) lowered DBP was observed in the groups treated with the serial doses of AIMLE as well as the reference drug when compared with the untreated HSF-only group at the end of the treatment period (Figure 2). The effects of AIMLE on serum nitric oxide (NO), antidiuretic hormone (ADH), and angiotensin-converting enzyme activity (ACE) in salt-induced hypertensive rats were investigated in this study. Administration of HSF significantly ($P<0.05$) increased ADH serum concentration and ACE activity as well as lowered NO levels. Treatment with AIMLE at doses of 500 and 1000 mg/kg body weight resulted in a significant dose-dependent decrease in serum antidiuretic hormone (ADH) concentration and ACE in activity, compared with the HSF-only group (Figures 3 and

4). The serum level of NO was observed to be significantly ($P<0.05$) increased in a dose-dependent pattern in groups treated with AIMLE, as well as the reference drug when compared with the HSF group (Figure 5).

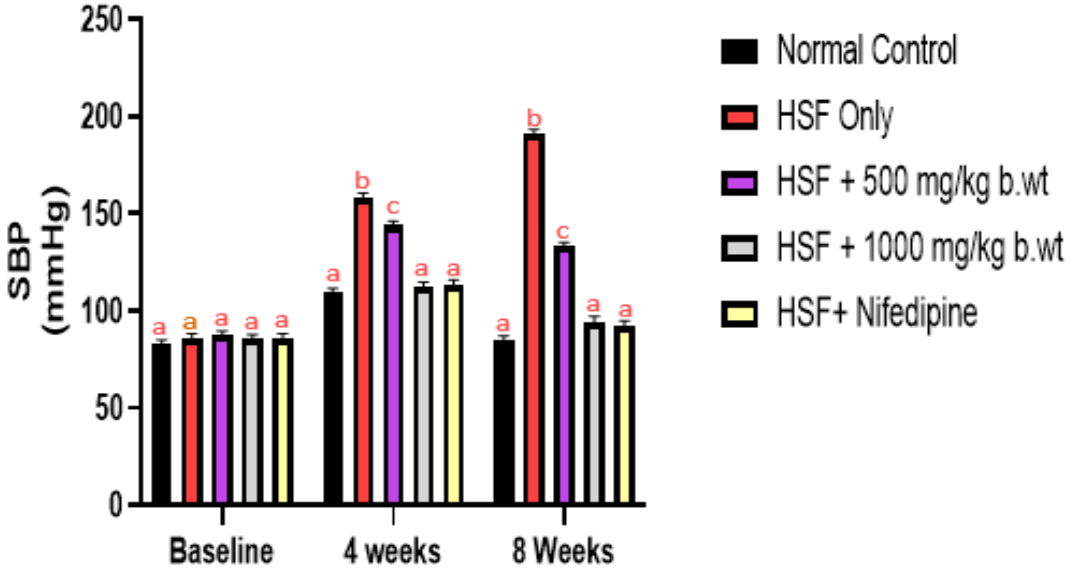

Figure 1: Effects of *Azadirachta indica* methanolic leaf extract on systolic blood pressure in salt-induced hypertensive rats. Data were expressed as mean ± SEM. Values with different letters are significantly different at $p<0.05$.

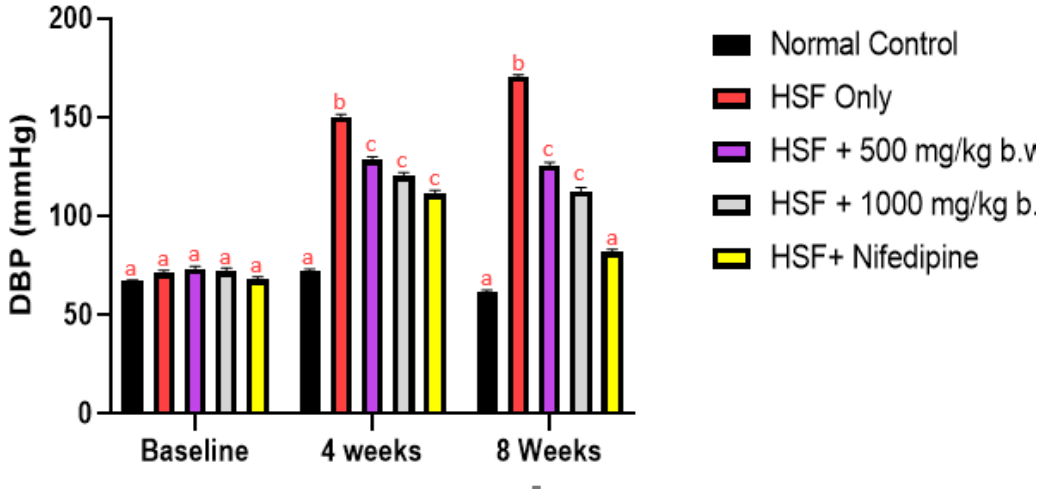

Figure 2: Effects of *Azadirachta indica* methanolic leaf extract on diastolic blood pressure in salt-induced hypertensive rats. Data were expressed as mean ± SEM. Values with different letters are significantly different at $p<0.05$.

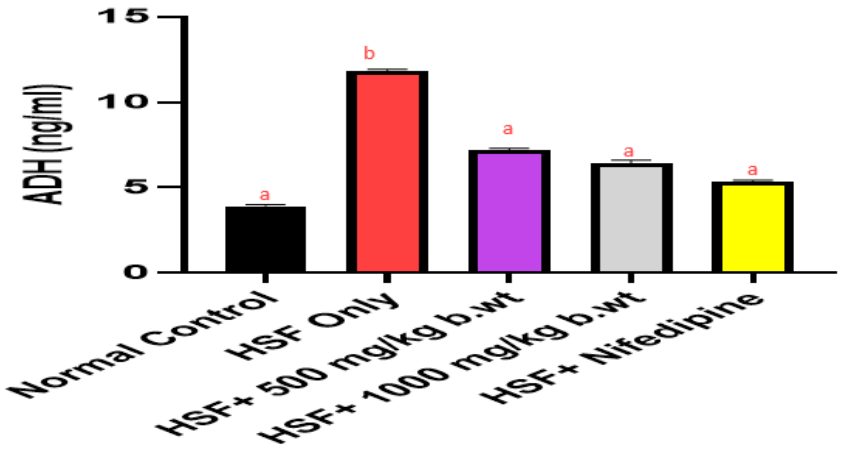

Figure 3: Effects of *Azadirachta indica* methanolic leaf extract on antidiuretic hormone in salt-induced hypertensive rats. Data were expressed as mean ± SEM. Values with different letters are significantly different at $p<0.05$.

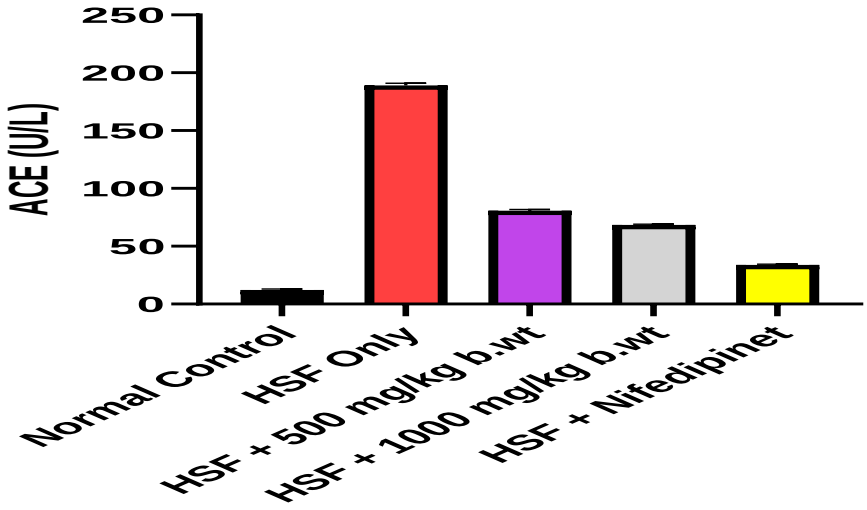

Figure 4: Effects of *Azadirachta indica* methanolic leaf extract on angiotensin-converting enzyme activity in salt-induced hypertensive rats. Data were expressed as mean ± SEM.

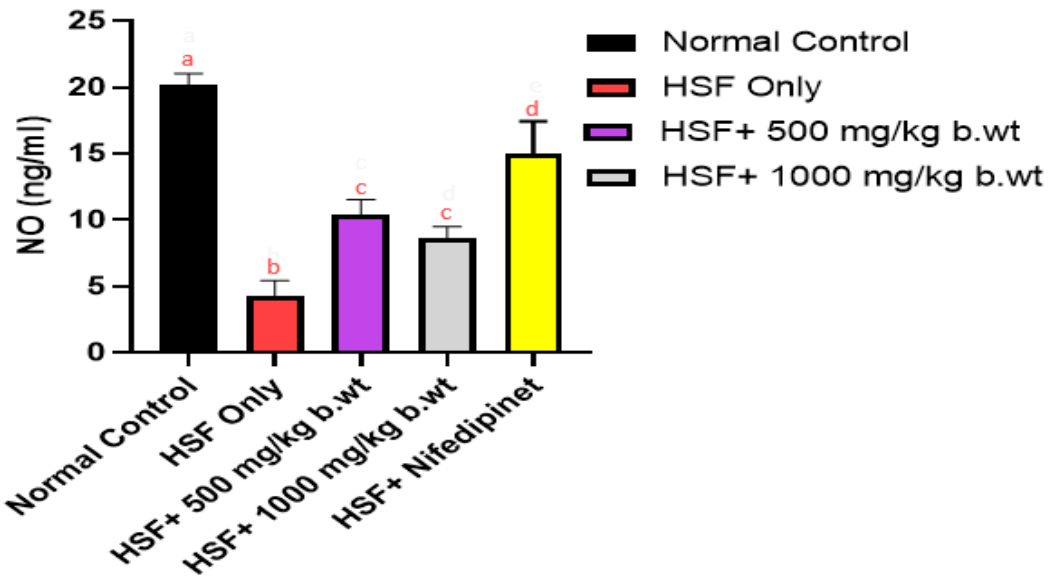

Figure 5: Effects of *Azadirachta indica* Methanolic Leaf Extract on Nitric Oxide Concentration in Salt-induced hypertensive rats. Data were expressed as mean ± SEM. Values with different letters are significantly different at p<0.05.

The effects of AIMLE on serum sodium, potassium, and bicarbonate concentrations in salt-induced rats were investigated in this study. A group of rats administered HSF showed significantly (P<0.05) higher concentrations of sodium, potassium, and bicarbonate ions when compared with the normal control group. Treatment with the serial doses of AIMLE as well as nifedipine, significantly (P<0.05) decreased the serum concentrations of sodium, potassium, and bicarbonate ions to near that of the normal control (Table 1).

Table 1: Effects of *Azadirachta indica* methanolic leaf extract on selected serum electrolytes in salt-induced hypertensive rats

Groups	**Potassium** (mmol/l)	**Bicarbonate** (mmol/l)	**Sodium** (mmol/l)
Normal control	2.452 ± 0.02701 ^{e}	18.60 ± 0.4948 ^{e}	84.24 ± 1.084 ^{e}
HSF only	5.015 ± 0.06790 ^{d}	66.16 ± 0.5309 ^{d}	182.0 ± 2.896 ^{d}
HSF + 500 AIMLE	3.005 ± 0.01224 ^{a}	34.30 ± 0.5417 ^{a}	134.9 ± 1.617 ^{a}
HSF + 1000 AIMLE	3.178 ± 0.01019 ^{b}	38.47 ± 1.023 ^{b}	144.7 ± 2.028 ^{b}
HSF + Nifedipne	2.723 ± 0.02372 ^{c}	22.87 ± 0.4505 ^{c}	108.1 ± 2.215 ^{c}

Data were expressed as Mean ± SEM. Values within the same column with different letters are significantly different at p<0.05**.**

DISCUSSION

Excessive sodium consumption, as defined by the World Health Organization, encompassing an intake exceeding 5 grams of sodium per day [23], has been unequivocally associated with a substantial elevation in blood pressure and has been linked to the onset of hypertension and its concomitant cardiovascular complications [23,24].

In the current study, the administration of high salt feed resulted in a progressive and significant increase in systolic and diastolic blood pressure beginning from 4 weeks till the end of treatment at 8 weeks, compared to the normal control group, validating the induction of hypertension. The observed increase in blood pressure may be attributed to the dysregulation of sodium metabolism, which can also be related to changes

in genes and receptors associated with mineralocorticoid synthesis and function [25]. The elevated blood pressure following oral administration of a high salt diet is in agreement with the previously reported findings that reported a progressive increase in blood pressure following prolonged high salt diet administration [26]. *Azadirachta indica*, a traditional therapeutic remedy, is one of such plant, containing bioactive compounds such as flavonoids and polyphenols, suggesting potential antioxidant and vasodilatory properties. The results of the present study demonstrate that oral administration of the serial doses of *Azadirachta indica* significantly reduced systolic and diastolic blood pressure in a dose-dependent manner with optimal efficacy at higher doses. This implies that AIMLE exhibits an antihypertensive effect in salt-induced hypertensive rats. The observed fall in blood pressure may be associated with the ability of the bioactive constituents of *Azadirachta indica* to mediate the activity of ACE and increase bioavailability of nitric oxide. The findings in this study agree with the study of Omobowale *et al*.[27] that reported a significant decrease in blood pressure in AIMLE treated rats in L-NAME induced hypertensive rats.

Nitric oxide is a key vasodilator also known as endothelium-derived vasorelaxant factor (EDRF) that helps relax blood vessels, thereby lowering blood pressure. Reduced concentrations of NO in plasma have been observed in patients with essential hypertension [22]. In this study, induction of hypertension using a high salt diet led to a significant decrease in the serum nitric oxide concentration, indicating a possible vascular constriction. This observation corroborates the earlier study of Dishy *et al*.[28] that reported a significant decrease in nitric oxide production in both salt-sensitive and salt-resistant normotensive subjects. However, *Azadirachta indica* extract significantly increases the bioavailability of nitric oxide. This suggests that neem leaf extract may enhance NO production, promoting vasodilation and potentially contributing to blood pressure reduction. This observation corroborates the earlier report of Yarmuhammadi *et al*.[29] that *Azadirachta indica* showed anti-hypertensive effects via up-regulation of endothelial nitric oxide synthase (eNOS). Angiotensin-converting enzyme regulates blood pressure through the renin-angiotensin-aldosterone system. The anti-diuretic Hormone (ADH), also known as vasopressin, plays a role in water retention and blood volume regulation, thereby regulating blood pressure. Elevated ADH and ACE levels can lead to increased blood pressure. In this study, induction of hypertension using a high salt diet caused a significant increase in ADH serum levels and ACE activity, suggesting a possible cause of high blood pressure. The observed increase in ADH following a high salt diet is in line with the report of Fressinaud *et al*.[30] that high salt intake is associated with a significant increase in antidiuretic hormone. In this study, AIMLE-treated groups showed a significant decrease in ADH concentration and ACE activity, potentially mitigating vasoconstriction and blood pressure elevation. This finding agrees with the study of Ahmad *et al*.[31,] who reported that ACE activity is associated with vasoconstriction and elevated blood pressure [31]. Potassium, bicarbonate, and sodium are pivotal elements in the intricate regulation of blood pressure [32]. It's noteworthy that a decrease in sodium intake is closely associated with a concurrent reduction in both systolic and diastolic blood pressure [32]. Conversely, potassium plays a significant role in the preservation of blood pressure through its impact on vascular tone and fluid balance [33]. In addition, bicarbonate is a critical component of the body's acid-base balance, and alterations in its levels can affect blood pH and, subsequently, blood pressure [33]. In this study, the induction of hypertension using a high-fat diet led to a significant increase in serum sodium, potassium, and bicarbonate concentrations, indicating a potential fluid and electrolytes imbalance. However, the administration of *Azadirachta indica* methanolic leaf extract significantly lowered the serum electrolytes concentrations.

Conclusion

Azadirachta indica methanolic leaf extract significantly lowered the elevated blood pressure caused by salt loading through increase in bioavailability of nitric oxide and down regulation of ACE and ADH activity, Thus, this study revealed that neem leaf shows promising effects as a potential natural anti-hypertensive agent.

Conflict of interest

The authors wish to declare that they do not possess any identifiable financial conflicts of interest or close personal associations that might conceivably influence the outcome of the research detailed in this study.

Source of funding

The research was self-funded by the authors, and no external funding was received.

Authors' contributions

Research Conception: MI, ODO, and AZL; Project design: SFO, SOS, KMA; Data collection:
MI, ODO, OHO, OO and HA; Drafting and Manuscript revision: MI, AZL, KBD, FKPI and HOJ.

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