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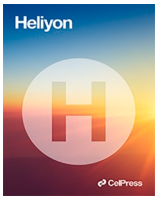

A pharmacological and toxicological biochemical study of cardiovascular regulatory effects of hibiscus, corn silk, marjoram, and chamomile

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ABSTRACT

Hypertension is one of the most typical causes of morbidity and mortality. The present study investigated the possible antihypertensive cardiovascular effects of an herbal mixture extract of Hibiscus, Corn silk, Marjoram, and Chamomile. HPLC analysis of the water extract prepared from the aerial parts of four plants and their mixture was done to detect the most predominant compounds. A safety study was done prior to the efficacy study to determine the dose and ensure the extract's safety in female rats. Hypertension was induced in ovariectomized and non-ovariectomized rats by oral administration of 50 mg/kg of LName for 30 days; the hypertensive rats were classified into non-ovariectomized and ovariectomized untreated groups, treated groups with high and low doses of the mixture(150,300 mg/kg) given to ovariectomized and non-ovariectomized hypertensive groups and a standard group treated with angiotensin-converting enzyme inhibitor. The untreated group showed significant elevation of blood pressure, heart rate, cholesterol, triglycerides, malondialdehyde, cyclic adenosine monophosphate, angiotensin-converting enzyme, C-reactive protein, and significantly lowered reduced glutathione, high-density lipoprotein, and endothelial nitric oxide synthase. Treatment significantly counteracted the effects of L Name. The mixture provides a promising natural cardiovascular regulating supplement owing to its high contents of flavonoids.

1. Introduction

Cardiovascular diseases, including elevated blood pressure "Hypertension"(HTN), are among the most typical causes of morbidity and death worldwide. Females are liable to develop hypertension after menstrual cessation due to the absence of the cardio-protective effects of female sex hormones [1,2]. Since the severe elevation of blood pressure may occur without any signs or complaints from patients and may dramatically worsen, ending in mortality, therefore it was termed "The Silent Killer" [3].

WHO guidelines for managing HTN 2021 recommended starting pharmacological treatment of diagnosed TN when systolic

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Abbreviation Meaning

ACE	angiotensin-converting enzyme
ACEI	angiotensin-converting enzyme inhibitors
ALT	alanine aminotransferase
IAEC	Animal Welfare and the Institutional Animal Ethical Committee
ARBs	antagonists of angiotensin-receptor
ARRIVE	Animal Research: Reporting of *in vivo* Experiments
AST	aspartate aminotransferase
$Ca+^2$	calcium
cAMP	cyclic adenosine monophosphate
CRP	C-Reactive Protein
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
HDL	high-density lipoprotein
HPLC	High-Performance Liquid Chromatography
HTN	hypertension
L-Name	NG-nitro-*l*-arginine methyl ester
MDA	malondialdehyde
Ovx	ovariectomized
K^+	potassium
RAAS	Renin-Angiotensin-Aldosterone system
R-GSH	reduced glutathione
RCF	relative centrifugal force
rpm	Rotations per minute
TC	total cholesterol
TG	triglycerides
WHO	World Health Organisation

pressure is 140 mmHg or more and diastolic 90 mmHg or more. This is because HTN is a seriously threatening condition worldwide. In the year 2021, about 1.4 billion adults around the world, about two-thirds of them are habitants of developing countries, have been reported to be suffering from hypertension. It is estimated that 23 % of hypertensive patients have one comorbidity, and 39 % have at least three comorbidities, such as strokes, hyperlipidemia, or diabetes mellitus. Some suffer from antihypertensive drug-induced comorbidities such as hyperkalemia and nephropathies. Therefore, laboratory investigation, besides blood pressure monitoring and electrocardiography, is helpful for follow-up of the disease's prognosis and treatment efficacy; they include lipid profiles, kidney function tests, and serum electrolytes [4].

Classes of medications used for controlling elevated blood pressure are variable. They include inhibitors of angiotensin-converting enzyme (ACEI), antagonists of angiotensin-receptor (ARBs), thiazides and thiazide-like diuretics, calcium channel blockers, alpha-blockers, and central adrenergic inhibitors. The occurrence of adverse effects of antihypertensive agents, such as cough, fatigue, sedation, orthostatic hypotension, and occurrence of depression with the intake of higher doses [3], was the motive to search for natural alternative supplementary products to help in the reduction of the side effects.

A sufficient supply of antioxidants in the diet via natural products is necessary for protection against cardiovascular disorders as they exert immune regulatory effects and protect cardiac endothelial cells through cell membrane stabilization [1].

Natural antioxidants are abundant in some herbal preparations that regulate blood pressure levels via different mechanisms. The list contains Hibiscus calyces' infusion, Marjoram, a member of the mint family and originated in Egypt. Chamomile dried flowers that contain mono, sesquiterpenoids, and flavonoids, which have a pivot role in its medicinal properties; and *Corn silk*. They all lower blood pressure through various mechanisms, mostly lowering blood lipids, thus preventing atherosclerosis and protecting the heart's cardiovascular conditions. The antihypertensive effects of Hibiscus, Corn silk, Marjoram, and Chamomile have been extensively studied and documented in various research. Each herbal component has demonstrated unique properties that contribute to blood pressure regulation. Hibiscus calyces infusion, for instance, is known to possess vasodilatory and diuretic properties, which can help lower blood pressure by promoting fluid excretion and relaxation of blood vessels. Corn silk has been found to inhibit the activity of angiotensin-converting enzyme (ACE), a key regulator of blood pressure, thus reducing vasoconstriction and promoting blood flow. As a member of the mint family, Marjoram possesses antioxidant and anti-inflammatory effects, which are linked to improved endothelial function and blood pressure regulation. Lastly, Chamomile's flavonoids and other bioactive compounds contribute to its blood pressure-lowering results by reducing cholesterol levels and atherosclerosis development. These herbal components, when combined in the current study's mixture, have the potential to exert synergistic antihypertensive effects, providing a promising natural alternative for managing elevated blood pressure [3,5–7]. There is a strong relation between protection against the occurrence of cardiovascular diseases and consumption of flavonoids in diet. It has been demonstrated in several studies that consuming flavonoids lowers the mortality rate due to cardiovascular diseases by 18 %, possibly because they reduce atherosclerosis [8].

However, when herbal preparations are used randomly for lowering elevated blood pressure without previous efficacy studies, they may cause serious problems. In previous studies, Hibiscus calyces, Corn silk, Marjoram, and Chamomile were studied for their effect on the cardiovascular system; each one was studied individually, but this is the first time to explore their products when mixed together. That's why the present study was designed to estimate the safety and blood pressure regulating the efficacy of an herbal mixture consisting of the four plants in various stages of rat life ranging from premenopausal to postmenopausal.

2. Materials and methods

Guideline ethics for Plant usage in the phytochemical study: The present study complies with local and national guidelines, as permission was obtained to collect plant material.

Guideline ethics for Experimental animal handling in the *in vivo* pharmacological study: The study was done in accordance with the guide for the care and use of laboratory animals. Experiments were performed according to the National Regulations of Animal Welfare and the Institutional Animal Ethical Committee (IAEC) in Egypt and are reported in accordance with Animal Research: Reporting of *in vivo* Experiments (ARRIVE) guidelines.

Ethical approval was obtained from the National Research Centre ethics committee under number **3411022022**.

2.1. Phytochemical study

2.1.1. Materials and methods

The phenolic and flavonoid compounds were extracted according to the method described by Hakkinen et al. (1998) and Mattila et al. (2000) [9,10]. HPLC analysis of the water extract prepared from the aerial parts of four plants (purchased from the market), *Hibiscus* calyces*, Corn* silk*, Marjoram,* and Chamomile, was carried out using an Agilent 1260 series. The separation was carried out using Eclipse C18 column (4.6 mm × 250 mm i.d., 5 μm). The mobile phase consisted of water (A) and 0.05 % trifluoroacetic acid in acetonitrile (B) at a flow rate of 1 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (82 % A); 0–5 min (80 % A); 5–8 min (60 % A); 8–12 min (60 % A); 12–15 min (85 % A) and 15–16 min (82 % A). The multi-wavelength detector was monitored at 280 nm. The injection volume was 10 μl for each of the sample solutions. The column temperature was maintained at 35 °C.

The mixture of the four plants was prepared by mixing equal weights from the four water extracts and then dissolving it in distilled water for oral administration to animals. The mixture syrup was freshly prepared daily.

2.2. In vivo pharmacological study

2.2.1. Materials

2.1.1. Animals. Female Wistar Albino rats weighing 180–200 g were used. The rats were obtained from the National Research Centre (NRC) animal house colony in Egypt. The animals were kept in standard plastic cages in an air-conditioned room at 22 ± 3 °C, 55 ± 5 % humidity, and supplied with a standard laboratory diet and water ad libitum.

2.1.2. Tested herbs. Mixture of extracts of Hibiscus calyces*, Corn* silk*, Marjoram,* and *Chamomile*.

2.1.3. Drugs and chemicals. Thiopental sodium, Diethyl ether, NG-nitro-*l*-arginine methyl ester (L-Name) purchased from Sigma Aldrich, USA. Cefotaxime (Cefotax®, sterile vial) purchased from Egyptian International Pharmaceutical Industries Company (E.I.P. I·Co) 10th of Ramadan City – industrial area B1, EGYPT. Angiotensin-converting enzyme (ACE) inhibitor(ACEI): Enalapril (Ezapril®) purchased from Multi-Apex Pharma (map), Egypt.

2.1.4. Diagnostic apparatus. Non-invasive BP monitor and thermostatically controlled heating cabinet (Ugo Basile, Varese, Italy). Spectrophotometer Co (JascoV-630; Jasco, Tokyo, Japan) and enzyme-linked immunosorbent assay apparatus (Thermo Fisher, Massachusetts, USA) were used for biochemical analysis.

2.1.5. Diagnostic kits. Kits for determination of total cholesterol, triglycerides, high-density lipoprotein (HDL), aspartate aminotransferase (AST), and alanine aminotransferase (ALT), urea, and creatinine were purchased from Biodiagnostic Company, Egypt. Elisa kits reagents were of the highest purity grade commercially available.

2.2.2. Methods

2.2.2.1. Ethical considerations. Approval of the experimental animal protocol by the Ethical Committee for Medical Research in National Research Centre, Egypt, was obtained under number 3411022022. Experiments were performed according to the National Regulations of Animal Welfare and the Institutional Animal Ethical Committee (IAEC).

2.2.2.2. Study design

2.2.2.2.1. Safety studies

2.2.2.2.1.1. Acute toxicity study

After the acclimatization period of female rats in their cages in the animal house for two weeks, ten rats were classified into two groups (five rats/group) as follows: Group I: The negative control group received distilled water orally.

Group II: The acute toxicity group that received the maximum soluble mixture dose (3 g/kg).

Animals were observed for 14 days for any changes in body weights, behavior, bowel habits, discoloration or loss of hair, and mortality. Then, blood samples were withdrawn for liver and kidney function tests analysis. The acute toxicity study followed the OECD test guideline 425 (2022) [11].

2.2.2.2.1.2. Sub-chronic toxicity study

When the mixture had proven to be tolerable in the acute toxicity study, a sub-chronic toxicity study was performed according to the method of El Fakir et al. (2021) [12], as follows:

Twenty-five female rats were employed in the study.

Group I: five rats served as a negative control, given distilled water daily orally for 60 days.

Sub-chronic toxicity groups:

Group II a: five non-ovariectomized(non-Ovx) rats were given the mixture (150 mg/kg) orally daily for 60 days.

Group II b: five non-Ovx rats were given the mixture (300 mg/kg) orally daily for 60 days.

Group III a: Five ovariectomized (Ovx) rats were given the mixture (150 mg/kg) orally daily for 60 days.

Group III b: five Ovx rats were given the mixture (300 mg/kg) orally daily for 60 days.

Animals were observed daily for the same signs of the acute toxicity study; then, blood samples were withdrawn for liver and kidney function tests analysis.

2.2.2.2.2. Efficacy study

After the mixture had proven safe in the sub-chronic toxicity study, the efficacy study was done. Sixty-four female rats were classified into eight groups (eight rats/group) as follows.

a) Control groups:

Group I: Negative control group: rats that received distilled water orally only throughout the study.

Group II: Positive Control group a: The untreated non-ovariectomized(non-Ovx) hypertension-induced group in which the rats were orally given NG-nitro-*l*-arginine methyl ester (L-Name 50 mg/kg) for 30 days [13].

Group III: Positive Control group b: The untreated ovariectomized (Ovx) hypertension-induced group in which the rats were ovariectomized, and this method by Ibrahim et al. (2017) [1] was modified by orally given L-Name(50 mg/kg) for 30 days.

b) Treated Groups:

Group IV represents the non-Ovx hypertensive group treated with 150 mg/kg of the herbal mixture.

Group V represents the non-Ovxhypertensive group treated with 300 mg/kg of the herbal mixture.

Group VI represents the Ovxhypertensive group treated with 150 mg/kg of the herbal mixture.

Group VII: This group represents the Ovx hypertensive group treated with 300 mg/kg of the mixture.

Group VIII: Reference group in which hypertensive rats were treated with 10 mg/kg of angiotensin-converting enzyme (ACE) inhibitor (ACEI): Enalapril treatment lasted for 30 days [14].

The administration of L-NAME for group II-VII was for 30 days, and an herbal mixture or reference drug was given in the last 30 Days.

The procedure of Ovariectomy: Rats were anesthetized with thiopental sodium (20 mg/kg; i. p.) and then bilaterally ovariectomized through dorsolateral incisions [15]. Care was taken to expose ovaries by doing the most diminutive-sized available incision. Internal muscle layers were sutured using absorbable catgut interrupted stitches, and interrupted silk sutures carefully sutured the site of external layers incision to avoid contamination. Finally, it was covered with sterile gauze, and the animal was given the antibiotic cefotaxime IV (90 mg/kg) after converting the human dose to the animal dose, according to Paget and Barnes (1964) [16]. The rats were rehydrated with saline infusion.

Blood pressure was measured at baseline for all rats, after induction of hypertension in both Ovx and non-Ovx rats, and after the last treatment dose. The systolic pressure and heart rate of animals were measured each week by a non-invasive blood pressure monitor (ML 125NIBP, AD Instruments, Australia). The animals were restrained in the tubes for 10–20min/day for 5 days prior to recording blood pressure in the tail-cuff technique; animals were warmed for 30 at 28 °C in a thermostatically controlled heating cabinet (Ugo Basile, Italy) for better detection of tail artery pulse, the tail was passed through a cuff and a tail-cuff sensor that was connected to an amplifier (ML 125 NIBP, AD Instruments, Australia). The amplified pulse was recorded during automatic inflation and deflation of the cuff. The blood pressure was measured by a tail-cuff method [17].

Biochemical parameters: At the end of the experimental period of safety studies that were done before the efficacy study, blood was obtained from all groups of rats that were kept fasting for 18 h after being lightly anesthetized with diethyl ether by puncturing retro-orbital plexus [18], the blood was allowed to flow into a clean, dry centrifuge tube and left to stand 30 min before centrifugation to avoid hemolysis. Then, blood samples were centrifuged for 15 min at 2500 rpm with RCF = 1048 gf to prevent hemolysis. The clear supernatant serum was separated and collected by Pasteur pipette into a dry clean tube to use for determination of serum levels of AST and ALT according to the method of [19], urea according to the method of [20], and creatinine according to the method of [21].

In the efficacy study, after the last treatment dose and blood pressure recording, blood collection followed the same procedures as the safety study. Serum was analyzed for levels of Triglycerides (TG) according to the method of [22], Total cholesterol (TC) according to the method of [23], High-density lipoprotein (HDL) according to the method of [24], Malondialdehyde (MDA) according to the method of [25], Reduced glutathione(R-GSH) according to the method of [26]. Endothelial nitric oxide synthase (e-NOS), C-reactive protein (CRP), Cyclic adenosine monophosphate (cAMP), and Angiotensin-converting enzyme (ACE) were quantified according to the method of manufacturer's kits using rat ELISA kits.

2.2.3. Statistical analysis

Before proceeding with the statistical analysis, data values were checked for normality using the Shapiro test. The data are presented as means ± S.E. Data were processed T-test for acute toxicity study statistical analysis while one-way ANOVA followed by the Tukey–Kramer Post hoc test other analyses. GraphPad Prism software (version 9, USA) was employed to perform the statistical analysis and establish the represented graphs. The significance level was set to $p < 0.05$ for all statistical tests.

3. Results and discussion

The objective of the phytochemical study in this experiment was to determine the phenolic compounds in the four plants, Hibiscus calyces, Corn silk, Marjoram, and Chamomile, and their mixture using the HPLC method to quantify several flavonoids in the water extract prepared from the aerial parts of four plant and their mixture. A simple, sensitive, and precise reversed-phase HPLC-DAD method was developed and validated to determine six main flavonoids simultaneously. Chlorogenic acid was the major phenolic compound, followed by gallic acid, naringenin, syringic acid, kaempferol, and caffeic acid. The contents of each extract was determined by using the area's corresponding calibration curves, and the calculated amounts are given in Table 1.

Since the four plant extracts showed considerable levels of flavonoids, therefore they were mixed together to study their biological effects as safety and efficacy on blood pressure when given as a mixture. Surprisingly, when the four plants are mixed, gallic, chlorogenic, catechin might not have dissolved completely.

The acute toxicity study results of the current work presented in Table 2 show non-significant changes in mixture tested groups compared to the negative control group, as serum levels of hepatic and renal function tests (AST, ALT, urea, and creatinine) of tested rats were nearly the same as the negative control rats. Also, no signs of toxicity or mortality were recorded during the 14-day duration of the study.

This denotes the safety of the mixture when given orally in doses of up to three gm/kg to rats. Accordingly, a dose of up to 300 mg/kg was selected for the sub-chronic toxicity and efficacy studies. The tested mixture doses for sub-chronic toxicity and efficacy studies were 150 and 300 mg/kg.

Sub-chronic toxicity study results shown in Table 3 reveal that the mixture could be used safely regarding the liver and kidney functions for continuous durations of up to two months in doses of 150 and 300 mg/kg, as there were non-significant changes in liver and kidney function tests of groups of non-ovariectomized rats given the mixture orally compared to groups that were given distilled water only for 60 days. Also, the sub-chronic toxicity study reported no mortalities or signs of toxicity. On the other hand, as shown in Table 4 ovariectomized groups showed significant elevation of AST, ALT, and creatinine compared to the negative control group. ALT and creatinine levels were significantly lower in the rats that received the high dose of the mixture (300 mg/kg) compared to those that received the low dose (150 mg/kg), which indicates that the elevated biomarkers' levels are most probably due to loss of hormonal protection of female sex hormones on liver and kidney against external factors rather than the tested mixture or may be due to diet or stress caused by exposure to the experiment. This effect was counteracted by giving the mixture in high doses. This may add to the value of the mixture for further future studies on its postmenopausal hepatic and renal protective effects.

Table 1
HPLC fingerprint of the four extract and the mixture.

	Conc.(μg/g)				
	mixture	Silk corn	marjorum	Hibiscus	camomile
Gallic acid	3016.8	131738.8	2547.9	9284.9	5661.2
Chlorogenic acid	3325.4	145213.2	21870.5	2641.4	532.4
Catechin	ND	ND	ND	112.2	1525.4
Methyl gallate	41.9	1828.6	648.5	106.7	313.1
Coffeic acid	228.0	9955.4	0.0	206.0	36.4
Syringic acid	316.3	13810.6	348.0	3462.1	734.3
Pyro catechol	ND	ND	749.3	ND	ND
Rutin	41.6	1815.1	ND	307.4	1810.0
Ellagic acid	268.3	11715.8	ND	ND	16.3
Coumaric acid	133.1	5811.6	155.3	54.5	85.0
Vanillin	ND	ND	ND	41.5	17.2
Ferulic acid	ND	ND	404.8	ND	210.7
Naringenin	802.1	35028.4	1077.6	192.9	926.4
Taxifolin	89.2	3896.3	2625.6	436.1	728.8
Cinnamic acid	24.9	1089.0	6.6	1.6	1571.6
Kaempferol	269.9	11785.8	108.7	12.3	47.2

Table 2
Acute Toxicity Study of the effect of mixture.

Parameter	Negative control	Mixture(3 gm/kg)
ALT (U/L)	15.50 ± 1.555	18.80 ± 3.109
AST (U/L)	27.67 ± 2.728	31.00 ± 1.155
Creatinine (mg/dl)	0.2200 ± 0.03464	0.2383 ± 0.05419
Urea (mg/dl)	42.25 ± 4.230	49.00 ± 2.309

Results are expressed as means (N = 5),of serum levels of Liver function tests(AST and ALT),and Renal function tests(Urea and Creatinine) ± SE, $p \leq 0.0001$, *t*-test was used for Acute toxicity study statistical analysis.

Table 3
Subchronic Toxicity study of the effect of mixture in Non-Ovx.

Parameter	Negative control	Mixture (150 gm/kg)	Mixture (300 gm/kg)
ALT (U/L)	24.33 ± 3.844	28.67 ± 2.028	28 ± 2.082
AST (U/L)	29 ± 2	32.5 ± 2.5	33.2 ± 1.53
Creatinine (mg/dl)	0.37 ± 0.026	0.4 ± 0.026	0.44 ± 0.027
Urea (mg/dl)	48 ± 2.517	47.5 ± 3.926	51 ± 1.732

Results are expressed as means (N = 5) of serum levels of Liver function tests(AST and ALT),and Renal function tests(Urea and Creatinine) ± SE, $p \leq 0.0001$.One way analysis of variance (ANOVA) followed Tukey Kramer's test for multiple comparison were used for statistical analysis.@Significantly different from Negative control group,* Significantly different from Mixture 150 mg/kg group.

Table 4
Subchronic Toxicity study of the effect of mixture in Ovx rats.

Parameter	Negative control	Mixture (150 gm/kg)	Mixture (300 gm/kg)
ALT (U/L)	17.85 ± 0.42	34.62 ± 1.11@	35.75 ± 0.32@
AST (U/L)	19.67 ± 1.667	28 ± 2.309$^{@}$	33.33 ± 0.882$^{@}$
Creatinine (mg/dl)	0.17 ± 0.023	0.52 ± 0.019$^{@}$	0.35 ± 0.021$^{@*}$
Urea (mg/dl)	45 ± 2.517	57.5 ± 1.5	51.33 ± 2.028

Results are expressed as means (N = 5) of serum levels of Liver function tests(AST and ALT),and Renal function tests(Urea and Creatinine) ± SE, $p \leq 0.0001$.One way analysis of variance (ANOVA) followed Tukey Kramer's test for multiple comparison were used for statistical analysis.@Significantly different from Negative control group,* Significantly different from Mixture 150 mg/kg group.

The body weights of animals in the mixture extract group in the acute toxicity study didn't show any significant change compared to the negative control group. During the sub-chronic toxicity study, the group that received a low dose of the mixture extract significantly increased compared to the negative group. All body weights were directly related to the amount of diet given to the rats daily. No abnormal behavior, bowel habits, hair discoloration, nasal discoloration, excessive salivation, or sudden mortalities were recorded during the safety studies (Table 5).

Based on the safety study of acute and sub-chronic toxicity studies, the efficacy study used 150 and 300 mg/kg of the mixture extract.

The *in vivo* pharmacological efficacy study focused on the blood pressure lowering effects of the herbal mixture extract, given in two doses to premenopausal (non-OVX) and postmenopausal (ovariectomized-OVX) rats, and the proposed mechanism of action could be

Table 5
Results of the Safety studies of the Mixture of *Hibiscus* calyces, *Corn* silk, *Marjoram* and *Chamomile*.

Signs	Groups				
	Acute Toxicity Study		Subchronic Toxicity Study		
	Negative Control	Mixture (3 gm/kg)	Negative Control	Mixture (150 mg/kg)	Mixture (300 mg/kg)
weekly food consumption(gm)	105	95	125	132	129
% change of Body weightfrom basal bwt(180-200 gm)	1.049 %± 0.008	1.081 %± 0.005	1.063 %± 0.002	1.142 %± 0.017$^{@}$	1.02± 0.014
Behaviour(irritability, Seizures, aggression,lethargy)	Normal	N/A	N/A	N/A	N/A
Bowel habits(Diarrhoea, constipation)	Normal	N/A	N/A	N/A	N/A
Hair Discolouration	Normal	N/A	N/A	N/A	N/A
Nasal discolouration	Normal	N/A	N/A	N/A	N/A
Excessive salivation	Normal	N/A	N/A	N/A	N/A

N = 5, results of body weights are expressed as means of %change of body weight± SE, $p \leq 0.005$. One way analysis of variance (ANOVA) followed Tukey Kramer's test for multiple comparison were used for statistical analysis.@Significantly different from Negative control group.

Table 6
Effect of Treatment of with a Mixture of *Hibiscus* calyces, *Corn* silk, *Marjoram* and *Chamomile* on Blood Pressure of Premenopausal (non-Ovx) and Postmenopausal (Ovx) Hypertensive rats.

Onset	Groups								
	Control groups			Treated Groups					Effect size (r^2)
	Negative Control	Positive Control L-name + Non- Ovx	Positive Control L-name + Ovx	Mixture (150 mg/kg) + HTN Non- Ovx	Mixture (300 mg/kg) + HTN Non- Ovx	Mixture (150 mg/kg) + HTN Ovx	Mixture (300 mg/kg) + HTN Ovx	ACEI (10 mg/kg) +HTN	
Baseline	77.65± 2.2	83.4± 3.8	82.62± 2.32	89.8± 3.15	82.12± 1.2	75.04± 3.77	83.63± 5.29	78.2± 2.84	0.1558
30 days (Induction of HTN)	69.1± 1.56	122.7± 3.02 ($^{@}$<0.0001)	120.4± 2.95 ($^{@}$<0.0001)	124.1± 5.02 ($^{@}$<0.0001)	122.7± 3.92 ($^{@}$<0.0001)	116± 3.59 ($^{@}$<0.0001)	124.1± 4.71 ($^{@}$<0.0001)	126.2± 4.53 ($^{@}$<0.0001)	0.8134
30 days (Treatment of HTN)	71.08± 2.19	101.3± 2.43$^{@}$ ($^{@}$<0.0001)	104.8± 2.87$^{@}$ ($^{@}$<0.0001)	92.58± 3.28$^{@}$ ($^{@}$<0.0001) ($^{\$}$<0.0445)	73.95± 2.82$^{*\#\&}$ (*<0.0001) ($^{\$}$<0.0001)	97.28± 2.33$^{@}$ ($^{@}$<0.0001)	75.22± 3.16$^{*\$\&}$ (*<0.0001) ($^{\$}$<0.0001) ($^{\&}$<0.0001)	100.3± 1.9$^{@}$ ($^{@}$<0.0001) (*<0.0001)	0.8255

N = 8. Results are expressed as means of systolic blood pressure± SE. One way analysis of variance (ANOVA) followed Tukey Kramer's test for multiple comparison were used for statistical analysis.$^{@}$Significantly different from Negative control group, * Significantly different from HTN Non Ovx Positive control group, $^{\$}$ Significantly different from HTN Ovx Positive control group, $^{\&}$ Significantly different from HTN Ovx group treated ACEI 10 mg/kg group. The exact P-value of each corresponding symbol is stated between brackets.

Table 7
Effect of Mixture of *Hibiscus* calyces, *Corn* silk, *Marjoram* and *Chamomile*on Heart rate in Premenopausal (non-Ovx) and Postmenopausal (Ovx) Hypertensive rats.

Groups Onset	Control groups			Treated Groups					effect size (r^2)
	Negative Control	Positive Control L-name + Non- Ovx	Positive Control L-name + Ovx	Mixture (150 mg/kg) + HTN Non- Ovx	Mixture (300 mg/kg) + HTN Non- Ovx	Mixture (150 mg/kg) + HTN Ovx	Mixture (300 mg/kg) + HTN Ovx	ACEI (10 mg/kg) +HTN	
Baseline	291± 4.45	303.1± 13.68	325.2± 9.05	299.5± 19.65	302.1± 14.03	294± 13.74	310.4± 12.89	332.8± 8.94	0.1897
30 days (Induction of HTN)	298.2± 8.54	401.2± 14.37$^{@}$ ($^{@}$<0.0001)	364.7± 30.18$^{@}$ ($^{@}$0.0120)	391.5± 6.83$^{@}$ ($^{@}$<0.0001)	375.3± 14.3$^{@}$ ($^{@}$0.0022)	413.3± 15.76$^{@}$ ($^{@}$<0.0001)	381.91± 12.96$^{@}$ ($^{@}$ = 0.0007)	385.3± 12.67$^{@}$ ($^{@}$ = 0.0004)	0.5763
30 days (Treatment of HTN)	288.3± 7.06	406.5± 4.66$^{@}$ ($^{@}$<0.0001)	373.5± 11.2$^{@}$ ($^{@}$<0.0001)	244.4± 8.83$^{@}$*$^{\&}$ ($^{@}$ = 0.0081) (*<0.0001) ($^{\&}$<0.0001)	283± 5.85*$^{\#}$$^{\&}$ ($^{@}$) (*<0.0001) ($^{\&}$<0.0001)	250.9± 7.9$^{@}$$^{\$}$*$^{\&}$ ($^{@}$ = 0.0381) (*<0.0001) ($^{\$}$<0.0001) ($^{\&}$<0.0001)	282.9± 7.2$^{\$}$*$^{\&}$ ($^{@}$) (*<0.0001) ($^{\$}$<0.0001) ($^{\&}$<0.0001)	347± 9.35$^{@}$* ($^{@}$ = 0.0002) (* = 0.0001)	0.9053

N = 8. Results are expressed as means of Pulse± SE. One way analysis of variance (ANOVA) followed Tukey Kramer's test for multiple comparison were used for statistical analysis. $^{@}$Significantly different from Negative control group, * Significantly different from HTN Non Ovx Positive control group, $ Significantly different from HTN Ovx Positive control group, & Significantly different from HTN Ovx group treated ACEI 10 mg/kg group. The exact P-value of each corresponding symbol is stated between brackets.

deduced via the biochemical analysis of rats' serum in all groups.

In the present efficacy study, administration of L-Name to OVX and non-OVX rats for 30 days and leaving rats untreated for an extra 30 days caused significant elevation of blood pressure, heart rate, ACE, cAMP, MDA, TC, TG, CRP, and decreased R-GSH, HDL, and eNOS significantly compared to the negative control. However, treatment with the herbal mixture extract for 30 days significantly ameliorated all these signs in a dose-dependent manner in treated groups compared to the positive (untreated group). The high dose of the mixture significantly reduced blood pressure compared to ovariectomized and non-ovariectomized rats treated with ACEI **(Tables 6–7) and (Figs. 1–4)**.

It is noteworthy to refer to the hypotensive and heart rate regulating effects of the mixture extract expressed in **(Tables 6–7)** of the vasodilator activity of flavonoids in the water extract prepared from the aerial parts of four plants.

In fact, the active ingredients in many medicinal plants are represented by bioactive substances such as flavonoids. Flavonoids have been tested for their potential therapeutic effects in various diseases [3,27–32].

To be specific, sixty-four flavonoids were evaluated for potential vasodilating properties using in vitro analysis along with an assessment of their mechanism. The mechanism of action used by all flavonoids in vitro includes, and can thus be arranged in descending order, and intra-erect potassium channel < activated low conductance calcium channel < 2-adrenergic receptor < potassium channel activated medium conductivity. Flavonoids that (1) have a planar structure of the C6–C3–C6 backbone in the presence of a (-C═O)-carbonyl group at the C4 position of the C ring, and compounds with (2) are believed to have similar substituents at the C5 and C7 positions of ring A, and the position of C3' on ring B provides a flavonoid structure with the most robust vasodilator properties. The relationship between structural and signaling pathways to vasodilators is profound [33].

In this work, the mixture contains high levels of flavonoids, mainly chlorogenic acid kaempferol, quercetin, and naringenin, that

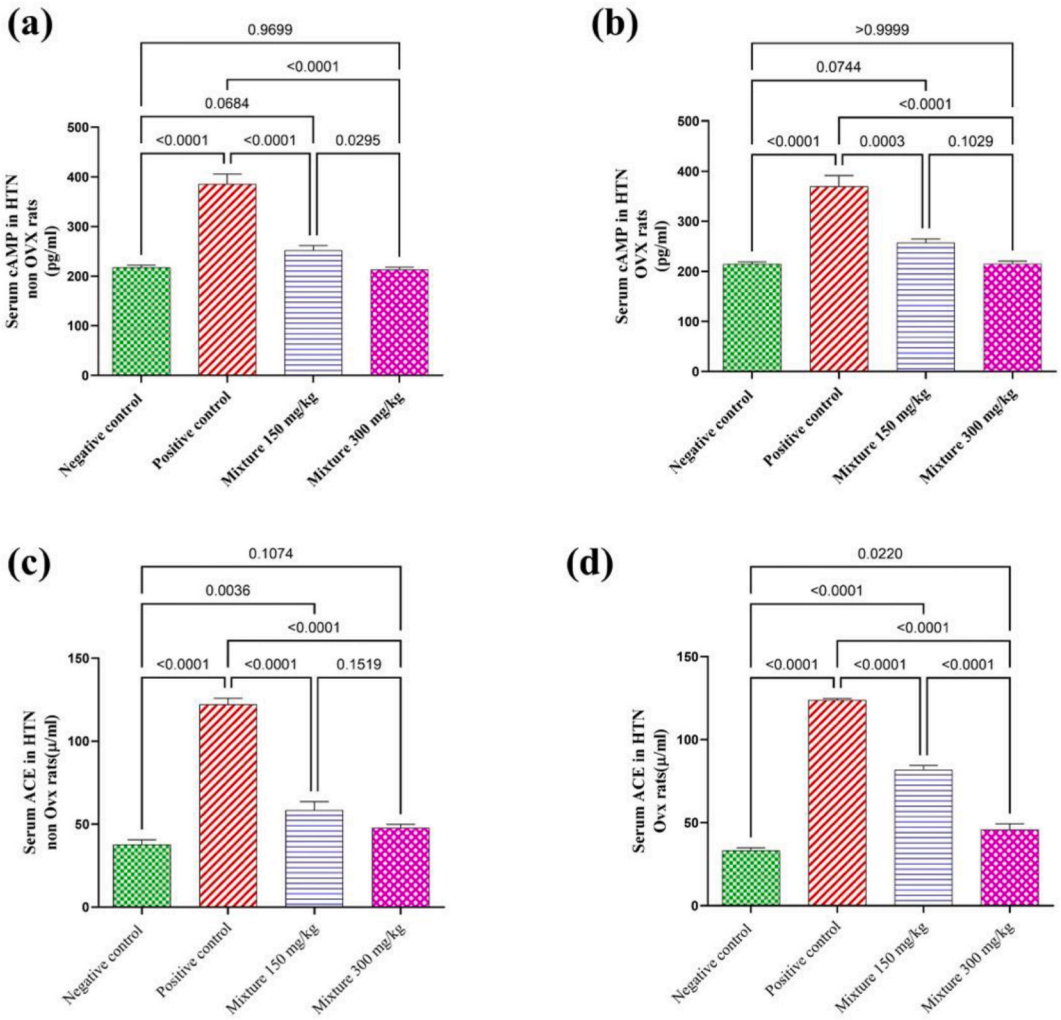

Fig. 1. Serum levels of (a & b) ACE and (c & d) cAMP in HTN non-Ovx and HTN Ovx rats Results are expressed as means of levels ±SE,N = 8,p ≤ 0.05.One way analysis of variance (ANOVA) with an effect size of (r^2) = 0.9481, 0.9365, 0.9687 & 0.9913, respectively) followed Tukey Kramer's test for multiple comparison were used for statistical analysis.

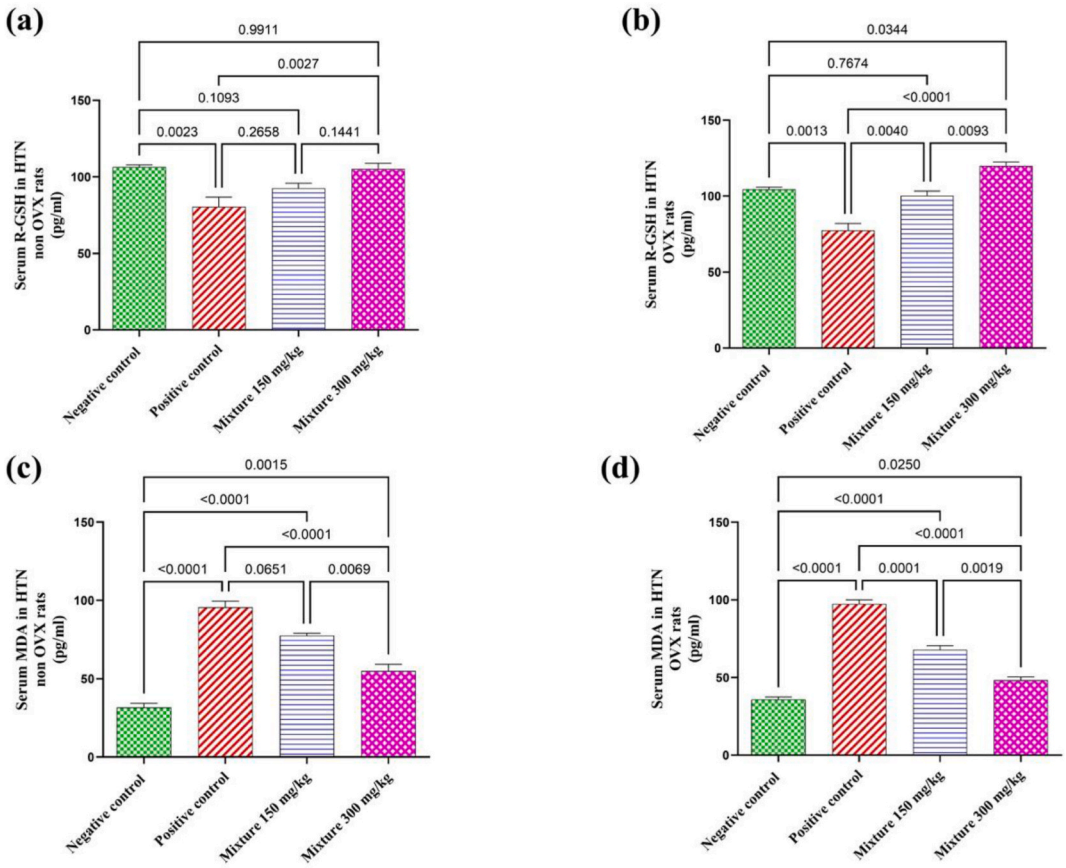

Fig. 2. Serum levels of (a & b) MDA and (c & d) R-GSH in HTN non-Ovx and HTN Ovx rats Results are expressed as means of levels ±SE, N = 8,p ≤ 0.0001.One way analysis of variance (ANOVA) with an effect size of (r^2) = 0.6840, 0.9215, 0.9158 & 0.9793, respectively) followed Tukey Kramer's test for multiple comparison were used for statistical analysis.

may be contributing to the antihypertensive activity as mentioned by Abou Baker (2022) [34].

Among all the flavonoids tested for their vasodilating effect in relation to the reported EC50 value, quercetin has structural properties that play an essential role in inducing potent vasodilating effects. There is a structural relationship of the flavonoid subclass with the manifested vaso-relaxation effect of quercetin. It was previously stated that *Chamomile, Hibiscus calyces, Corn silk, and Marjoram* are rich in quercetin [35–38].

ACE inhibitors (ACEI) bind a zinc atom to the active site of ACE; this action slows the conversion of angiotensin I to angiotensin II, thus preventing vasoconstriction caused by the potent angiotensin II. The blood pressure-lowering effect of quercetin is due to its ability to inhibit the Renin-Angiotensin-Aldosterone system (RAAS) [39]; it also inhibits vaso-constriction by chelating zinc and thus acting as ACEI [40]. It also improves endothelial function, which explains the significantly low ACE level in all groups treated with the mixture in our study(Fig. 3). It postulates that the mechanism of action of the mixture is due to the ACE inhibitory effect. Subsequently, the mixture's inhibitory effect on ACE is dose-dependent on blood pressure. It can be concluded that the compound's main chemical structure enforced the mixture's ability to prohibit any vasoconstriction; thus, it lowered the elevated blood pressure and regulated cardiac rate in our study (**Tables 6–7**). Moreover, administration of the mixture extract to rats significantly reduced the levels of cAMP to near normal levels (Fig. 1). cAMP is a second messenger whose increase leads to increased cardiac contractility, heart rate, and conduction velocity [41]. This mechanism of action of the mixture causes a synergistic blood pressure lowering effect due to the negative inotropic effect due to reduced cAMP, which consequently leads to reduced cardiac output and heart rate (**Tables 6–7**), resulting in blood pressure reduction.

Dyslipidemia, oxidative stress, and inflammation decrease eNOS production and consequently lead to endothelial dysfunction, which ends in reduced functional effectiveness of the arterial wall and is associated with cardiovascular disorders such as hypertension [39]. In our study, the mixture extract significantly lowered TC, TG, MDA, and CRP and elevated HDL, R-GSH, and, in turn, elevated eNOS (Figs. 2–4), suggesting their efficacy in maintaining arterial wall elasticity and vasodilation.

In the study of Yan et al.; (2020), they stated that gallic acid (GA) could protect against hypertension and its associated cardiovascular complications by inhibiting Angiotensin II activity and improving endothelial function by inhibiting eNOS degradation; it also abolished the oxidative stress induced in the aorta [42]. These findings are in agreement with the conclusions of our study, where the mixture extract that was rich in GA levels showed a reduction in the levels of ACE (Fig. 1) and MDA (Fig. 2), thus inhibiting the

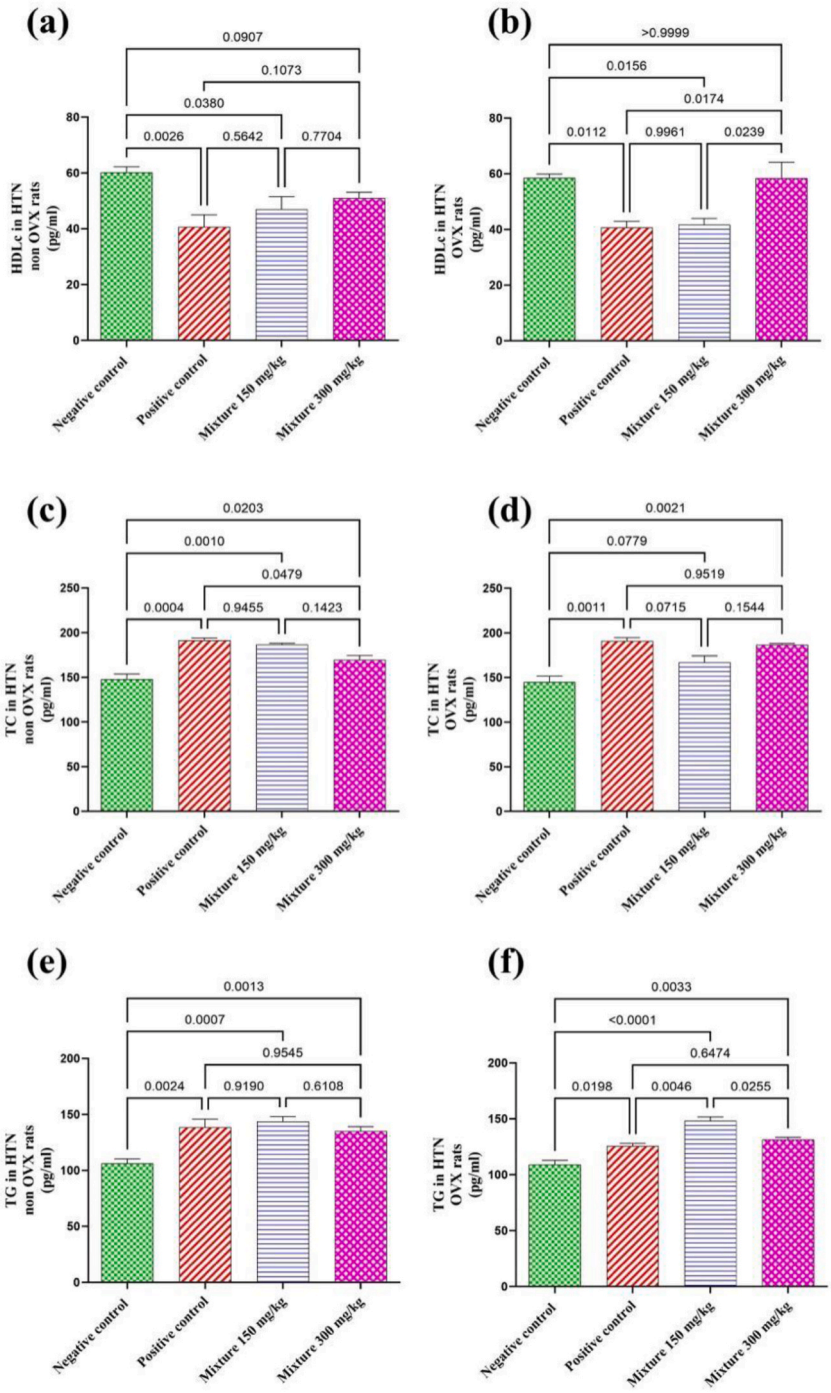

Fig. 3. Serum levels of (a & b) HDLc, (c & d) TC, and (e & f) TG in HTN non-Ovx and HTN Ovx rats Results are expressed as means of levels ±SE,N = 8,p ≤ 0.0001.One way analysis of variance (ANOVA) with an effect size of (r^2) = 0.6397, 0.7706, 0.7672, 0.8336, 0.7560, & 0. 0.8990, respectively) followed Tukey Kramer's test for multiple comparison were used for statistical analysis.

production of angiotensin II, moreover, it elevated R-GSH and eNOS; subsequently, it reduced the elevated blood pressure and heart rate in both OVX and non OVX rats significantly compared to the positive control group (**Tables 6–7**).

Postmenopausal women often experience hormonal changes and remarkably decreased estrogen levels, which can influence cardiovascular health. Estrogen has been associated with vasodilation and improved endothelial function, contributing to blood pressure regulation [1–3]. Therefore, the absence of cardio-protective effects of estrogen in postmenopausal women could be relevant in our study's population and may impact the response to the herbal mixture.

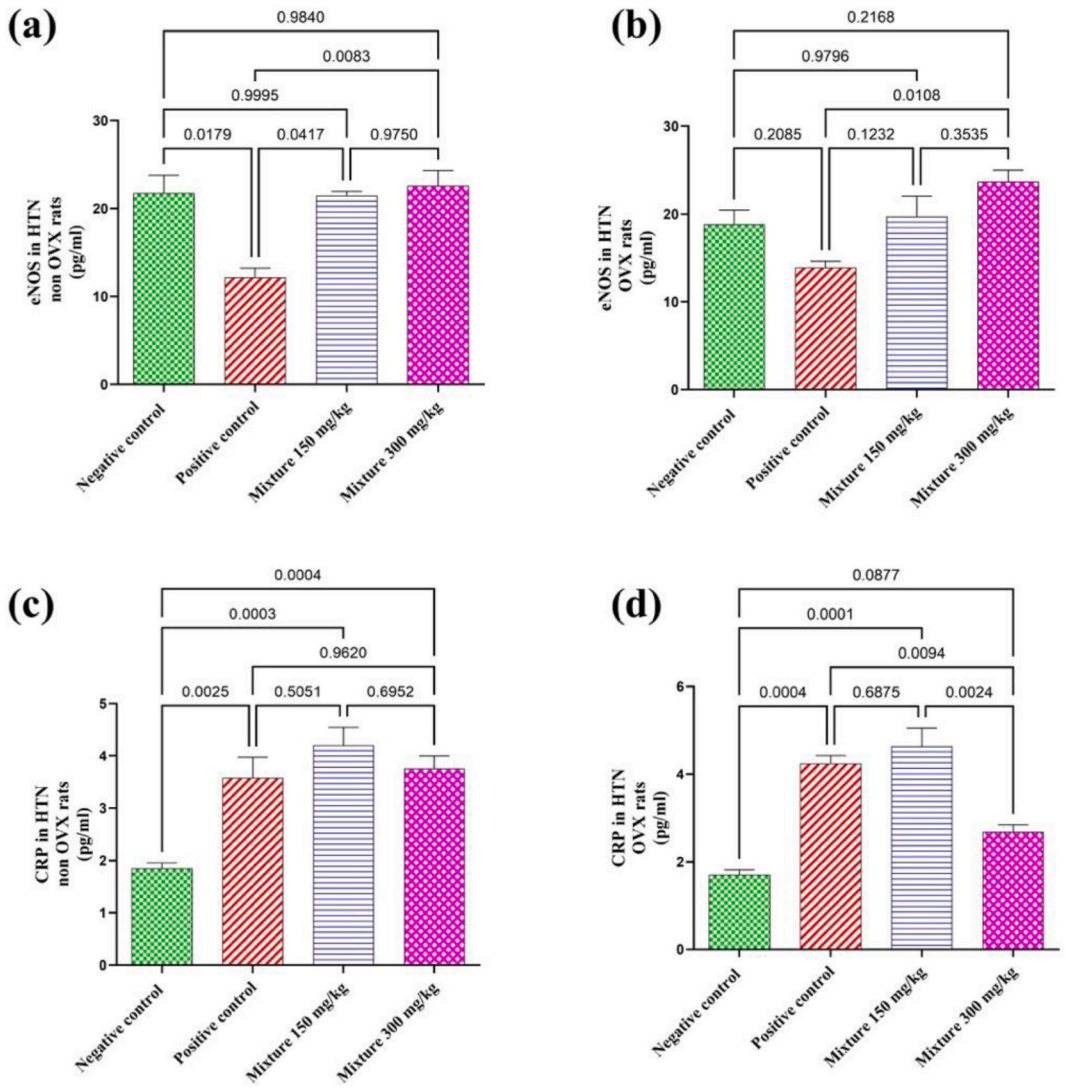

Fig. 4. Serum levels of (a & b) e-NOS and (c & d) CRP in HTN non-Ovx and HTN Ovx rats Results are expressed as means of levels ±SE,N = 8,p ≤ 0.0001.One way analysis of variance (ANOVA) with an effect size of (r^2) = 0.5715, 0.7034, 0.7586 & 0.9193, respectively) followed Tukey Kramer's test for multiple comparison were used for statistical analysis.

Additionally, nitric oxide plays a critical role in blood pressure control as a potent vasodilator. Reduced nitric oxide bioavailability has been linked to hypertension [43–45]. Therefore, it is essential to investigate if the herbal mixture extract modulates nitric oxide production or availability, as this may be one of the mechanisms contributing to its antihypertensive effects.

Naringenin and kaempferol are other components of the mixture. Naringenin acts via the activation of voltage-gated K^+ channels and Ca^{2+}-activated K^+ channels, thus causing vaso-relaxation; it also has antioxidant properties so that it can modulate nitric oxide (NO) levels [43,45]. Kaempferol acts by elevating endothelium-derived NO and as an anti-inflammatory. It suppresses the aortic rings' phosphorylated myosin light chain (MLC) and protein kinase C, thus inducing endothelium-dependent vasorelaxation [46]. This explains the elevated led levels of eNOS and reduced levels of CRP (Fig. 4), which contributed to a significant reduction in blood pressure in our study **((Table 6)**. Kluknavsky et al., 2016 stated that catechin significantly reduced oxidative stress in spontaneously hypertensive rats and increased eNOS activity [46,47], which enforces our study results.

4. Conclusions

In the present study, the mixture of hibiscus calyces*, Corn* silk*, Marjoram,* and *chamomile extract* exerted promising antihypertensive and heart-regulating effects in premenopausal and postmenopausal rats equally dose-dependent. Its effect was significantly better than the standard ACEI drug used in the study, which suggests that it can be used as a natural food supplement besides medicinal treatment in hypertensive cases associated with cardiac rhythm disorders.

The mixture modulates the Renin-Angiotensin-Aldosterone system and inhibits ACE; also, it acts as a hypolipidemic, anti-inflammatory, and antioxidant natural product.

Moreover, it reduced the cAMP, which most probably added to its hypotensive effect via reducing cardiac contractility, thus reducing cardiac output and heart rate and lowering the elevated blood pressure.

5. Limitation of the current study

The study investigating the antihypertensive cardiovascular effects of the herbal mixture extract has several limitations. Firstly, using rats as the experimental model may not directly translate to human responses. Additionally, the study's scope was limited to specific cardiovascular parameters in rats with induced hypertension, potentially overlooking other health effects or interactions with other systems. Human trials were not conducted, hindering validation of the herbal mixture's safety and efficacy in real-world scenarios. The sample size of animals in each group may not be sufficient for robust statistical significance.

Moreover, the short duration of the study and its focus on female rats limit its applicability to long-term and gender-specific effects. Optimal dosage and dose-response effects were not thoroughly explored, and comparative data with other antihypertensive medications or interventions were lacking. Lastly, the herbal mixture toxicity study did not include information about hematological parameters. While the study provides valuable insights, further research involving human trials with larger samples and longer durations is needed to comprehensively assess its clinical relevance and safety.

Guideline ethics for plant usage in the phytochemical study

The present study complies with local and national guidelines, as permission was obtained to collect plant material.

Guideline ethics for experimental animal handling in the *in vivo* pharmacological study

The study was done in accordance with the guide for the care and use of laboratory animals. Experiments were performed according to the National Regulations of Animal Welfare and the Institutional Animal Ethical Committee (IAEC) in Egypt and are reported in accordance with Animal Research: Reporting of *in vivo* Experiments (ARRIVE) guidelines.

Ethical approval was obtained from the National Research Centre ethics committee under number **3411022022**.

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Data availability

Data will be made available on request.

CRediT authorship contribution statement

Bassant MM. Ibrahim: Writing – original draft, Methodology. **Marawan A. Elbaset:** Writing – original draft, Methodology. **Doha H. Abou Baker:** Writing – original draft, Methodology. **Emad N. Zikri:** Validation. **Souad El Gengaihi:** Writing – review & editing, Supervision, Project administration, Investigation, Conceptualization. **Mouchira Abdel Salam:** Visualization, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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