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ORIGINAL ARTICLE

Antihypertensive Effect of Methanol Leaf Extract of *Azadirachta indica* is Mediated through Suppression of Renal Caspase 3 Expressions on Nω-Nitro-L-Arginine Methyl Ester Induced Hypertension

Temidayo Olutayo Omóbòwálé, Ademola Adetokunbo Oyagbemi[1], Olumuyiwa Abiola Adejumobi, Fisayo Ugbor, Ebunoluwa Racheal Asenuga[1], Temitayo Olabisi Ajibade[1], Jeremiah Moyinoluwa Afolabi, Blessing Seun Ogunpolu, Olufunke Olubunmi Falayi[2], Idayat Titilayo Gbadamosi[3], Olufunke Eunice Ola-Davies[1], Adebowale Bernard Saba[2], Anofi Ashafa[4], Momoh Audu Yakubu[5], Adeolu Alex Adedapo[3], Oluwafemi Omoniyi Oguntibeju[6]

Department of Veterinary Medicine, Faculty of Veterinary Medicine, University of Ibadan, [1]Department of Veterinary Physiology and Biochemistry, Faculty of Veterinary Medicine, University of Ibadan, [2]Department of Veterinary Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Ibadan, [4]Department of Botany, Faculty of Science, University of Ibadan, Nigeria, [5]Department of Environmental and Interdisciplinary Sciences, College of Science, Engineering and Technology, Vascular Biology Unit, Center for Cardiovascular Diseases, COPHS, Texas Southern University, Houston, TX, USA, [6]Phytomedicine and Phytochemistry Group, Department of Biomedical Sciences, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, Bellville, [3]Faculty of Natural and Agricultural Sciences, Qwaqwa Campus, University of the Free State, South Africa

ABSTRACT

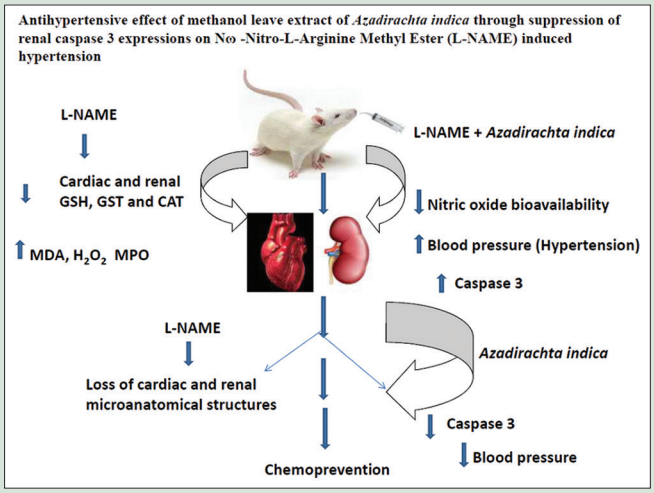
Background: *Azadirachta indica* (AI) Adr Juss (*Meliaceae*), known as neem, has been used traditionally for the treatment of various disease conditions including obesity and hypertension. **Objective:** The antihypertensive effect and mechanism of action of modulatory effect of AI were investigated after the induction of hypertension using Nω-nitro-L-arginine methyl ester (L-NAME). **Materials and Methods:** Five groups of ten rats divided as follows: Control; L-NAME (40 mg/kg); L-NAME + 100 mg/kg AI; L-NAME and 200 mg/kg AI; and L-NAME and Enalapril (25 mg/kg) were used. **Results:** following the application of L-NAME, hypertension (elevated systolic, diastolic, mean arterial blood pressures) and increased levels of oxidative stress markers were observed in rats. Immunohistochemistry showed increased caspase-3 expressions in hypertensive rats compared to normotensive rats. Conversely, AI treatment resulted in restoration of physiological antioxidant status and normotension, comparable to the standard antihypertensive agent enalapril. **Conclusion:** AI leaf is a good candidate for the management of high blood pressure.

Key words: Antioxidant, apoptosis, *Azadirachta indica*, hypertension, oxidative stress, phytotherapy

SUMMARY

- The methanol leaf extract of *Azadirachta indica* was evaluated for its antihypertensive and renoprotective effects in drug-induced hypertension. Finger-print of *Azadirachta indica* was determined with GC-MS. Evaluation of markers of oxidative stress and antioxidant defense were done biochemically, while immunohistochemistry was used to assess markers of apoptosis. Linolenic acid and linoleic acid were observed in *Azadirachta indica* with GC-MS. Leaf extract of *Azadirachta indica* also restored normotension and physiological antioxidant treated rats.

Abbreviations Used: L-NAME: Nω-nitro-L-arginine methyl ester; AI: *Azadirachta indica*; NO: Nitric oxide; DOCA: deoxycorticosterone acetate; CDNB: 2-dichloro-4nitobenzene; DTNB: 5,5-dithiobis-2-nitrobenzoic acid; TCA: Trichloroacetic acid; TBA: Thiobarbituric acid; GSH: Reduced glutathione; H_2O_2: Hydrogen peroxide; NaOH: Sodium hydroxide; HRP: Horseradish peroxidase GCMS: Gas chromatography mass spectrometry; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; MAP: Mean arterial pressure; NPSH: Nonprotein thiol; DAB: Diaminobenzidine; MPO: Myeloperoxidase; MA: Malondialdehyde; CAT: Catalase; SOD: Superoxide dismutase; ROS: Reactive oxygen species; GST: Glutathione S-transferase; GPx: Glutathione peroxidase; NOS: Nitric oxide synthase.



Correspondence:
Dr. Ademola Adetokunbo Oyagbemi,
Departments of Veterinary Physiology, Biochemistry and Pharmacology, Faculty of Veterinary Medicine, University of Ibadan, Ibadan, Nigeria.
E-mail: ashafaaot@ufs.ac.za
DOI: 10.4103/pr.pr_10_20

Access this article online
Website: www.phcogres.com
Quick Response Code:

Cite this article as: Omóbówálé TO, Oyagbemi AA, Adejumobi OA, Ugbor F, Asenuga ER, Ajibade TO, *et al*. Antihypertensive effect of methanol leaf extract of *Azadirachta indica* is mediated through suppression of renal caspase 3 expressions on Nω-Nitro-l-arginine methyl ester induced hypertension. Phcog Res 2020;12:460-5.

Submitted: 14-Feb-2020 **Revised:** 09-Apr-2020
Accepted: 15-Jul-2020 **Published:** 23-Jan-2021

INTRODUCTION

The hypertensive state, which may be defined as the elevation in the blood pressure beyond the normal range, signifies a deviation of the cardiovascular system functioning from the physiological state with consequent development of other diseases such as stroke and myocardial infarction.[1] Although several factors may predispose an individual to the development of the hypertensive state and other cardiovascular diseases, other risk factors such as smoking, sedentary lifestyle, old age, and obesity have also been implicated.[2] The deficiency of nitric oxide which is produced within the vascular endothelium leads to a malfunction of the vasodilatory mechanism in blood vessels and is one of the primary causes of hypertension, alteration of cellular proliferation and thrombosis.[3-5] Experimentally, hypertension may be induced by drugs such as L-NAME which selectively inhibit nitric oxide synthase activity in vascular beds.[6] As a result, there is inadequate vasodilation and increased tension in the vascular wall and elevated blood pressure.

Different parts of several medicinal plants, as well as phytochemicals are currently used traditionally or are being researched for experimental validation of their folkloric use for managing hypertension.[7] The medicinal plant *Azadirachta indica* Adr Juss (AI) which belongs to the family *Meliaceae* reportedly has diverse medicinal efficacies in the prevention or treatment of several diseases.[8] AI has been reported to be effective in experimental hypertension associated with increased water retention and fluid overload.[9] In this study, we investigated the probable modulatory roles and mechanism of AI in L-NAME-induced hypertension in rats.

Animals and study design

The experimental animals used in this study were 50 rats (male, Wistar strain, 175–200 g). The source of the rats was the breeding unit at animal house of the Faculty of Veterinary Medicine, University of Ibadan, Nigeria. Plastic cages were used to keep the rats for a 4-week acclimatization period and throughout the experiment. Pelletized rat feed, water and natural photoperiod 12 h light/day were provided optimally for the rats throughout the experimental period.

Five groups of animals were used with each group having ten animals each and designated as follows: Control (normotensive); 40 mg/kg L-NAME (hypertensive); AI_1 (100 mg/kg)-treated hypertensive; AI_2 (200 mg/kg)-treated hypertensive; and enalapril-treated hypertensive. All administrations were done by oral gavage, using a bulb steel needle, for 21 consecutive days.

AI extraction

Naturally grown AI tree was the source of freshly harvested AI leaves used for this study. Following identification and authentication, AI was extracted with methanol for 72 h, filtered, and subsequently evaporated to dryness on a water bath. A yield of 4.68 percent was obtained from the dry leaves of AI.

Ethical standard

The authors assert this study was conducted following the approval of the scientific committee concerned with the use of animals for research purposes (ACUREC) at the University of Ibadan, Oyo State, Nigeria.

Separation of *Azadirachta indica* phytochemicals with gas chromatography-mass spectrometry

This was done as previously described in our recent paper.[10]

Measurement of blood pressure parameters

The systolic, diastolic and mean arterial blood pressures of the rats were recorded on the last day of the experimental period by an indirect method using an automated plethysmograph (Kent Scientific, USA).

Biochemical assays

Twenty-four hours after the last administration, retro-orbital venous puncture was done to obtain fresh blood. The puncture was done carefully with capillary tubes into non-heparinised sample bottles that were devoid of anticoagulants in order to obtain the serum. Immediately following the sacrifice of the experimental rats, the hearts and kidneys were harvested on ice and subsequently processed for enzymatic and nonenzymatic biochemical evaluations. The Biuret method was used for the evaluation of protein,[11] whereas nitric oxide (NO),[12] reduced glutathione[13] and catalase (CAT) were measured as previously described.[14] The enzymes glutathione S-transferase (GST), glutathione

Table 1: GC-MS analysis of methanol leaf extract of *Azadirachta indica*

Retention Time	Compound name	Formula	Molecular weight
14.575	2-Chlorobenzoic acid	$C_7H_5ClO_2$	156
17.407	Lauric acid	$C_{12}H_{24}O_2$	200
19.674	Myristic acid	$C_{14}H_{28}O_2$	228
20.525	Oleyl alcohol	$C_{16}H_{32}O$	240
20.526	Neophytadiene	$C_{20}H_{38}$	278
20.592	Myristyl aldehyde	$C_{14}H_{28}O$	212
20.783	Pentadecanal	$C_{15}H_{30}O$	226
20.980	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296
20.983	Lyngbyoic acid	$C_{13}H_{24}O_2$	212
21.432	Methyl Palmitate	$C_{17}H_{34}O_2$	270
21.849	Palmitic acid	$C_{16}H_{32}O_2$	256
21.850	2,6-Di-O-palmitoyl-L-ascorbic Acid	$C_{38}H_{68}O_8$	625
23.108	Linoleic acid methyl ester	$C_{19}H_{34}O_2$	294
23.167	Methyl linolenate	$C_{19}H_{32}O_2$	292
23.167	11,14,17-Eicosatrienoic acid methyl ester	$C_{21}H_{36}O_2$	320
23.275	Phytol	$C_{20}H_{40}O$	296
23.401	Methyl stearate	$C_{19}H_{38}O_2$	298
23.401	Stearic acid	$C_{18}H_{36}O_2$	284
23.625	Linolenic acid	$C_{18}H_{30}O_2$	278
23.817	Oleic acid	$C_{18}H_{34}O_2$	282
23.817	Octadecanoic Acid, 2-(2-Hydroxyethoxy) Ethyl Ester	$C_{22}H_{44}O_4$	372
24.167	1-Tridecanol	$C_{13}H_{28}O$	200

Table 2: Effect of *Azadirachta indica* (AI) on serum nitric oxide and myeloperoxidase in L-NAME-induced hypertensive rats

Groups	Control	L-NAME	L-NAME + AI1	L-NAME + AI2	L-NAME + ENALAPRIL
NO	0.12±0.01	0.08±0.01[a]	0.10±0.01[b]	0.15±0.03[b]	0.14±0.01[b]
MPO	18.70±3.32	59.66±3.60[c]	24.96±1.95[d]	29.85±1.08[d]	15.75±0.63[d]

[a]Significant (P<0.05) decrease compared with control within row. [b]Significant (P<0.05) increase compared with L-NAME within row; [c]Significant (P<0.05) increase compared with control within row; [c]Significant (P<0.05) decrease compared with control within row. NO (Nitric oxide (μmole/L), MPO (Myeloperoxidase (Units/mg protein)

peroxidase (GPx), and superoxide dismutase (SOD) were measured using standard methods.[15-17] Also, malondialdehyde (MDA), hydrogen peroxide, sulfhydryl protein thiol (PSH) and non-protein thiol (NPSH), and serum myeloperoxidase (MPO) activity were measured as previously described.[18-21]

Immunohistochemical evaluation of caspase 3

This was obtained from paraffin embedded renal and cardiac tissues as earlier described.[22] The integrated optical density was measured with the software Image J.

Statistical evaluation

Requisite statistical analyses were done on data expressed as mean ± standard deviation with one-way analysis of variance with Dunnett's post-test. Confidence limit was set at 95%.

RESULTS

The phytochemicals obtained with the gas chromatography mass spectrometry (GC-MS) in this study are shown in Table 1. Significant reduction (P < 0.05) in the NO level was observed in hypertensive rats relative to the normotensive control but reduction in NO was not significant following treatment with AI (AI_1 and AI_2) which dose dependently increased the NO levels from 0.08 ± 0.01 μmole/L (L-NAME) to 0.1 ± 0.01 μmole/L (AI_1 + L-NAME) and 0.15 ± 0.03 μmole/L (AI_2 + L-NAME) and enalapril [Table 2]. This is indicative of augmentation of serum NO bioavailability by AI leaf extract. Also, hypertensive rats showed MPO activity elevation which became ameliorated by AI and enalapril administration [Table 2].

Furthermore, H_2O_2 increased significantly in both renal and cardiac tissues of the hypertensive animals compared with normotensive ones [Table 3], but observed reduction of H_2O_2 level by AI_2 unlike AI_1 was significant. Also, reduction of GSH and thiols of hypertensive rats relative to the normotensive, AI and enalapril-treated rats was observed [Table 3]. In contrast, MDA concentration increased significantly in hypertensive rats compared with AI and enalapril-treated rats [Table 3].

Our results also showed that the activity of GST in hypertensive rats decreased significantly (P < 0.05) relative to AI- and enalapril-treated rats, but GPx increased significantly in hypertensive rats [Table 4]. The administration of L-NAME which precipitated hypertension significantly reduced CAT in cardiac/renal tissues of the hypertensive animals; an effect ameliorated by AI in a dose-responsive manner [Table 4]. Similar to what was observed for CAT activity, treatment of hypertensive rats with AI_2 and enalapril led to increases in the activity of SOD. The haemodynamic parameters increased significantly (P < 0.05) in hypertensive rats, but AI treatment normalised blood pressure parameters to near normotensive values [Table. 5]. Immunohistochemistry revealed an increased expression of caspase 3 in L-NAME group relative to the AI group. [Figures 1 and 2].

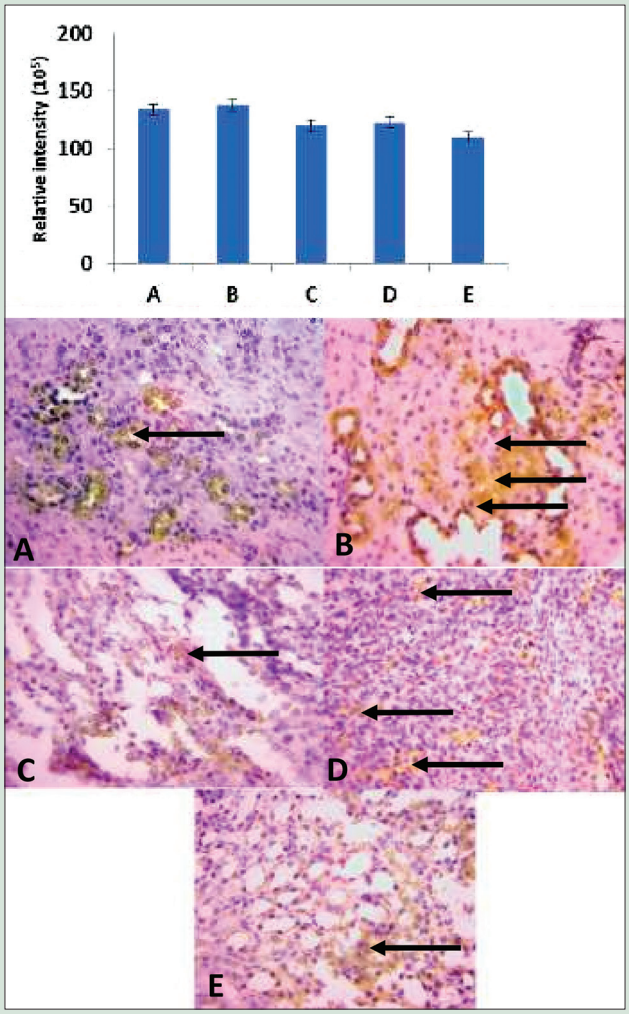


Figure 1: Renal caspase 3 activity. Group A (control), Group B (N$^{\omega}$-nitro-L-arginine methyl ester; 40 mg/kg), Group C (N$^{\omega}$-nitro-L-arginine methyl ester + *Azadirachta indica*) (100 mg/kg), Group D (N$^{\omega}$-nitro-L-arginine methyl ester + *Azadirachta indica*) (100 mg/kg) and Group E (Nω-nitro-L-arginine methyl ester + enalapril (25 mg/kg). Quantification was carried out image J software

DISCUSSION

The GC-MS analysis carried out in this study identified 22 compounds in AI, with several of the compounds being chemically and biologically important and may account for the high ethnopharmacological relevance of AI in the management of several diseases.[23] For instance, linolenic acid, one of the identified constituents of AI is reported to improve prognostic outcomes and reduce deaths in cardiovascular diseases including myocardial infarction and stroke.[24] Phytol (an acyclic diterpene alcohol), linolenic acid (an essential fatty acid [FA]), homo-γ-linolenic acid (a polyunsaturated constituent of the cholesteryl

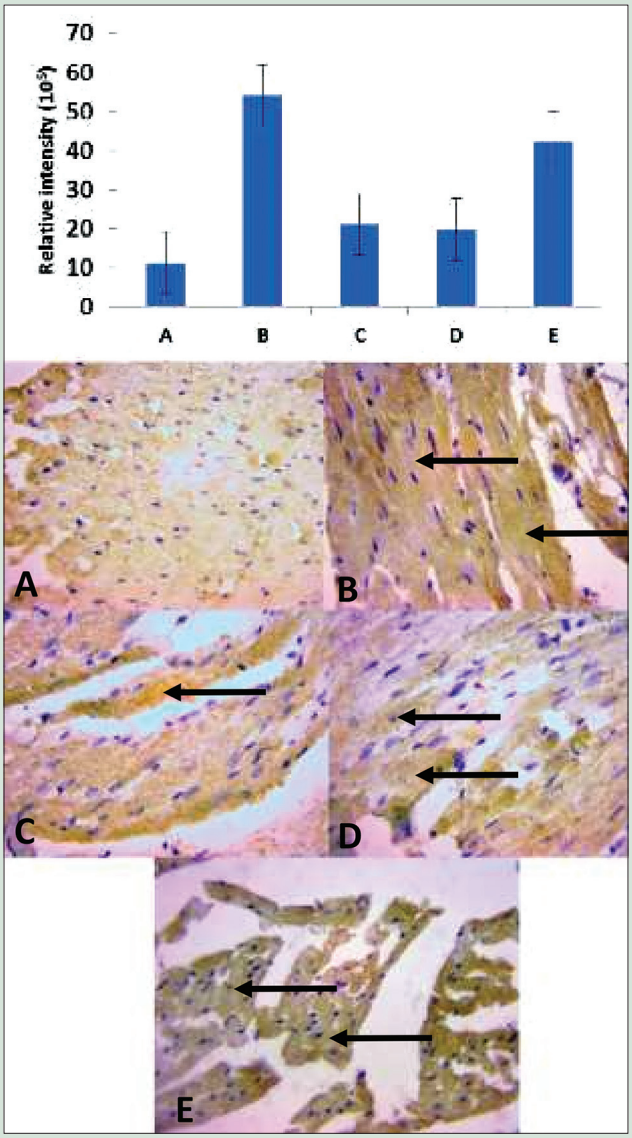


Figure 2: Cardiac caspase 3 activity. Group A (control), Group B (N$^{\omega}$-nitro-L-arginine methyl ester; 40 mg/kg), Group C (N$^{\omega}$-nitro-L-arginine methyl ester + *Azadirachta indica*) (100 mg/kg), Group D (N$^{\omega}$-nitro-L-arginine methyl ester + *Azadirachta indica*) (200 mg/kg) Group E (N$^{\omega}$-nitro-L-arginine methyl ester + Enalapril (25 mg/kg). Quantification was carried out image J software

esters isolated from swine adrenals), palmitic acid (saturated FA) and Tridecylic acid (which is also a saturated FA) have been reported as the five main components of AI leaf.[25] Similarly, hexadecanoic acid, methyl ricinoleate, ricinoleic, as well as the esters of oleic, stearic, and linoleic acids are also abundant constituents of *A. indica* gum exudates.[26]

There was a significant (P<0.05) reduction NO concentration in rats that were administered L-NAME when compared with those of AI treated rats. This observation is due to the competitive interaction of L-NAME with NO synthase with consequent inhibition of NO production.[27] Since NO is an endogenous vasodilator, reduction in its production leads to elevated tension in blood vessel walls due to reduced surface area available for blood to exert force on the blood vessel wall.[28] In many instances, hypertension has cardiomyopathy and alteration in renal function due to increased glomerular filtration rate as co-existing morbidities.[29-31] Observations in our study corroborates earlier reports of Birben *et al*.[32] that L-NAME administration induces oxidative stress in addition to hypertension in rats. Oxidative stress, due to elevation of reactive oxygen species (ROS) such as NAD(P)H oxidase and NO synthase, causes macromolecules to become oxidised with resultant depletion of the physiological antioxidant stores.[33,34] The results of this study corroborate earlier report of Hamilton *et al*.[35] that the inhibition of NAD(P)H oxidase by antioxidants such as SOD improves endothelial function in rat and human blood vessels.[35] Imbalance between NO and ROS levels in favour of ROS reportedly predisposes to hypertension in mammalian systems.[36,37] Improvements in the antioxidant defense system, signified in this study by increased GSH, PSH, NPSH, GST and CAT suggest potent ability of AI to scavenge free radicals, conserve the physiological antioxidants in various tissues and organs in the cardiovascular and renal systems with an overall effect of positive modulation of the hypertensive state. Antihypertensive drugs such as lisinopril and amlodipine in addition to reducing blood pressure in experimental hypertension, have been reported to improve the antioxidant status in mammalian subjects.[38,39] Likewise, the antihypertensive drug enalapril, an angiotensin converting enzyme inhibitor exerts a renoprotective effect by modulating the systemic antioxidants.[40,41]

AI and enalapril significantly reduced the activity of MPO in hypertensive rats; an observation that suggests inhibition of inflammation, as well as antioxidative potential for AI. MPO, as well as its oxidant products (e.g., hypochlorous acid) act as critical modulators in the initiation and propagation of cardiovascular diseases.[42] The observable antihypertensive effect of AI, indicated in this study by significant decreased blood pressure parameters in AI treated rats might be due to the presence of linolenic acid and other phytonutrients in the

Table 3: The effect of *Azadirachta indica* on cardiac and renal markers of oxidative stress

Groups	Control	L-NAME	L-NAME + AI1	L-NAME + AI2	L-NAME + enalapril
Cardiac MDA	0.19±0.05	0.38±0.07[a]	0.21±0.02[b]	0.19±0.06[b]	0.14±0.05[a,b]
Renal MDA	0.09±0.01	0.13±0.02[a]	0.13±0.01[a]	0.13±0.03[a]	0.12±0.03[a]
Cardiac H_2O_2	11.73±0.90	13.92±1.4[a]	13.64±0.38[a]	11.35±0.14[b]	12.98±1.30[a]
Renal H_2O_2	14.39±0.52	18.89±0.38[a]	17.73±2.46[a]	14.54±0.85[b]	14.81±1.81[b]
Cardiac PT	114.00±3.81	106.33±7.42*	144.75±8.49[#]	139.00±14.92[#]	120.67±13.59[#]
Renal PT	140.83±37.94	115.25±5.51*	201.50±25.25[#]	165.75±42.41[#]	179.33±2.92[#]
Cardiac NPT	35.50±3.04	25.38±2.30*	27.88±0.53[#]	22.25±1.06	22.88±2.26
Renal NPT	32.50±3.54	27.50±1.10*	32.00±2.27	31.56±1.74	33.50±2.26
Cardiac GSH	72.00±4.85	63.25±1.43	69.06±2.44	64.63±2.67	69.13±2.17
Renal GSH	85.06±1.20	73.00±1.93	79.38±1.09	76.17±2.74	77.31±1.88

[a]Significant (P<0.05) increase compared with control within row; [b]Significant (P<0.05) decrease compared with L-NAME within row; *Significant (P<0.05) decrease compared with control group; [#]Significant (P<0.05) increase compared with L-NAME within row. GSH: Reduced glutathione (μmol/mg protein); L-NAME: N$^{\omega}$-nitro-L-arginine methyl ester; MDA: Malondialdehyde (μmole of MDA formed/mg protein); H_2O_2: Hydrogen peroxide generation (μmol/mg protein); PT: Protein thiol (μmol/mg protein); NPT: Nonprotein thiol (μmol/mg protein)

Table 4: The effect of *Azadirachta indica* on cardiac and renal antioxidant enzymes

Groups	Control	L-NAME	L-NAME + AI1	L-NAME + AI2	L-NAME + enalapril
Cardiac GPx	174.83±0.38	178.94±1.63	175.06±0.60	175.25±2.21	178.13±0.60
Renal GPx	177.38±1.53	184.8±0.91	179.00±2.26	189.06±0.24	182.17±2.47
Cardiac GST	25.06±4.13	15.67±1.87[a]	24.45±3.71[b]	26.13±5.19[b]	21.44±0.94
Renal GST	16.38±1.22	14.06±1.12[a]	15.09±2.17	18.15±0.14[b]	22.49±0.50[b]
Cardiac CAT	3.06±0.67	1.75±0.60[a]	4.65±0.48[b]	6.05±2.00[b]	6.68±1.62[b]
Renal CAT	1.76±0.80	0.57±0.02[a]	1.33±0.70[b]	1.63±0.01[b]	1.16±0.18[b]
Cardiac SOD	0.10±0.06	0.26±0.06[a]	0.28±0.08	0.20±0.05	0.40±0.03
Renal SOD	0.25±0.04	0.20±0.02	0.20±0.02	0.23±0.07	0.27±0.12

[a]Significant (P<0.05) increase compared with control within row; [b]Significant (P<0.05) decrease compared with L-NAME within row; *Significant (P<0.05) decrease compared with control group; #Significant (P<0.05) increase compared with L-NAME within row. GPx: Glutathione peroxidase (units/mg protein); GST: Glutathione S-transferase (mmole 1-chloro-2, 4-dinitrobenzene-GSH complex formed/min/mg protein); CAT: Catalase activity (μmoles of H_2O_2 consumed/min/mg protein); SOD: Superoxide dismutase (units/mg protein); GSH: Reduced glutathione; L-NAME: N$^{\omega}$-nitro-L-arginine methyl ester

Table 5: Effect of *Azadirachta indica* on blood pressure of rats

Groups	Control	L-NAME	L-NAME + AI1	L-NAME + AI2	L-NAME + enalapril
SBP (mmHg)	118.14±5.52	162.57±7.85[a]	147.38±7.94[b]	144.33±7.94[b]	121.17±7.83[b]
DBP (mmHg)	82.00±4.80	129.00±18.16[a]	110.00±11.46	86.17±9.33[b]	96.17±6.71[b]
MAP (mmHg)	93.71±4.68	137.00±15.00[a]	122.00±12.40[b]	105.33±6.89[b]	104.33±7.00[b]

[a]Significant (P<0.05) increase compared with control; [b]Significant (P<0.05) decrease compared with L-NAME. SBP: Systolic blood pressure; DBP: Diastolic blood pressure; MAP: Mean arterial blood pressure; L-NAME: N$^{\omega}$-nitro-L-arginine methyl ester

AI leaf extract. This observation from our study is in agreement with an earlier report that suggests an inverse relationship between blood pressure and linolenic acid intake[43] Other studies have also reported the anti-inflammatory and cardioprotective effects of some essential oils from AI.[44] Recently, Rhee *et al.* reported that intakes of tuna and dark fish, α-linolenic acid, and marine omega-3 FAs were not associated with risk of major cardiovascular disease.[45] However, attention has been focused recently on beneficial effects of omega-3 FA in cardiovascular disease prevention.[46] Similarly, the anti-atherosclerotic properties of ω-6 (18:2) and ω-3 (18:3) FAs have also been reported.[47] Therefore, the reduction of blood pressure, observed in this study, in the AI treated hypertensive rats might be associated with the presence of linolenic acid in the leaf extract of AI.

Previous studies elsewhere have shown that the pathogenesis of hypertension involves apoptosis and inflammation.[48] Three important enzymes (caspase 8,9,10) are involved in the activation of caspase-3 which is a principal enzyme in the execution phase of apoptosis.[49] Lower expressions of caspase-3 in this study in AI treated rats suggest inhibition of the apoptotic mechanism. In this study, the higher caspase-3 expressions in the tissues of the hypertensive rats suggest apoptosis because caspase-3 is effected in both the extrinsic and intrinsic apoptotic pathways.[50] However, decreased caspase-3 expressions observed in AI treated rats is suggestive of anti-apoptotic property of AI in renal apoptosis associated with hypertension.

CONCLUSION

Methanol extract of *A. indica* therapy has potent antihypertensive effects as shown by the reduction in the blood pressure and cardiorenal oxidative stress elicited via enhancement of the systemic antioxidants and serum bioavailability of NO and reduced expressions of caspase 3 in the kidney of rats. Combining all, leaf extract of *A. indica* may provide viable therapeutic option for hypertension treatment and management especially in poor-resource setting like Africa. Further studies are required for the isolation, characterisation and structural elucidation of antihypertensive agent from *A. indica* leaf extract.

Financial support and sponsorship

The authors acknowledge the financial support received from the Nigerian National Research Foundation of the Tertiary Education Trust Fund. Authors also acknowledged financial support from Cape Peninsula University of Technology (CPUT) granted to Prof OO Oguntibeju.

Conflicts of interest

There are no conflicts of interest.

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