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Ajuga bracteosa Exerts Antihypertensive Activity in L-NAME-Induced Hypertension Possibly through Modulation of Oxidative Stress, Proinflammatory Cytokines, and the Nitric Oxide/Cyclic Guanosine Monophosphate Pathway

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Cite This: *ACS Omega* 2022, 7, 33307−33319 **Read Online**

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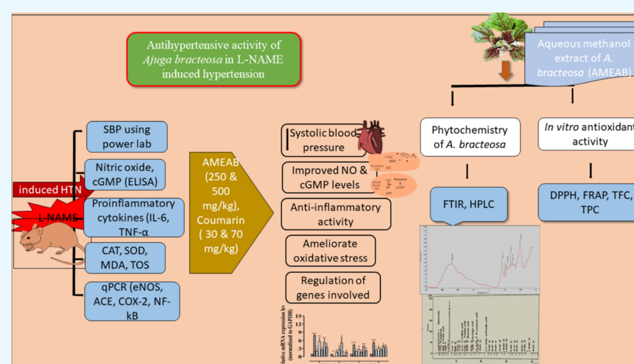

ABSTRACT: *Ajuga bracteosa* has been used in traditional medicine to treat hypertension and other ailments. The present study has been designed to investigate the beneficial effects of *A. bracteosa* in L-nitro arginine methyl ester (L-NAME)-induced hypertensive rats. Hypertension was induced by intraperitoneal injection of L-NAME (185 μmol kg^{-1} i.p.). The aqueous methanol extract of *A. bracteosa* (AMEAB, 250 and 500 mg kg^{-1}) and coumarin (30 and 70 mg kg^{-1}) were administered orally from day 8 to day 35 of the study. *In vivo* antihypertensive activity was assessed by measuring the blood pressure using a PowerLab data system. The effects of the AMEAB and coumarin on nitric oxide (NO), cyclic guanosine monophosphate (cGMP), interleukin-6 (IL-6), the tumor necrosis factor (TNF-α), and oxidative stress markers were also assessed using kit methods. Phytochemical profiling of the AMEAB was carried out through high-performance liquid chromatography (HPLC) where quercetin, gallic acid, caffeic acid, vanillic acid, benzoic acid, syringic acid, *p*-coumaric acid, and ferulic acid were labeled as plant constituents including coumarin. The AMEAB and coumarin significantly reduced blood pressure at the tested doses of 500 and 70 mg kg^{-1}, respectively. Serum levels of NO and cGMP were found to be significantly increased in AMEAB- and coumarin-treated groups when compared with only L-NAME-challenged rats. In addition, a marked decrease was noticed in the serum concentrations of proinflammatory cytokines (IL-6 and TNF-α) in AMEAB- and coumarin-treated rats. Moreover, in AMEAB- and coumarin-treated animals, a noticeable improvement was observed in the levels of antioxidant enzymes including catalase, superoxide dismutase, and malonaldehyde, and the total oxidant status when compared with those of only L-NAME-challenged rats. The data of real-time polymerase chain reaction (RT-PCR) experiments supported that the antihypertensive and anti-inflammatory activities of the AMEAB and coumarin are possibly mediated through modulation of endothelial nitric oxide synthase (eNOS), angiotensin-converting enzyme (ACE), nuclear factor (NF)-kB, and COX-2 gene expressions. This study concludes that *A. bracteosa* possesses an antihypertensive effect mediated through the modulation of the antioxidant, anti-inflammatory, and NO/cGMP pathways, thus providing a rationale to the antihypertensive use of *A. bracteosa* in traditional medicine.

1. INTRODUCTION

Cardiovascular diseases affect 1.39 billion adults and cause around 10.4 million deaths annually. Among these ailments, hypertension is the major contributing factor.[1] Hypertension is augmented by multiple variables including genetic, sociodemographic, and behavioral factors.[2] Alarmingly, in the developing world, data represent that there will be an expected increase of 30% in the prevalence of hypertension by the year 2025.[2] During recent years, a modest decrease in the prevalence of hypertension has been observed in high-income countries, while low- and middle-income countries have experienced a marked increase.[3] Uncontrolled hypertension may lead to cardiovascular complications including development of stroke, myocardial infarction (MI), ischemia, retinal, and renal problems. The use of modern medicines to treat clinical hypertension is usually associated with several side effects including tiredness, bradycardia, postural hypotension, cold

Received: June 21, 2022
Accepted: August 30, 2022
Published: September 8, 2022

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Table 1. *In Vitro* Antioxidant Activities of the Aqueous Methanol Extract of *A. bracteosa* (AMEAB)[a]

TPC mg GAE g^{-1} dry weight	TFC mg catechin g^{-1} dry weight	DPPH IC_{50} ($\mu g\ mL^{-1}$)		FRAP assay (nmol Fe^{+2} equiv mg^{-1} dry extract)
AMEAB	AMEAB	AMEAB	ascorbic acid	AMEAB
488.41 ± 2.65	12.3 ± 0.32	92.5 ± 2.14	4.02 ± 1.66	1693.58 ± 27.52

[a]Results were presented as a mean ± standard error of the mean (SEM) ($n = 3$).

Table 2. FTIR Spectrum Peak Characterization of the Aqueous Methanol Extract of *A. bracteosa*

no	wavenumber cm^{-1} (observed)	wavenumber range cm^{-1} (reference)	primary structure	possible compound	refs
1	3208.00	3000–3600	O–H and N–H stretch	alcohol, phenol, carbohydrates, peroxide	Caunii et al.,[54] Cao et al.[55]
2	1603.42	1600–1706	proteins amide I, C–O, C–N, CNN	proteins	Hands et al.[56]
3	1518.43	1500–1600	aromatic and N–H bending vibrations	amino acids	Caunii et al.,[54]
4	1442.32	1300–1450	primary/secondary O–H bending, phenol or tertiary alcohol	phenyl groups	Coates,[57] Caunii et al.[54]
5	1338.72	1300–1380	CH_3 bending	lipid	Baker et al.[58]
6	1281.27	1280–1350	C–N stretch	aromatic secondary amines	Coates[57]
7	1028.82	1008–1230	stretch of C–O deoxyribose/ribose, DNA, RNA (PO_2^-), C–C stretch, C–H bend	deoxyribose/ribose, DNA, RNA	Hands et al.[56]

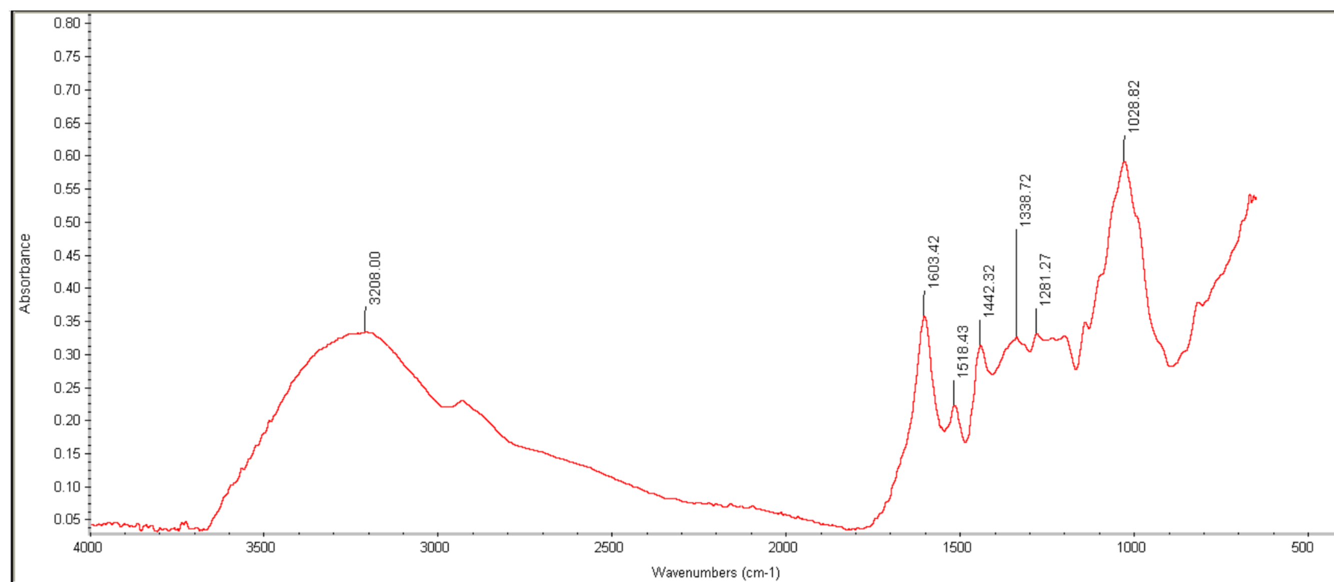

Figure 1. FTIR spectrum of the aqueous methanol extract of *A. bracteosa* measured in an array of 500–4000 cm^{-1} with a resolution of 4 cm^{-1}.

extremities, depression, and nausea.[4] Modification of lifestyle and dietary habbits including the use of natural products can serve as a substitute for synthetic drugs when treating mild to moderate hypertension.[5] The ethnobotanical studies play a pivotal role in the research and development of novel agents, as the literature shows that around 40% of the medications consumed in the modern world originate from natural sources; primarily, these are plant-derived.[4]

The vascular tone is modulated by varied functional factors including nitric oxide (NO), renin-angiotensin-aldosterone system (RAAS), sympathetic nervous system (SNS), reactive oxygen species (ROS), potassium channels, and calcium ions. An imbalance in these contributing factors may lead to increased blood pressure. NO, cardiac output, and peripheral vascular resistance (PVR) play a pivotal role in the pathogenesis of hypertension.[5] A decrease in the NO level causes endothelial dysfunction and oxidative stress. NO deficiency is induced in experimental animals using L-NAME.[6]

Ajuga bracteosa (local name: kori booti), belonging to the family Lamiaceae/Labiatae, is a perennial hairy herb distributed in subtropical regions from Kashmir to Bhutan, including Pakistan. *A. bracteosa* has diverse health benefits in toothaches, malaria, amenorrhea, rheumatism, palsy, and gout.[7] Traditionally, it has been used as astringent, anthelmintic, anti-inflammatory, antimicrobial, blood purifier, carminative, anticough, antiasthma, antijaundice, cooling agent, sore throat, acne, pimples, and headache medication. *A. bracteosa* has also been used as a cooling, diuretic,[8] and blood pressure-lowering agent.[9,10] *A. bracteosa* has been reported to exhibit anti-inflammatory,[11] antiplasmodial,[12] cholinesterase inhibiting,[13] antidiabetic,[14] antioxidant, and antibacterial activities.[15] *In vitro* anti-Alzheimer, cytotoxic, and antileshminial potentials have also been reported.[16] This plant is also known to contain several phytocompounds such as neo-clerodanediterpenoids, flavonol and iridoid glycosides, ergasterol-5-8-endoperoxide, phytoecdysones,[10,17] gamma sitosterol, beta sitosterol, triacontanyldocosanoate, and tetracosanoic acid.[18] Many polyphenols have been reported in the extract of *A. bracteosa* including caffeic acid, chlorogenic acid, *p*-coumeric acid, sinapic acid, gallic acid, salicylic acid, kaempferol, quercetin,

https://doi.org/10.1021/acsomega.2c03888
ACS Omega 2022, 7, 33307–33319

coumarin, resorcinol, ferulic acid, vanillic acid, rutin, and catechin. Pyrocatechol and *trans*-cinnamic acid have also been found in *A. bracteosa*.[7] In addition, this plant has also been reported to contain ajuganane, 3,4′-dihydroxy-3,6,7-trimethoxyflavone, 7-hydroxy-3,6,3′,4′-tetramethoxyflavone, and ursolic acid as phytoconstituents,[17] thus exhibiting enrichment with the diverse nature of phytoingredients.

Although its multiple pharmacological effects have been reported in the literature, the antihypertensive action of *A. bracteosa* has not been studied yet. The objective of the current study is to explore the therapeutic potential of *A. bracteosa* as an antihypertensive agent in L-NAME-induced hypertension rat model. To probe the target, this study covers the chemical characterization of *A. bracteosa* followed by a series of *in vitro* and *in vivo* experiments and molecular docking analysis.

2. RESULTS

2.1. *In Vitro* Antioxidant Potential of *A. bracteosa*. The total phenolic content (TPC) and total flavonoid content (TFC), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ferric reducing antioxidant power (FRAP) assays were carried out based on standard regression lines used for gallic acid, quercetin, ascorbic acid, and $FeSO_4.7H_2O$, as seen in Table 1.

2.2. Fourier Transform Infrared Spectroscopic (FTIR) Analysis of *A. bracteosa*. FTIR peaks with wavenumber ranges and functional groups of the AMEAB were identified as reported previously (Table 2). The FTIR spectrum (Figure 1) revealed absorption signals for seven wavenumbers, which were identified as probable functional groups in the samples, namely, carbohydrates at 3208.00 cm^{-1} (O−H, N−H, and C−O), lipids at 1338.72 cm^{-1} (CH_3), protein at 1603.42 cm^{-1} (amide I of proteins and C−N), amino acid at 1518.43 cm^{-1} (aromatic, N−H), phenyl groups at 1442.72 cm^{-1} (O−H), aromatic secondary amine at 1281.27 cm^{-1}, and deoxyribose/ribose, DNA, and RNA at 1028.82 cm^{-1}.

2.3. High-Performance Liquid Chromatography (HPLC) Analysis of Phenolic Compounds in the Aqueous Methanol Extract of *A. bracteosa* (AMEAB). The AMEAB was analyzed for the presence of different phenols and flavonoids using HPLC. Varied compounds with their retention time were identified and are shown in Table 3.

Table 3. HPLC Analysis of *A. bracteosa*

no	compound name	retention time (min)	area (mV s^{-1})	concentration (ppm)
1	quercetin	2.840	161.885	8.58
2	coumarin	2.990	94.824	132.72
3	gallic acid	4.567	97.340	3.51
4	caffeic acid	12.413	151.697	6.98
5	vanillic acid	13.387	46.884	2.91
6	benzoic acid	14.527	203.861	21.52
7	syringic acid	16.667	266.173	6.65
8	*p*-coumaric acid	18.100	594.092	7.73
9	ferulic acid	22.060	938.079	6.34

Phenolic compounds including gallic acid, caffeic acid, vanillic acid, benzoic acid, syringic acid, *p*-coumeric acid, ferulic acid, and coumarin were identified, while quercetin was expressed as flavonoid. Coumarin (132.72 mg kg^{-1} of the dry plant material) was found to be a major compound in the test material followed by benzoic acid (21.52 mg kg^{-1} of the dry plant material) and quercetin (8.58 mg kg^{-1} of the dry plant material). Vanillic acid (2.91 mg kg^{-1} of the dry plant material) was found in a lesser proportion. The obtained chromatogram of the AMEAB is shown in Figure 2a, while that of coumarin is shown in Figure 2b.

2.4. Molecular Docking Analysis to Support the Antihypertensive Activity of *A. bracteosa*. The affinity among the protein targets and the ligands was investigated using molecular docking. The AutoDock Vina program was used for the docking analysis through the PyRx user interface. The *E*-value (kcal mol^{-1}) was used to assess the affinity of the protein and best-docked pose complex. It has provided a prediction of binding free energy and binding constant for docked ligands (Table 4). Ajuganane, coumarin, and flavone formed stable complexes with nitric oxide synthase (PDB ID: 1M9K) and displayed binding energies of −7.2, −7.7, and −9.5 kcal mol^{-1} in comparison to that of 7-nitroindazole (−6.5 kcal mol^{-1}), respectively. Binding interactions of ajuganane, coumarin, flavone, and 7-nitroindazole with different amino acid residues of the binding site of nitric oxide synthase (PDB ID: 1M9K) are presented in the Supporting Information (Figures S2−S5). Furthermore, ajuganane, coumarin, and flavone formed stable complexes with the angiotensin-converting enzyme (PDB ID: 1O86) and displayed binding energies of −6.1, −6.0, and −7.9 kcal mol^{-1} in comparison to that of captopril, −5.3 kcal mol^{-1}, respectively. Binding interactions of ajuganane, coumarin, flavone, and captopril with different amino acid residues of the binding site of the angiotensin-converting enzyme (PDB ID: 1O86) are presented in the Supporting Information (Figures S6−S9).

2.5. Effect of *A. bracteosa* Administration on Sytolic Blood Pressure (SBP) and Heart Rate (HR). SBP measurements of different animal groups are presented in Figure 3. A significant increase ($p < 0.001$) in SBP (201.47 ± 3.16 mm Hg) of L-NAME-challenged animals was observed compared to SBP (127.63 ± 1.46 mm Hg) of animals receiving distilled water. Captopril (25 mg kg^{-1}), coumarin (30 and 70 mg kg^{-1}), and AMEAB (250 and 500 mg kg^{-1}) caused a marked ($p < 0.001$) reduction in SBP values by 39.09, 38.52, 45.21, 34.76, and 38.16%, respectively, when compared to only hypertensive animals. When recording the heart rate (Figure 4), it is observed that the AMEAB attenuated ($p < 0.01$) the heart rate in treatment groups compared to that in hypertensive animals.

2.6. Effect of *A. bracteosa* on Biochemical Markers. L-NAME administration to animals caused a significant increase in cholesterol, triglyceride (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), createnine, and urea (Table 5). The treatment of animals with the AMEAB and coumarin resulted in marked ($p < 0.001$) reductions in cholesterol, TG, AST, ALT, creatinine, and urea levels, similar to the effect of the standard drug (captopril), when compared with only L-NAME-induced animals as seen in Table 5.

2.7. Effect of *A. bracteosa* on Serum NO and Cyclic Guanosine Monophosphate (cGMP) Levels. Serum levels of NO and cGMP were significantly ($p < 0.001$) decreased in L-NAME-induced hypertensive rat models compared to the assessed NO levels of animals in the normotensive group. Interestingly, such a decrease in the serum levels of NO and cGMP was markedly ($p < 0.05$) prevented by treatment with AMEAB and coumarin as seen in Figure 5a,b, respectively.

2.8. Effect of *A. bracteosa* on the Serum Levels of Proinflammatory Cytokines (IL-6 and TNF-α). Results

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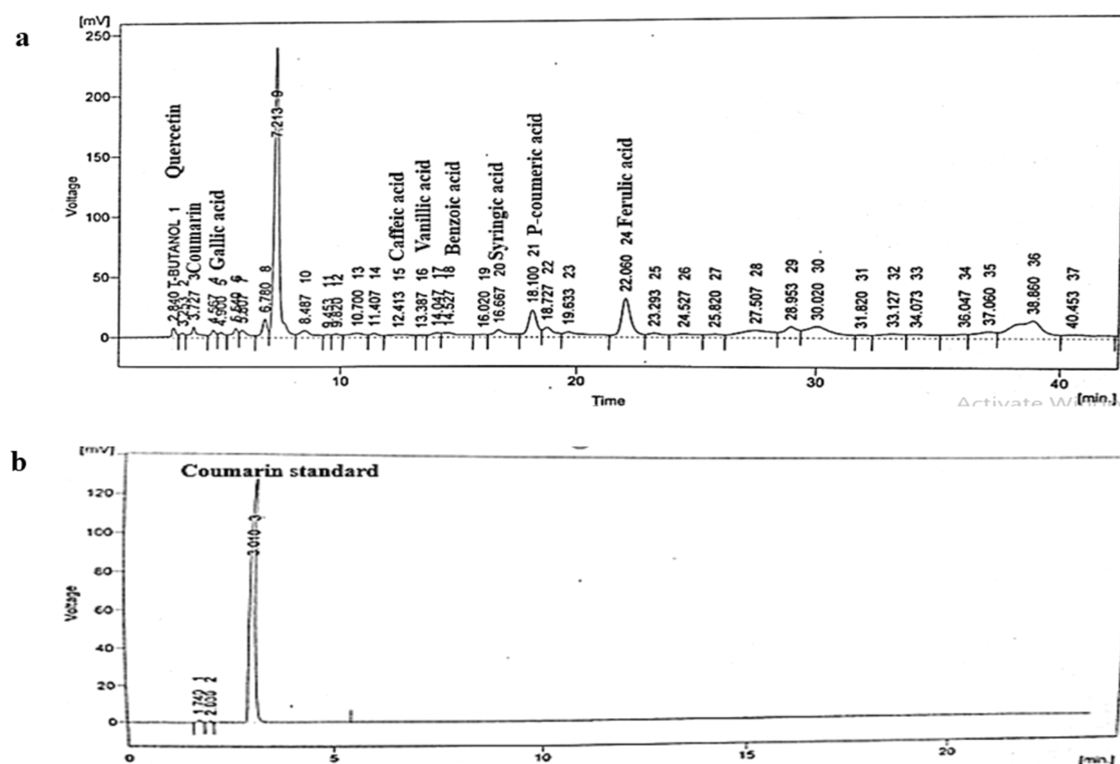

Figure 2. High-performance liquid chromatography fingerprint of the aqueous methanol extract of *A. bracteosa* (AMEAB) (a) and high-performance liquid chromatography fingerprint of coumarin (standard) (b).

Table 4. Binding Affinities of Ligands for Nitric Oxide Synthase (PDB ID: 1M9K) and Angiotensin-Converting Enzyme (PDB ID: 1O86)

compound	nitric oxide synthase (PDB ID: 1M9K)	angiotensin-converting enzyme (PDB ID: 1O86)
ajuganane	−7.2	−6.1
coumarin	−7.7	−6.0
tetramethoxyflavone	−9.5	−7.9
7-nitroindazole	−6.5	
captopril		−5.3

revealed that administration of animals with L-NAME caused a significant ($p < 0.001$) increase in IL-6 and TNF-α concentrations ($p < 0.001$) compared to the data of animals in the normotensive group. However, treatment with AMEAB (250 and 500 mg kg^{-1}) and coumarin (30 and 70 mg kg^{-1}) resulted in a marked ($p < 0.001$) decrease in the respective serum levels of IL-6 and TNF-α compared to the only L-NAME-induced hypertensive animal group as detailed in Figure 6a,b.

2.9. Estimation of Oxidative Stress Biomarkers in the Heart, Liver, and Kidney Tissue Homogenates.

The enzymatic activities of catalase (CAT), sodium oxide dismutase (SOD), malonaldehyde (MDA), and total oxidant status (TOS) were assessed where the activities of CAT and SOD were significantly ($p < 0.001$) decreased, while levels of MDA and TOS were noticeably ($p < 0.001$) increased in the tissue homogenates of L-NAME-induced animals compared to the data of animals in the normotensive control group (Figure 7). Administration of coumarin (30 and 70 mg kg^{-1}) and AMEAB (250 and 500 mg kg^{-1}) significantly ($p < 0.001$) upregulated CAT and SOD activities (Figure 7a,b), while it downregulated MDA and TOS in the selected vital organs of

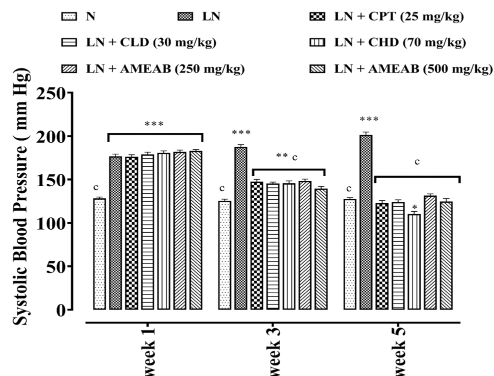

Figure 3. Effect of the aqueous methanol extract of *A. bracteosa* (AMEAB) treatment on systolic blood pressure (SBP) in L-NAME-induced hypertension, where N: normal control; LN: hypertensive control; LN + CPT: L-NAME with captopril (25 mg kg^{-1}); LN + CLD: L-NAME with coumarin (30 mg kg^{-1}); LN + CHD: L-NAME with coumarin (70 mg kg^{-1}); LN + AMEAB (250 mg kg^{-1}): L-NAME with the aqueous methanol extract of *A. bracteosa* (250 mg kg^{-1}); and LN + AMEAB (500 mg kg^{-1}): L-NAME with the aqueous methanol extract of *A. bracteosa* (500 mg kg^{-1}). Values are expressed as mean ± SEM ($n = 6$), ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ compared to the normotensive control group and $^{c}p < 0.001$ compared to the hypertensive control group. Statistical analysis was conducted by performing two-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test.

interest as seen in Figure 7c,d. Administration of coumarin and AMEAB significantly upregulated the CAT activity in the organ (heart, liver, and kidney) homogenates (Figure 7a). Both coumarin and AMEAB at both tested doses caused a considerable increase in the SOD activity of heart and liver tissue homogenates, whereas in the kidney homogenate, SOD

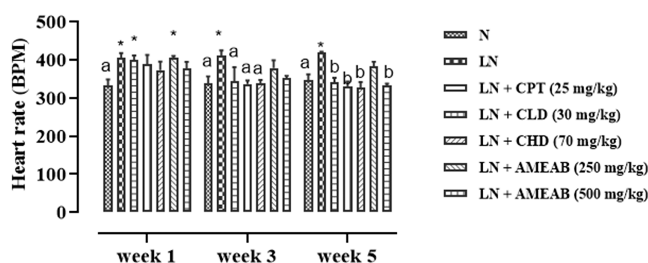

Figure 4. Effect of the aqueous methanol extract of *A. bracteosa* (AMEAB) treatment on the heart rate (HR) beats min^{-1} in L-NAME-induced hypertension, where N: normal control; LN: hypertensive control; LN + CPT: L-NAME with captopril (25 mg kg^{-1}); LN + CLD: L-NAME with coumarin (30 mg kg^{-1}); LN + CHD: L-NAME with coumarin (70 mg kg^{-1}); LN + AMEAB (250 mg kg^{-1}): L-NAME with the aqueous methanol extract of *A. bracteosa* (250 mg kg^{-1}); and LN + AMEAB (500 mg kg^{-1}): L-NAME with the aqueous methanol extract of *A. bracteosa* (500 mg kg^{-1}). Values are expressed as mean ± SEM ($n = 6$), $^{*}p < 0.05$ compared to the normotensive control group and $^{a}p < 0.05$ and $^{b}p < 0.01$ compared to the hypertensive control group. Statistical analysis was performed by applying two-way ANOVA followed by the Bonferroni post hoc test.

activity was found significantly ($p < 0.001$) increased in the coumarin-treated group at both tested doses, while a relatively lesser improvement ($p < 0.05$) was found at only a higher dose (500 mg kg^{-1}) of AMEAB-treated animals compared to the data of hypertensive animals. The observed increase in SOD activity proposes that these treatments had an effective defending mechanism in response to ROS (Figure 7b). Interestingly, the distressing effects of lipid peroxidation in L-NAME-induced hypertension animals were decreased significantly ($p < 0.001$) with administration of the AMEAB and coumarin (Figure 7c). The levels of TOS in the heart, liver, and kidney homogenates were increased ($p < 0.001$) significantly (Figure 7d).

2.10. Effect of *A. bracteosa* Administration on Endothelium Dysfunction. The vascular response to acetylcholine, an endothelium-dependent vasodilator, was measured in aortic rings to access the integrity of the endothelium. L-NAME-induced hypertensive animal models exhibited a significant decrease in acetylcholine-mediated relaxation of aortic rings, while the treatment of the AMEAB (500 mg kg^{-1}) and coumarin (70 mg kg^{-1}) caused a considerable ($p < 0.01$) improvement in acetylcholine-mediated vasorelaxation when compared with only hypertensive animals (Figure 8).

2.11. Effect of *A. bracteosa* Treatment on mRNA Expression Levels. The expression of genes involved in the regulation of blood pressure was evaluated by RT-PCR. GAPDH was designated as an internal control. The expression of mRNA levels of the angiotensin-converting enzyme (ACE), COX-2, and NF-kB were pronouncedly ($p < 0.001$) increased, while the expression of endothelial nitric oxide synthase (eNOS) was markedly ($p < 0.01$) decreased in in L-NAME-challenged hypertensive rats compared to normal rats. The values of ACE, COX-2, and NF-kB gene expressions in animals at a high dose of coumarin (70 mg kg^{-1}) and AMEAB (500 mg kg^{-1}) were significantly ($p < 0.001$) decreased, while the expression of eNOS genes was markedly ($p < 0.001$) increased compared to the data of the hypertensive animal group. The resultant modulation in the studied gene expression was also found to be in line with that observed in animals on captopril (Figure 9).

3. DISCUSSION

Herbal remedies are being increasingly consumed by the public in Eastern and Western countries due to their frequent availability, acceptance, affordability, and relative safety. Modern-day revival of herbal remedies for the management of hypertension is well documented. This study is conducted to evaluate the antihypertensive activity of *Bracteosa* in L-NAME-induced hypertensive rats. Its effects on proinflammatory cytokines, oxidative stress biomarkers, endothelial modulating, and NO/cGMP pathways were studied. Hypertension in experimental settings results in a decrease in the NO/cGMP level and an increase in renin-angiotensin-aldosterone and sympathetic activities, which result in increased resistance for blood flow through the vessels, thus promoting the development of hypertension.[37] After induction of hypertension with L-NAME, a significant increase in blood pressure compared to that in normotensive rats was observed, which was found consistent with the previous studies. L-NAME-induced hypertension is associated with reduced endothelial relaxations, damage to cardiac and aortic tissues,

Table 5. Effect of the Aqueous Methanol Extract of *A. bracteosa* on Serum Biochemical Biomarkers in L-NAME-Induced Hypertensive Rat Models[a]

groups	cholesterol (mg dL^{-1})	triglyceride (mg dL^{-1})	ALT (U L^{-1})	AST (U L^{-1})	creatinine (mg dL^{-1})	urea (mg dL^{-1})
normal	79.87 ± 1.43^{c}	80.15 ± 1.36^{c}	43.91 ± 1.03^{c}	73.00 ± 1.25^{c}	0.85 ± 0.07^{a}	29.5 ± 1.68^{c}
L-NAME hypertensive	$97.29 \pm 3.41^{***}$	$104.76 \pm 2.71^{***}$	$61.04 \pm 3.75^{***}$	$121 \pm 6.43^{***}$	$1.6 \pm 0.34^{*}$	$56.6 \pm 4.21^{***}$
LN + CPT (25 mg kg^{-1})	82.34 ± 1.54^{c}	85.11 ± 2.14^{c}	47.05 ± 2.84^{b}	$89.02 \pm 2.36^{*c}$	0.94 ± 0.22	31.3 ± 1.84^{c}
L-NAME + CLD (30 mg kg^{-1})	84.14 ± 2.86^{b}	91.67 ± 3.28^{a}	53.15 ± 3.22	$94 \pm 3.78^{**c}$	1.40 ± 0.22	39.2 ± 5.34^{b}
L-NAME + CHD (70 mg kg^{-1})	81.32 ± 2.56^{c}	87.53 ± 2.35^{b}	49.58 ± 1.79^{a}	86.24 ± 2.66^{c}	1.10 ± 0.08	36.34 ± 4.42^{b}
L-NAME + AMEAB (250 mg kg)	86.41 ± 1.34^{b}	$93.70 \pm 5.82^{*}$	$58.00 \pm 2.92^{**}$	$106.34 \pm 4.32^{***}$	1.05 ± 0.12	$43.2 \pm 2.80^{*a}$
L-NAME + AMEAB (500 mg kg^{-1})	83.5 ± 1.89^{c}	89.07 ± 2.73^{b}	$55.55 \pm 1.98^{*}$	$98.04 \pm 5.52^{***b}$	0.96 ± 0.15	38.6 ± 1.60^{b}

[a]N: normal control; LN: hypertensive control; LN + CPT: L-NAME with captopril (25 mg kg^{-1}); LN + CLD: L-NAME with coumarin (30 mg kg^{-1}); LN + CHD: L-NAME with coumarin (70 mg kg^{-1}); LN + AMEAB (250 mg kg^{-1}): L-NAME with the aqueous methanol extract of *A. bracteosa* (250 mg kg^{-1}); and LN + AMEAB (500 mg kg^{-1}): L-NAME with the aqueous methanol extract of *A. bracteosa* (500 mg kg^{-1}). Values are expressed as mean ± SEM ($n = 6$), $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$ as compared to the normotensive control group and $^{c}p < 0.001$, $^{b}p < 0.01$, and $^{a}p < 0.05$ as compared to the hypertensive control group. Statistical analysis was carried out by one-way ANOVA followed by the Dunnett post hoc test.

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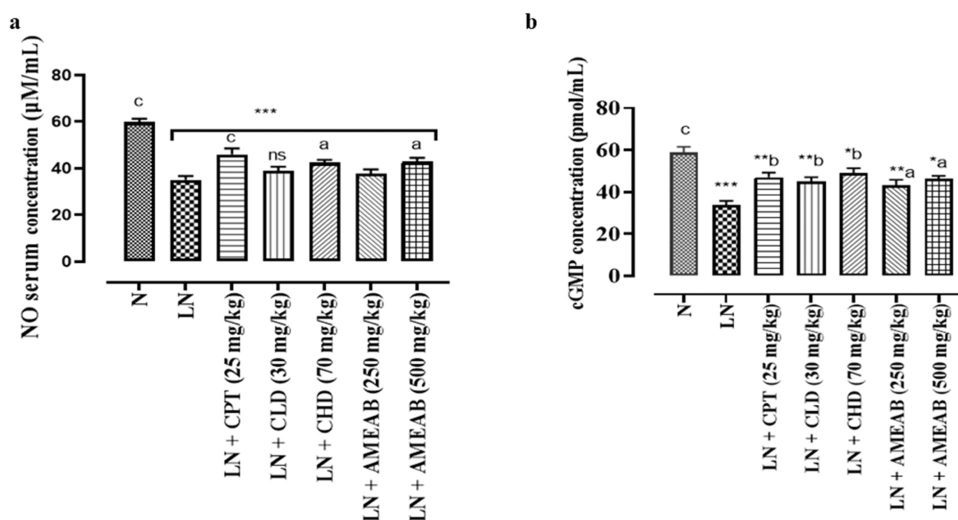

Figure 5. Effect of the aqueous methanol extract of *A. bracteosa* (AMEAB) treatment on serum NO (a) and cGMP (b) in L-NAME-induced hypertension, where N: normal control; LN: hypertensive control; LN + CPT: L-NAME with captopril (25 mg kg^{-1}); LN + CLD: L-NAME with coumarin (30 mg kg^{-1}); LN + CHD: L-NAME with coumarin (70 mg kg^{-1}); LN + AMEAB (250 mg kg^{-1}): L-NAME with the aqueous methanol extract of *A. bracteosa* (250 mg kg^{-1}); and LN + AMEAB (500 mg kg^{-1}): L-NAME with the aqueous methanol extract of *A. bracteosa* (500 mg kg^{-1}). Values are expressed as mean ± SEM ($n = 6$), $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$ compared to the normotensive control group and $^{c}p < 0.001$, $^{b}p < 0.001$, $^{a}p < 0.05$, and ns = nonsignificant compared to the hypertensive control. Statistical analysis was performed using one-way ANOVA followed by the Dunnett post hoc test.

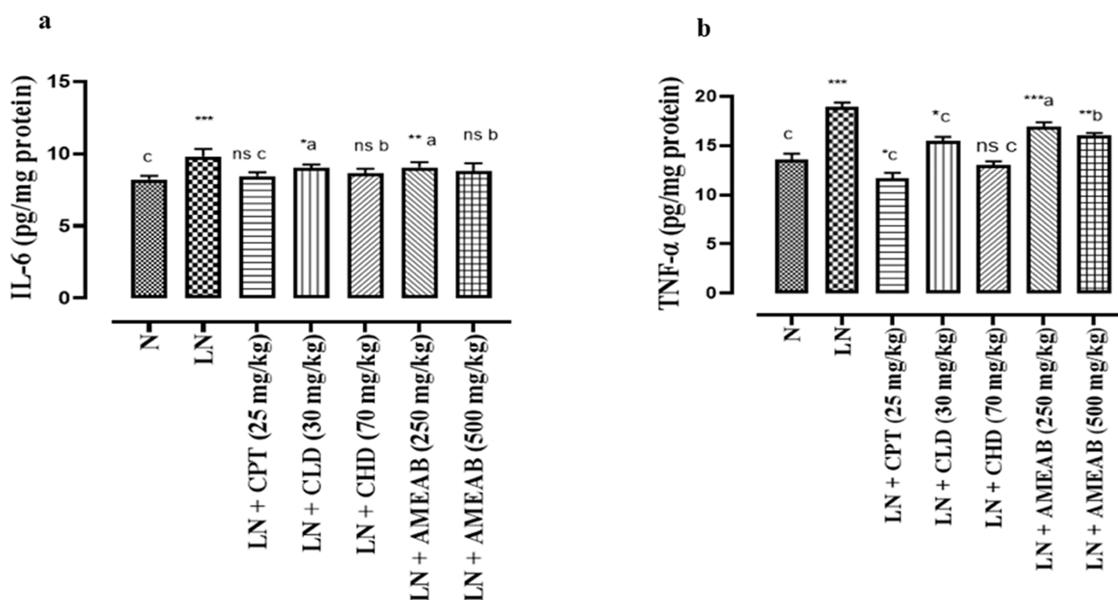

Figure 6. Effect of the aqueous methanol extract of *A. bracteosa* (AMEAB) treatment on proinflammatory cytokine: IL-6 (a) and TNF-α (b) in L-NAME-induced hypertension, where N: normal control; LN: hypertensive control; LN + CPT: L-NAME with captopril (25 mg kg^{-1}); LN + CLD: L-NAME with coumarin (30 mg kg^{-1}); LN + CHD: L-NAME with coumarin (70 mg kg^{-1}); LN + AMEAB (250 mg kg^{-1}): L-NAME with the aqueous methanol extract of *A. bracteosa* (250 mg kg^{-1}); and LN + AMEAB (500 mg kg^{-1}): L-NAME with the aqueous methanol extract of *A. bracteosa* (500 mg kg^{-1}). Values are expressed as mean ± SEM ($n = 6$), $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, and ns = nonsignificant compared to the normotensive control group and $^{c}p < 0.001$, $^{b}p < 0.01$, and $^{a}p < 0.05$ compared to the hypertensive control. Statistical analysis was performed by applying one-way ANOVA followed by the Dunnett post hoc test.

and fibrosis in the renal vascular system.[38] The observed protection ability of *A. bracteosa* against L-NAME-induced hypertension indicates that it may affect directly or indirectly the NO production or its activity. In molecular docking analysis, *A. bracteosa* constituents were docked against the potential targets of hypertension including NOS and ACE. Principal secondary metabolites of the plants also showed compatibility with these targets. Molecular docking studies supported the existence of pharmacological effects of the phytoconstituents on hypertension.

FTIR analysis is a useful and nondestructive method to obtain the basic information about functional groups in different phytochemicals of the plant extract. The absorption band for *A. bracteosa* appearing in the range of 500−4000 cm^{-1} is due to the presence of functional groups such as carboxylic acid, phenolics, esters, and saccharides.[39] Polyphenols are known to have beneficial effects on hypertension by acting as

https://doi.org/10.1021/acsomega.2c03888
ACS Omega 2022, 7, 33307−33319

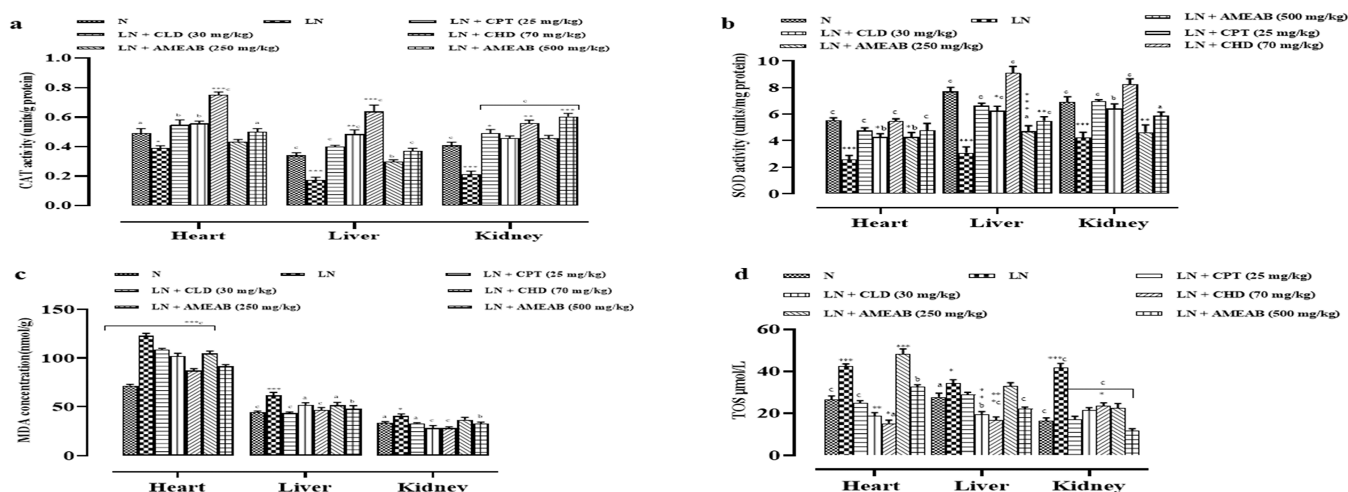

Figure 7. Effect of the aqueous methanol extract of *A. bracteosa* (AMEAB) treatment on CAT (a), SOD (b), MDA (c), and TOS (d) in L-NAME-induced hypertension. Where N: normal control; LN: L-NAME hypertensive control; LN + CPT: L-NAME with captopril (25 mg kg^{-1}); LN + CLD: L-NAME with coumarin (30 mg kg^{-1}); LN + CHD: L-NAME with coumarin (70 mg kg^{-1}); LN + AMEAB (200 mg kg^{-1}): L-NAME with the aqueous methanol extract of *A. bracteosa* (250 mg kg^{-1}); and LN+ AMEAB: L-NAME with the aqueous methanol extract of *A. bracteosa* (500 mg kg^{-1}). Values expressed as mean ± SEM ($n = 6$), $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$ compared to the normotensive control and $^{c}p < 0.001$, $^{b}p < 0.01$, and $^{a}p < 0.05$ compared to the hypertensive control. Statistical analysis was carried out by applying one-way ANOVA followed by the Dunnett post hoc test.

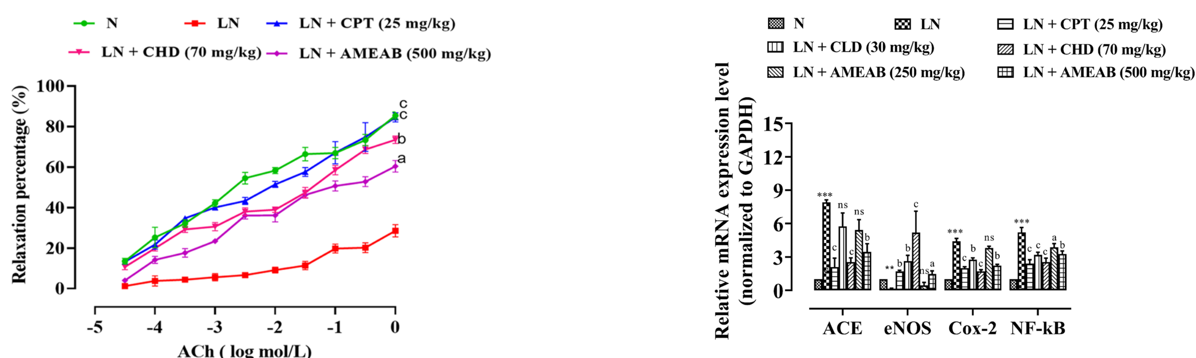

Figure 8. Effect of the aqueous methanol extract of *A. bracteosa* (AMEAB) treatment on the cumulative dose response to acetylcholine relaxation of aortic rings against phenylephrine preconstruction, where N: normal control; LN: hypertensive control; LN + CPT: L-NAME with captopril (25 mg kg^{-1}); LN + CHD: L-NAME with coumarin (70 mg kg^{-1}); and LN + AMEAB (500 mg kg^{-1}): L-NAME with the AMEAB (500 mg kg^{-1}). The results are expressed as mean ± SEM, where $^{a}p < 0.05$, $^{b}p < 0.01$, and $^{c}p < 0.001$ *vs* hypertensive group.

Figure 9. Effect of the aqueous methanol extract of *A. bracteosa* (AMEAB) treatment on ACE, eNOS, COX-2, and NF-kB mRNA expression in L-NAME-induced hypertension, where N: normal control; LN: hypertensive control; LN + CPT: L-NAME with captopril (25 mg kg^{-1}); LN + CLD: L-NAME with coumarin (30 mg kg^{-1}); LN + CHD: L-NAME with coumarin (70 mg kg^{-1}); LN + AMEAB (250 mg kg^{-1}): L-NAME with the AMEAB (250 mg kg^{-1}); and LN + AMEAB (500 mg kg^{-1}): L-NAME with the AMEAB (500 mg kg^{-1}). Values are expressed as mean ± SEM ($n = 6$), $^{***}p < 0.001$ and $^{**}p < 0.01$ as compared to the normotensive control and $^{c}p < 0.001$, $^{b}p < 0.01$, $^{a}p < 0.05$, and ns = nonsignificant compared to the hypertensive control. Statistical analysis was performed by one-way ANOVA followed by the Dunnett post hoc test.

antioxidant, anti-inflammatory, and endothelium-modulating agents[40] The HPLC analysis of the *A. bracteosa* extract identified qurecetin, gallic acid, caffeic acid, vanillic acid, benzoic acid, syringic acid, *p*-coumaric acid, ferulic acid, and coumarin, which forms the basis for the use of *A. bracteosa* as an antihypertensive agent. As mentioned, the *A. bracteosa* extract used in the present study contained flavonoids including quercetin. Previous studies have shown that quercetin was able to improve the endothelial function by increasing NO production.[41] Gallic acid has also been shown to reduce blood pressure through improving oxidative stress.[42] In addition to gallic acid, coumarin also offers antioxidant and vasorelaxant activities.[43] Additionally, *p*-coumaric acid may also act as an anti-inflammatory agent.[44] The presence of such phytoconstituents with a diverse pharmacological profile also support the determined antihypertensive potential of *A. bracteosa*.

The current study suggests that *A. bracteosa* with varied bioactive compounds might provide a basis to *A. bracteosa* as a candidate for the prevention of cardiovascular-related ailments in L-NAME-induced hypertension models. To date, this is the first study providing the therapeutic capacity of *A. bracteosa* against hypertension and associated oxidative stress, inflammation, and endothelial dysfunction. Results of the present study showed that administration of L-NAME to experimental animals caused a marked elevation in systolic blood pressure and developed dyslipidemia, which is in agreement with earlier findings on this model.[6] Concomitant treatment of animals

with L-NAME and *A. bracteosa*/coumarin moderated the increase in systolic blood pressure, decreased the total cholesterol level, and increased the diminished NO level observed when compared with only hypertensive animals. These observations suggest that the *A. bracteosa* extract and coumarin ameliorated L-NAME-induced hypertension in rats. NO plays a major role in the activation of cGMP-dependent protein kinase through guanylyl cyclase (sGC), hence causing relaxation of the smooth muscles of the blood vessels.[45] The mechanism of action of L-NAME involves elevation of serum free fatty acid concentrations by lowering the activity of the enzymes responsible for oxidizing fatty acids. Carnitine palmitoyltransferase helps in the development of hyperlipidemia. Reduced fatty acid oxidation may explain the increase in serum triglycerides and cholesterol.[41] In the present study, the antihyperlipidemic effects of *A. bracteosa* and coumarin were also investigated. It is therefore possible that the antihyperlipidemic potential of *A. bracteosa* is partly due to stimulation of fatty acid oxidation, and the presence of phytoconstituents may play an additional role in lipid metabolism regulations, as hypolipidemic activity of coumarin is possibly mediated through vasodilation in addition to oxidation.[46]

Chronic vascular inflammation is one of the key factors contributing to the development of hypertension. Through generation of reactive oxygen species (ROS), inflammation promotes endothelial dysfunction and atherosclerosis. Also, proinflammatory cytokines including IL-6 and TNF-α are released massively, while the availability of NO remains limited.[47] By inhibiting ROS, the endothelial function is improved, resulting in a reduction in blood pressure through increased NO production.[48] In our study, the L-NAME-induced hypertensive group exhibited reduced NO/cGMP levels in comparison to that in treatment groups. *A. bracteosa* and coumarin attenuated L-NAME-induced hypertension possibly through the NO/cGMP-mediated pathway.

The pathogenesis of hypertension has been linked to oxidative stress. Several studies have shown that hypertensive animals possess increased levels of lipid peroxidation and low levels of endogenous antioxidant enzymes. The influence of oxidative stress on animal models of L-NAME-induced hypertension is one of the possible mechanisms. The link between ROS production and RAS activation has been demonstrated in rats treated with L-NAME, where eNOS uncoupling has been demonstrated as a major source of superoxide production.[49] It has been found that NO-deficient hypertensive rats have low levels of antioxidant enzymes, including superoxide dismutase and catalase.[31] Polyphenols are involved in providing fortification against diabetes, cardiovascular, and neurodegenerative diseases. ROS is produced by NADPH oxidase. High levels of ROS contribute to vascular diseases including hypertension, vascular hypertrophy, and dysfunction by decreasing the NO availability. Natural phenolic compounds have high antioxidant ability to inhibit NADPH oxidase activity. Quercetin improves the endothelial function by improving endothelial NO synthase (eNOS) activity.[50] Coumarin produced antioxidant effects through the hydrogen atom transfer mechanism by removing free oxygen radicals. In some cardiovascular diseases, there is an imbalance between the calcium influx and potassium efflux. Natural coumarin relieved the cytoplasmic Ca^{2+} overload by maintaining the calcium level and mitochondrial stability. This might result in biological effects such as blood pressure lowering and antiarrhythmic and negative ionotropic activities.[51] Altered serum lipids and oxidative stress are well associated with the pathogenesis of cardiovascular diseases. Coumarin has been reported to have antioxidant and lipid lowering effects.[46]

During our study, there was a marked change in the gene expression (ACE, eNOS, COX-2, and NF-kB) of experimental rats treated with L-NAME. Administration of *A. bracteosa* and coumarin led to the downregulation of ACE and upregulation of eNOS expression levels. Inhibition of ACE is possibly due to the antioxidant activity of plant phytoconstituents. These phytoconstituents form bonds with the zinc atom present on the catalytic site of ACE. The finding of our study that the extract of *A. bracteosa* contains compounds such as caffeic acid and quercetin possessing effective antioxidant activity supports this hypothesis. Also, caffeic acid has been reported to inhibit ACE activity.[52] Compounds with antioxidant activity such as *p*-coumaric acid, benzoic acid, vanillic acid, and caffeic acid may possibly increase the expression of eNOS.[53] Similarly, the observed overexpression of COX-2 and NF-kB in the heart tissue might be responsible for the production of reactive oxygen species in the hypertensive rats. Treatment with *A. bracteosa* and coumarin suppresses COX-2 and NF-kB, which may also be contributing to its blood pressure lowering effect through the anti-inflammatory pathway. Our results were found to be consistent with those of an earlier study, showing that *A. bracteosa* possesses anti-inflammatory activity that might have been facilitated through cyclooxygenase inhibition.[11]

4. CONCLUSIONS

This study revealed that *A. bracteosa* and coumarin possess antihypertensive effects when tested in L-NAME-induced hypertension. These effects were found to be possibly mediated through the endothelial modulatory NO/cGMP pathway with an additional influence on oxidative stress (CAT, SOD, MDA, and TOS) and inflammatory biomarkers (IL-6 and TNF-α). The observed downregulation of candidate genes such as ACE, NF-kB, and COX-2 and upregulation of eNOS also provide sound pharmacological basis to the use of *A. bracteosa* in hypertension. Thus, this study proves *A. bracteosa* to be a potential candidate for the treatment of hypertension and related pathologies.

5. MATERIALS AND METHODOLOGY

5.1. Chemicals and Drugs. Analytical-grade chemicals and drugs were used. Captopril, coumarin, methanol, *N*(*G*)-nitro-L-arginine methyl ester (L-NAME), acetylcholine (ACh), and coumarin were purchased from Sigma Aldrich.

5.2. Collection of the Plant Material. The plant material was collected and identified by Dr. Sardar Irfan Mehmood, Department of Botany, Government Boys' Degree College Abbasapur, Poonch, Azad Kashmir, Pakistan (Voucher specimen no: AJKH.3001).

5.3. Preparation of the Extract. The whole plant was airdried under the shade away from direct sunlight. The plant material was ground into a coarse powder. A subsequent maceration of the powder was performed in aqueous methanol (80% v/v) for 3 days with occasional stirring. To filter the soaked material, muslin cloth and Whatman filter paper were used. Using a rotary evaporator at 40 °C under reduced pressure, the combined filtrate was evaporated to obtain the required plant extract.[19]

5.4. Animals and Diets. Wistar albino rats of age 6–8 weeks; 220–250 g body weight, were used in the study. The animals were kept in a controlled environment at 22–25 °C. The rats were acclimatized for at least a week before starting any experimentation. Food and water were made available to the animals freely. Laboratory animals were housed following the principles of laboratory animal housing (NIH publication no. 85-23, revised in 1985). All the experimental procedures were approved by the Ethical Committee for Animal Experimentation of Government College University, Faisalabad, Pakistan (IRB: ref no. GCUF/ERC/2263/20-11-20).

5.5. Total Phenolic Content (TPC). By following the Folin and Ciocalteu method,[20] a 1 mL sample, 5 mL of Folin–Ciocalteu, and 4 mL of 20% sodium carbonate were mixed and incubated for an hour. A blue color complex was formed, and absorbance was measured at a wavelength of 765 nm. Gallic acid solution in methanol at different concentrations (0.01–0.10 mg mL^{-1}) was used for the preparation of the standard curve. Next, 1 mL aliquots of each concentration in methanol were mixed with 4 mL of sodium carbonate and 5 mL of reagent. Absorbance was measured at 765 nm after a 1 h incubation period. The total phenolic content was estimated using the standard curve method.[21]

5.6. Total Flavonoid Content (TFC). A total of 0.5 mL (25, 50, and 100 μg mL^{-1}) of catechin (standard), 1.5 mL of ethanol (95%), 0.1 mL of aluminum chloride (10%), 0.1 mL of potassium acetate (1M), and 2.8 mL of distilled water (D.W.) were incubated at room temperature for 30 min. Using a spectrophotometer at a wavelength of 415 nm, absorption of standard mixtures was observed. Similarly, 0.5 mL of *A. bracteosa* extract solution was reacted with $AlCl_3$ for the estimation of the flavonoid content. For the blank, $AlCl_3$ was replaced with distilled water.[22]

5.7. Antioxidant Activity of the AMEAB. *5.7.1. DPPH Assay.* Stock solutions of the *A. bracteosa* extract (10 mg mL^{-1}), ascorbic acid, and DPPH (200 μmol L^{-1} in methanol) were prepared. Different concentrations of the *A. bracteosa* extract (200, 100, 50, 25, 12.5, and 6.25 μg mL^{-1}) were prepared. A total of 100 μL of ascorbic acid dilutions and a sample along with DPPH were added to a 96-well plate and incubated for 30 min in the dark. After the incubation period, absorbance was measured at 517 nm through an ELISA reader (DIA source, Belgium). The IC_{50} value was calculated. Results were expressed as percentage scavenging activity.[22]

5.7.2. FRAP. A 50 μL (sample) + 150 μL FRAP working solution (acetate buffer, TPTZ in HCl, $FeCl_3.6H_2O$, and FRAP reagent) was mixed and incubated for 8 min at room temperature. Absorbance was measured at 600 nm. Scavenging activity was measured against $FeSO_4.7H_20$ as a standard.[22]

5.8. Fourier Transform Infrared Spectroscopic (FTIR) Fingerprint of *A. bracteosa*. *A. bracteosa* extract (1 mg) was weighed and mixed with 100 mg of potassium bromide (KBr). Afterward, this was pressed under 10 psi mechanical pressure to form a tablet and kept in a Petri dish containing desiccants (silica gel). The tablet was fixed in the transmission sample holder of a FTIR instrument (ThermoScientific Nicolet, 6700) with a resolution of 4 cm^{-1} and wavelength range of 500–4000 cm^{-1}. The spectra attained were considered useable only when at least 60% transmission was attained.[23]

5.9. HPLC Analysis of the *A. bracteosa* Extract for Phenolic Compounds and Coumarin. A sample of the *A. bracteosa* extract was prepared for high-performance liquid chromatography analysis by mixing a 50 mg sample in 24 mL of methanol followed by 16 mL of distilled water and 10 mL of 6 M HCl followed by an incubation period at 95 °C of 2 h. The solution was filtered through a 0.45 μm nylon membrane filter. A gradient HPLC Shimadzu, Japan, was used for the separation of phenolics from plant samples using a C118 (shim-pack CLC-ODS) 25 cm × 4.6 mm, 5 μm column. Separation was carried out on a gradient mobile phase (A: water and acetic acid, B: acetonitrile). The flow rate was 1 mL min^{-1}. The gradient used for solvent B was 15% for 0–15 min, 45% for 15–30 min, and 100% for 35–45 min. The HPLC instrument was attached to an UV–visible detector at a wavelength of 280 nm. Results were interpreted by comparing the retention time and the UV–visible peaks previously obtained by injection of standards. The quantification was carried out by external standardization.[24] The established method used for the separation of phenolics and flavonoids *via* HPLC was used as described previously.[25] Similarly, coumarin standardization was done in the isocratic mode using acetonitrile (40)/water (60); v/v. An injection volume of 20 μL at a flow rate of 1 mL min^{-1} was used at 274 nm UV detection. Coumarin quantification in the plant sample was performed by an external standard method by comparing with coumarin (Sigma-Aldrich) as a standard. The stock solution was prepared by mixing 224 mg of the dry extract in a 50 mL solution of methanol/water (80:20).[26]

5.10. Molecular Docking Analysis. The antihypertensive activity was inspected in computational modeling of phytochemicals for their antihypertensive prospective by docking analysis using the Autodock Tools program. The three-dimensional (3D) X-ray crystallized structures of nitric oxide synthase (protein data bank (PDB) ID: 1M9K) and angiotensin-converting enzyme (PDB ID: 1O86) were recovered from the RSCB Protein Data Bank (http://www.rscb.org). The proteins were energy-minimized, and Gasteiger charges were added and saved in the .pdbqt format. The hydrophobicity and Ramachandran graphs were generated using Discovery Studio 4.1 Client (2012). The protein architecture and statistical percentage values of helices, β-sheets, coils, and turns were accessed using VADAR 1.8.[27]

The 3D conformers of ajuganane (CID: 28289865), coumarin (CID: 323), and 7-dihydroxy-3,6,3,4-tetramethoxy-flavone (CID: 96118) were drawn from the ChemSpider and PubChem database, respectively. The compounds were drawn in Discovery Studio Client and saved in the .pdb format as ligands after energy minimization. Autodock tools were used for the preparation of ligands in their most stable conformations. The ligands were saved in the .pdbqt format after the addition of the Kolman and Gasteiger charges. A molecular docking experiment was used for all the synthesized ligands against nitric oxide synthase and angiotensin-converting enzyme using the PyRx virtual screening tool with the Auto Dock VINA Wizard approach.[28]

The grid box center values for nitric oxide synthase (PDB ID: 1M9K) (center X = 15.861, center Y = −8.806, center Z = −22.278) and size values were adjusted (X = 88, Y = 60, Z = 96). The grid box center values for the angiotensin-converting enzyme (PDB ID: 1O86) (center X = −17.328, center Y = 71.184, center Z = 26.348) and size values were adjusted (X = 92, Y = 94, and Z = 112) for a better conformational position in the active region of the target protein. Phytoconstituents were docked individually against nitric oxide synthase and the angiotensin-converting enzyme with a default exhaustiveness value of 50. The predicted docked complexes were evaluated

https://doi.org/10.1021/acsomega.2c03888
ACS Omega 2022, 7, 33307–33319

based on the lowest binding energy values (kcal mol^{-1}). The 3D graphical depictions of all the docked complexes were accomplished using Discovery Studio (2.1.0) (Discovery Studio Visualizer Software, Version 4.0., 2012). Structural analysis of target protein nitric oxide synthase (PDB ID: 1M9K) consisted of 34% helices (275 residues), 24% β-sheets (193 residues), 41% coils (333 residues), 10% turns (84 residues), and a total of 801 amino acid residues. The *R*-value of the particular protein seemed to be 0.256, and the resolution was 2.01 Å. Unit cell dimensions for the lengths were observed to be a = 69.786, b = 91.573, and c = 156.096 with 90° angle for α, β, and γ. The Ramachandran plot confirmed that 98% of the amino acids were in the allowed regions for the phi (φ) and psi (ψ) angles. Similarly, the angiotensin-converting enzyme (PDB ID: 1O86) consisted of 61% helices (354 residues), 6% β-sheets (36 residues), 32% coils (185 residues), 20% turns (120 residues), and a total of 575 amino acid residues. The *R*-value of the selected protein appeared to be 0.220, and the resolution was 2.00 Å. Unit cell dimensions for the lengths were observed to be a = 56.47, b = 84.9, and c = 133.99 with 90° angle for α, β, and γ. The Ramachandran plot confirmed that 98.5% of the amino acids were in the allowed regions for the phi (φ) and psi (ψ) angles. The Ramachandran plots for the target proteins are presented in the Supporting Information (Figure S1).

5.11. Blood Pressure Monitoring in Conscious and Anesthetized Rats. Hypertension was induced by injecting 185 μmol kg^{-1} L-NAME intraperitoneally as an inhibitor of NOS in Wistar albino rats for a duration of 1 week. Only animals with blood pressures greater than 160 mm Hg were selected for further study.[29] Rats were randomly divided into different groups depending on their treatment: 1: albino rats (normotensive) were given normal saline, 2: L-NAME (185 μmol kg^{-1} i.p. twice daily)-induced hypertensive group, 3: L-NAME with captopril (20 mg kg^{-1}) from the 8th day, 4: L-NAME with coumarin (30 mg kg^{-1}), 5: L-NAME with coumarin (70 mg kg^{-1}), 6: L-NAME with the AMEAB (aqueous methanol extract of *A. bracteosa*) (250 mg kg^{-1}), and 7: L-NAME with the AMEAB (500 mg kg^{-1}).

Noninvasive blood pressure (NIBP) and invasive measurement techniques were used to measure blood pressure (BP) using a PowerLab data acquisition system (AD Instrument, Australia). Before the start of the experiment, the animals were trained for 7−10 days. To elude any effect of the circadian cycle, the BP was measured at the same time of the day (11 am to 1 pm). Around 6−7 readings were recorded on average for every trial. Systolic blood pressure (SBP) was measured on day 0, followed by after week 1, 3, and 5. Throughout the experiment, the animals were given a standard diet and water.[30] To anaesthetize, on the terminal day (35th) of the treatment, sodium thiopental (70−90 mg kg^{-1}, i.p.) was administered to the rats. Transducers coupled to chart data systems were used for the blood pressure measurement after the tracheostomy and carotid artery cannulations. Heparinized saline (100 IU mL^{-1}) was injected into a transducer to avoid clotting. The animal's basal blood pressure and heart rate were measured after stabilization.[19]

5.12. Biochemical Measurements. At the end of the scheduled treatment, blood samples were drawn by cardiac puncture and immediately centrifuged at 4000*g* for 10 min. Serum was separated for biochemical analysis of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, urea, triglycerides (TG), and total cholesterol (TC) using biochemical kits (Biosystem, Spain) using a bioanalyzer.[19]

5.13. Estimation of cGMP, NO, and Proinflammatory (IL-6 and TNF-α) Biomarkers. NO and cGMP levels were measured in serum samples using a colorimetric NO assay kit and cGMP enzyme-linked immunosorbent assay kits (ELISA) kits (Elabscience), respectively, as per the manufacturer's protocols.[31] Similarly, the levels of IL-6 and TNF-α in serum were measured by ELISA (Elabscience).[30,32]

5.14. Estimation of Oxidative Stress Markers in Tissue Homogenates. After extracting the blood, the animals were sacrificed with isoflurane. The abdominal area was opened and the heart, liver, and kidneys were removed. The organs were washed with 0.9% of normal saline and kept at −80 °C till further analysis.[33] Next, 100 mg of each organ was homogenized in 0.1 M phosphate buffer solution. The organ homogenates were centrifuged at 9000*g* for 10 min at 4 °C. The supernatant was transferred to clean microcentrifuge tubes and stored at −80 °C until further analysis.[30]

5.15. Total Oxidant Status (TOS). In an acidic environment, the ferrous ion is converted into a ferric ion by xylenol orange with the help of oxidant species. The following test format was used: 225 μL of reagent 1 (R1), 35 μL of sample volume, and 11 μL of reagent 2 (R2). Primary absorbance was taken at 560 nm and a secondary wavelength of 800 nm after 4 min. Sample blank absorbance was taken as the first absorbance before mixing RI and R2. The composition of R1 was 150 μM xylenol orange, 140 mM NaCl, and 1.35 M glycerol in 25 mM hydrogen sulfate solution at pH 1.75 and R2, 5 mM ferrous ion, and 10 mM *o*-dianisideine in 25 mM H_2SO_4 solution. TOS was measured as μM H_2O_2 equiv L^{-1}.[34]

5.16. Estimation of Catalase (CAT) and Superoxide Dismutase (SOD) Activities. CAT activity was calculated at 240 nm using a spectrophotometer (Cecil Instruments, U.K.). The mixture contains 0.05 mL of the supernatant, 1 mL of 30 mM hydrogen peroxide, and 1.95 mL of 50 mM phosphate buffer (pH: 7). A standard curve was prepared using different concentrations of bovine serum albumin (BSA). Similarly, SOD activity was measured at 325 nm spectrophotometrically, with 0.1 mL of the supernatant with 2.8 mL of 0.1 M phosphate buffer (pH: 7.4). Different concentrations of SOD were used to plot the SOD standard curve.[35]

5.17. Estimation of the Malonaldehyde (MDA) Level. To estimate the level of lipid peroxidation (LPO), 0.1 mL of heart, liver, and kidney homogenates was mixed with 0.2 mL of aqueous sodium dodecyl sulfate (8.1%) and 1.5 mL of aqueous thiobarbituric acid (0.8%). The volume was made up to 4 mL using distilled water and heated on a water bath for 60 min at 95 °C. The mixture was cooled by adding 1 mL of distilled water with 5 mL of *n*-butanol and pyridine mixture in a ratio of 15:1 (v/v). Then, the mixture was briskly shaken and centrifuged for 10 min at 3000 rpm. The upper red layer was extracted, and its absorbance was measured at a wavelength of 532 nm. The LPO was expressed in nmol g^{-1} protein.[36]

5.18. *Ex Vivo* Vascular Reactivity. To determine the function/integrity of the endothelium, the *ex vivo* vascular reactivity technique was employed. The aorta of each rat was dissected and cleaned from the surrounding tissues and then cut into rings measuring 3 mm long. Care was taken to avoid any damage to the endothelium. Two stainless-steel wires were inserted into the lumen of the rings, which were held in place using a clip and a transducer. To facilitate the measurement of

https://doi.org/10.1021/acsomega.2c03888
ACS Omega 2022, 7, 33307−33319

isometric force, a resting tension of 1 g was applied. The organ chamber was filled with 10 mL of Krebs−Henseleit (composition in mM: 118 NaCl, 4.7 KCl, 25, $NaHCO_3$, 1.18 $MgSO_4$, 1.18 KH_2PO_4, 2.5 $CaCl_2$, and 5.5 glucose, pH 7.4, maintained at 37 °C and gassed with carbogen: 95% O_2 and 5% CO_2). After every 15 min, the Krebs solution was changed and allowed to equilibrate for approximately 1 h. Then, the aortic rings were contracted with 10^{-6} M phenylephrine, and when the constant contraction was attained, accumulative additions of acetylcholine (10^{-8} to 10^{-4}) were made to measure relaxation.[19]

5.19. Real-Time Polymerase Chain Reaction. RNA was isolated from the heart tissue using the TRIZOL reagent (Invitrogen, Thermo Fisher Scientific) and was quantified using a NanoDrop instrument. The cDNA synthesis kit (molecular biology by ThermoScientific) was used to transcribe RNA into cDNA using the manufacturer's protocol. RT-PCR was performed on a Quant studio 3 detection system, and amplification was undertaken using SYBR green (molecular biology by ThermoScientific). ACE, eNOS, NF-kB, and COX-2 were the genes of interest, which were quantified against GAPDH through real-time PCR. The primer sequence and product size are shown in Table 6. A total volume of 15 μL was used for amplification, which contained 7.5 μL of SYBR green, 0.75 μL of each primer, and 1 μL of cDNA. The RNA amount was relatively normalized to the amount of the endogenous control and $^{\Delta}$Ct method.[32]

Table 6. List of Biomarkers Utilized in RT-PCR

biomarkers	forward/ reverse	sequence	product size
rACE	forward	GCTTGACCCTGGATTGCAGC	145
	reverse	CTCCGTGATGTTGGTGTCGT	
eNOS	forward	ATCCTGCTGCCCTCTTCGTAT	192
	reverse	GTGTTGGGTTGGGCATCTCAT	
COX-2	forward	ATCAGGTCATCGGTGGAGAG	196
	reverse	CTCGTCATCCCACTCAGGAT	
NF-kB	forward	AAGATGTGGTGGAGGACTTG	148
	reverse	GGTGGTTGATAAGGAGTGCT	
rGAPDH	forward	GAAGGTCGGTGTGAACGGAT	192
	reverse	ATGAAGGGGTCGTTGATGGC	

5.20. Statistical Analysis. For data analysis Graphpad prism, version 8.4.3 was used. One-way ANOVA followed by Dunnett's and two-way ANOVA followed by Bonferroni post-doc tests were used to test the significance of the results. Data were expressed as mean ± SEM. $P < 0.05$ was considered statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c03888.

Ramachandran plots for target proteins; phytoconstituents binding interaction with proteins; and docking figures (PDF)

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Author Contributions

B.S. was involved in the concept design, data acquisition, analysis, and article writing. M.H.M. supervised this project, provided guidance for data acquisition, and performed article review and submission. B.M. was involved in article design and writing. F.J. provided assistance in article writing. M.T.K. was involved the *in silico* molecular docking and revision of the article. M.F. and R.R. were involved in the revision of the article. M.S. provided lab facilities and assistance for PCR.

Funding

The present work was partially supported by a grant from the HEC of Pakistan, under the indigenous fellowship programme to B.S. (pin no: 315-12394-2BS3-134(50034182)).

Notes

The authors declare no competing financial interest.
The data that support the findings of this study are available from the corresponding author, upon reasonable request.

■ ACKNOWLEDGMENTS

M.H.M. and B.S. are thankful to the Higher Education Commission (HEC) of Pakistan for providing partial financial assistance for research work.

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