

Anti-Hypertensive Activity of *Punica granatum* peels Ethyl Acetate Extract on Fludrocortisones Induced Hypertension in Wistar Rats

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ABSTRACT

Pomegranate (*Punica granatum* Linn.) has several medicinal properties. Pomegranate juice has been reported to be an effective source of high amounts of phyto-constituents with many health and nutritional benefits. This work assessed the possible effect on blood pressure (B.P) and heart rate of *P. granatum* peel ethyl acetate extract (PGE) at a dosage of 400 mg / kg / day on hypertension-induced fludrocortisone Wistar rats. PGE therapy substantially lowered the B.P of hypertensive rats treated with salt fludrocortisones, but the mean blood pressure and heart rate in normotensive rats were not altered. In isolated aortic strip, the contractile reactions to noradrenalin of PGE-treated hypertensive rats have been substantially reduced. The active fraction was purified by successive chromatographic techniques resulted in isolation of quercetin and ursolic acid, this bioactive mixture induced a significant decrease in the blood pressure of hypertensive rats as compared to hypertensive control rats at a concentration of 10 mg/kg.

Key words: *Punica granatum*, Hypertension, Ursolic acid, Quercetin.

INTRODUCTION

Chronic increase of peripheral vascular resistance owing to functional (changes in vascular reactivity) and structural (increased wall-to-lumen arteriolar ratio) abnormalities, the main hemodynamic alteration in the established phase of essential human hypertension is considered.^{1,2} The sympathetic central nervous system plays a key role in regulating vascular resistance. Furthermore, an elevated sympathetic control of vascular tone as factors in the development of hypertension was supported by several experimental and clinical evidences. High B.P is also associated with elevated circulating levels of pro-inflammatory cytokines which possible to change both the vascular expression of enzymes for e.g. inducible nitric oxide synthase (iNOS), vascular tone regulation for e.g. iNOS, and vascular tone regulation during this pathology.^{3,4}

In fact, increased iNOS vascular action and/or protein expression were described in the hypertension.⁵ Others have previously studied the function of iNOS-derived NO in responses to vasoconstrictors and endothelium-dependent vasodilators in lipopolysaccharides or interleukin-1b stimulated arteries.⁶⁻⁸ Oxidative stress can influence the vascular reactivity through various mechanisms. As second messengers, reactive oxygen species activate multiple signaling molecules and act a significant position in vascular physiopathology.^{9,10} Various sources of superoxide anion (O₂⁻) were characterized within vessels. O₂⁻ can be produced in different conditions, including xanthine oxidase, uncoupled NOS and COX. However, it is well known at the vascular level that

the key source of O₂⁻.¹¹ In the present study, the animal hypertension model shares several characteristics that are similar to human hypertension. That most of these methods were constructed using etiological factors that are assumed to be responsible for human hypertension such as excessive salt intake, renin angiotensin-aldosterone (RAAS) hyperactivity. Additionally, there is enhanced secretion of the vasopressin and altered (RAAS) activity resulting in increased sympathetic activity.¹² Side-effects from antihypertensive medications have encouraged researchers to find novelhypotensive drugs in metabolites or extracts from edible and medicinal plants to control hypertension and that cause less side effects.¹³

MATERIALS AND METHODS

Preparation of Pomegranate (*Punica granatum* Linn.) extract (PGE)

Pomegranate fruits (3 kg) were purchased from the local market in Abhur, Jeddah, KSA. The fruits were cleaned, shadow dried and stored. The peel and the seed powders were extracted with 70% ethanol for one week concentrated under vacuum at 60°C and then dried. Vacuum-dried ethanolic extract (800 g) was macerated in ethyl acetate in the solvent: solute ratio of 3: 1 for 48 h with frequent shaking. The ethyl acetate fraction was dried under vacuum using rotary evaporator IKA- RV10, USA.

Isolation of bioactive compounds of active fraction

Vacuum-dried ethyl acetate extract (150 g) was chromatographed on Silica gel 60 using gradient

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elution of mobile phase (*n*-hexane: acetone). Active fraction which eluted with (*n*-hexane: acetone) (8:2) was purified by combination of column chromatography on silica gel G-60, Chromatorex OD successively to yield active compounds 1,2 which were identified as ursolic acid and quercetin, respectively.

Animals

Male albino Wistar rats 100-150 gm were used. The study was conducted according to the National Institutes of Health guidelines for the care and use of laboratory animals. All animal care and experimental procedures were carried out with the ethics approval of the local regulatory authority. The animals were kept at room temperature with a 12 h/12 h dark /light cycle, which allowed us to perform experiments in the active phase of the animals. Rats were habituated to laboratory conditions. Rats received a standard diet and water.

Drugs and chemicals

PGE was prepared from peels and seeds of *P.granatum*. Noradrenalin and Fludrocortisone acetate were purchased by Sigma-Aldrich, USA. All other chemicals used in the study were of analytical grade. PGE were suspended in deionized water and administered orally.

Mineralocorticoid induced hypertension

Groups of rats were kept on a diet high in sodium chloride and drinking water was replaced by 2 % sodium chloride solution. After they attain a weight of about 250 gm, they were given fludrocortisones dissolved in sesame oil at dose of 10 mg/kg once daily for 3 weeks.¹⁴

Hypotensive activity in normotensive rats

Normotensive rats of either sex were randomly assigned into 3 groups (n = 5). Group 1 represented normal control, group 2 and 3 received 200 and 400 mg/kg p.o. of PGE, respectively. Basal blood pressure and heart rates were measured at 0, 2, 4 and 6 h using non-invasive blood pressure recorder apparatus (Ugo basile instruments, Varese, ITALY). Each rat was placed in restrainer and appropriate cuff with sensor was mounted on its tail and warmed to about 33- 35 ° C. The tail cuff was inflated to a pressure above 200 mmHg, systolic blood pressure; diastolic blood pressure and heart rate were measured directly by the tail cuff and pulse sensor.¹⁵

Antihypertensive activity in hypertensive rats

Three groups (n = 5) of Hypertensive rats (250 ± 10 gm) were treated orally once daily with deionized water, 10 ml/kg (control), PGE 200 mg/kg and PGE 400 mg/kg for three weeks. The systolic blood pressure and heart rate were measured by non-invasive blood pressure recorder apparatus before and after treatment. The rats were initially trained for blood pressure measurement on at least three separate occasions to establish a baseline blood pressure.

Vascular reactivity experiments

Vascular reactivity was studied in aortic segments by isometric tension.¹⁶ Briefly, two parallel stainless steel pins were introduced through the lumen of the segments: one was fixed to the organ bath wall and the other one was connected to a force transducer (Ugo basile, Italy), which in turn was connected to an amplifier. Segments were incubated in an organ bath containing 25 ml of Krebs–Henseleit solution at 37 ± 0.5 °C, continuously bubbled with a 95% O₂–5% CO₂ mixture (pH 7.4). An optimal resting tension of 1.5 g was applied to all aortic segment.¹⁷ This tension was adjusted every 15 min during a 60-min equilibration period before adding drugs. The vessel rings were equilibrated for 1 hour with the tension of 2.0 g and pre-contracted with KCl (60 mM) to produce the maximal KCl-induced contractile plateau. Subsequently the cumulative dose–response curve for noradrenaline (NA) (10⁻¹⁰-

10⁻⁵M) was obtained. The values of the NA-induced contraction were expressed as a percentage of maximal contraction induced by KCl. During 2 hours of stabilization the tissue was washed every 15 minutes. The dose response curve of nor-adrenalin (8.47 ×10⁻⁷ to 1.73 × 10⁻⁴) was studied on the isolated aortic strips of hypertensive rats and hypertensive rats treated with VAD. Contractile response to cumulative addition of agonist was recorded isometrically using Ugo basile transducer. Complete cumulative dose-response curves to NE were successively obtained at 30 min intervals in the absence and presence of the studied compound added 5 min before the second curve. Developed tension was plotted versus the log of NE concentration and concentration of NE producing half-maximal contraction was estimated graphically.¹⁸

Data analysis and statistics

Results are expressed as mean ± s.e.m. of the number of rats indicated; differences were analyzed using Student’s t-test. P > 0.05 was considered as statistically significant. Vasoconstrictor responses induced by Noradrenalin were expressed as a percentage of the tone generated by 75 mM KCl. Straight lines (log dose response curves) was drawn by linear regression.

RESULTS

Hypotensive activity in normotensive rats

In normotensive rats, baseline values of systolic blood pressure and HR were (118 ± 1) mmHg and (376 ± 10) bpm, respectively. Both of the used PGE at doses of 200 mg / kg and 400 mg / kg displayed no major hypotensive impact and did not affect HR on normotensive rats with respect to rats taking vehicle-only (Tables 1 and 2).

Anti-hypertensive activity

After 3 weeks of administration, oral fludrocortisone administration once daily triggered a substantial increase in blood pressure. Everyday oral administration of the various PGE doses led to a variable reduction in B.P. After 3 weeks of treatment, PGE at a dose of 200 mg / kg and 400 mg / kg developed substantial (p > 0.05) antihypertensive effect (Table 3).

At a dose of 200 mg / kg PGE showed a marked drop in heart rate at week 2 and week 3. At high dose (400 mg / kg) PGE reported a substantial decrease (p > 0,05) in the heart rate of hypertensive rats at the end of the third week compared to the control group (Table 4).

Table 1: Effect of PGE on the systolic blood pressure of normotensive rats.

Time	Normotensive rats (SBP mmHg ± SEM)		
	Normal control group	Treatment	
		200 mg/kg	400 mg/kg
0 hr	126 ± 1.11	118 ± 1.08	123 ± 1.02
2 hr	116 ± 1.09	115 ± 1.10	115 ± 1.18
4 hr	124 ± 1.10	121 ± 0.99	117 ± 0.77
6 hr	122 ± 1.19	112 ± 0.87	122 ± 0.81

SBP: Systolic blood pressure, all values are mean of 5 observations + SE.

Table 2: Effect of PGE on the heart rate of normotensive rats.

Time	Normotensive rats (Heart beats/min ± SEM)		
	Normal control group	Treatment	
		200 mg/kg	400 mg/kg
0 hr	379 ± 3.51	385 ± 2.99	372 ± 3.22
2 hr	395 ± 3.43	371 ± 3.01	420 ± 3.84
4 hr	389 ± 3.85	451 ± 3.21	397 ± 3.44
6 hr	392 ± 3.75	402 ± 3.88	375 ± 3.72

All values are mean of 5 observations + SE.

Antihypertensive activity of isolated fractions

Non polar fraction at dose of 10 mg/kg induced a significant decrease in the blood pressure of hypertensive rats as compared to hypertensive control rats while polar fractions showed no change in the blood pressure of hypertensive rats at the same dose (Figure 1 & Table 4).

Contractile response of vascular ring to NA

The vascular dysfunction is associated with increased vasoconstriction and a reduced diastolic function. We are therefore interested in deciding where the vascular where the vascular feature switches to a physiological modulator by detecting the vascular reactivity of the aortic rings, noradrenaline (NA). In isolated aortic rings, the cumulatively added NA (10-10-10-5 M) caused concentration-dependent contractile responses. We found that the group treated with DOCA these significantly increased, PGE treatment reduced the vasoconstrictive effect than the treated group (Figure 1). In addition, the PGE treated group's contractile responsivity to NA was significantly lower than the control group (Figure 1). PGE treatment (400 mg) significantly changed the dose-response curve of noradrenalin in isolated aortic strips to the right, As compared to the DOCA salt-treated hypertensive animals dose-response curve.

Identification of active compounds 1 and 2

Compound 1 was obtained as white powder that showed purple color on TLC after spraying with vanillin H₂SO₄. The ¹H and ¹³C NMR spectral data were given in Table 6 The ¹H and ¹³C NMR spectra of compound 1 (Figures 2 and 3) exhibited signals of an olefinic proton at δ_H 5.08 (H-12, s; δ_C 126.19), seven methyl signals at δ_H 0.85 (3H-23, s; δ_C 28.75), δ_H 0.84 (3H-24, s; δ_C 16.32), δ_H 0.64 (3H-25, s; δ_C 15.89), δ_H 0.71 (3H-26, s; δ_C 17.50), δ_H 0.99 (3H-27, s; δ_C 23.95), δ_H 0.75 (3H-29, d, J = 6.8 Hz; δ_C 17.65) and δ_H 0.82 (3H-30, d, J = 6.0 Hz; δ_C 21.43), nine methylene signals at δ_H 1.50 (2H-1, m; δ_C 39.45), δ_H 1.41 (2H-2, m; δ_C 28.23), δ_H 1.41 (2H-6, m; δ_C 19.02), (δ_H 1.80 (H-7_b, m) and δ_H 1.28 (H-7_a, m; δ_C 33.92), δ_H 1.80 (2H-11, m; δ_C 23.89), δ_H 1.41 (2H-15, m; δ_C 28.69), δ_H 1.50 (2H-16, m; δ_C 24.95), δ_H 1.41 (2H-21, m; δ_C 31.35) and δ_H 1.50 (2H-22, m; δ_C 37.56), five methine signals at δ_H 0.66 (H-5, d, J = 5.6 Hz; δ_C 56.14), δ_H 1.41 (H-9, m; δ_C 48.22), δ_H 2.09 (H-18, d, J = 12.8; δ_C 53.83), δ_H 1.50 (H-19, m; δ_C 39.85) and δ_H 1.41 (H-20, m; δ_C 39.80) and one oxygenated methine signal at δ_H 3.00 (H-3, dd, J = 4.8, 10.8 Hz; δ_C 78.55). In addition, the ¹³C-NMR spectrum showed signals that were allocated to one olefinic quaternary carbon (C-13, δ_C 139.26), one carboxylic acid (C-28, δ_C 178.54) and five quaternary carbons (C-4, δ_C 32.74), (C-8, δ_C 40.34), (C-10, δ_C 37.71), (C-14, δ_C 42.82) and (C-17, δ_C 48.43).

Table 3: Effect of PGE on systolic BP of hypertensive rats on continuous therapy for 21 days.

Treatment	Hypertensive rats (SBP ± SEM)			
	Days			
	0	7	14	21
Normal control	110.50 ± 3.64	110.83 ± 3.38	90.50 ± 0.81	112.30 ± 1.89
Hypertensive control	145.16 ± 0.79*	144.83 ± 1.47*	155.40 ± 1.41*	140.11 ± 1.63*
PGE 200mg/kg	143.50 ± 1.31	152.40 ± 1.89	131.20 ± 0.89**	123.90 ± 1.29**
PGE 400mg/kg	146.10 ± 1.38	143.20 ± 0.83	128.40 ± 1.12**	119.26 ± 0.89**

SBP: Systolic Blood Pressure, All values are mean of 5 observations + SE, *P < 0.05 compared to normal control group, **P < 0.05 compared to hypertensive control group.

Table 4: Effect of PGE on heart rate of hypertensive rats on continuous therapy for 21 days.

Treatment	Hypertensive rats (Heart beats/min ± SEM)			
	Days			
	0	7	14	21
Normal control	411.33 ± 3.64	387.3 ± 3.45	378.7 ± 3.64	418.14 ± 3.89
Hypertensive control	445.16 ± 2.79	444.73 ± 4.47*	455.10 ± 4.41*	440.11 ± 3.63
PGE 200mg/kg	443.50 ± 3.31	452.30 ± 3.89	431.20 ± 3.89	423.90 ± 3.29
PGE 400mg/kg	446.40 ± 3.38	443.40 ± 3.83	435.40 ± 1.12	376.25 ± 2.89**

*P < 0.05 compared to Normal control group, **P < 0.05 compared to hypertensive control group, all values are mean of 5 observations + SE

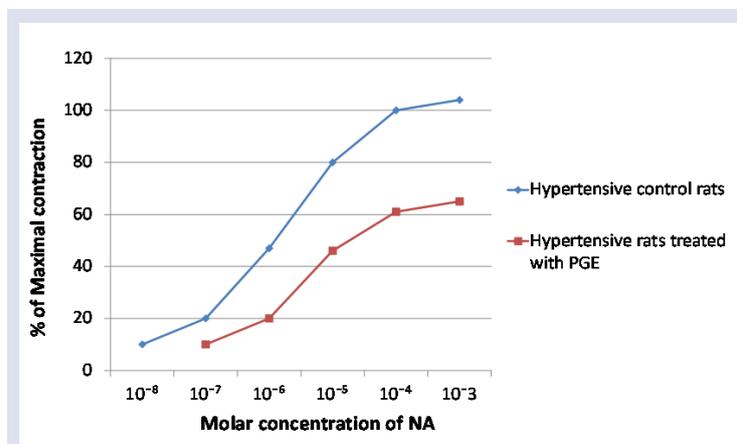
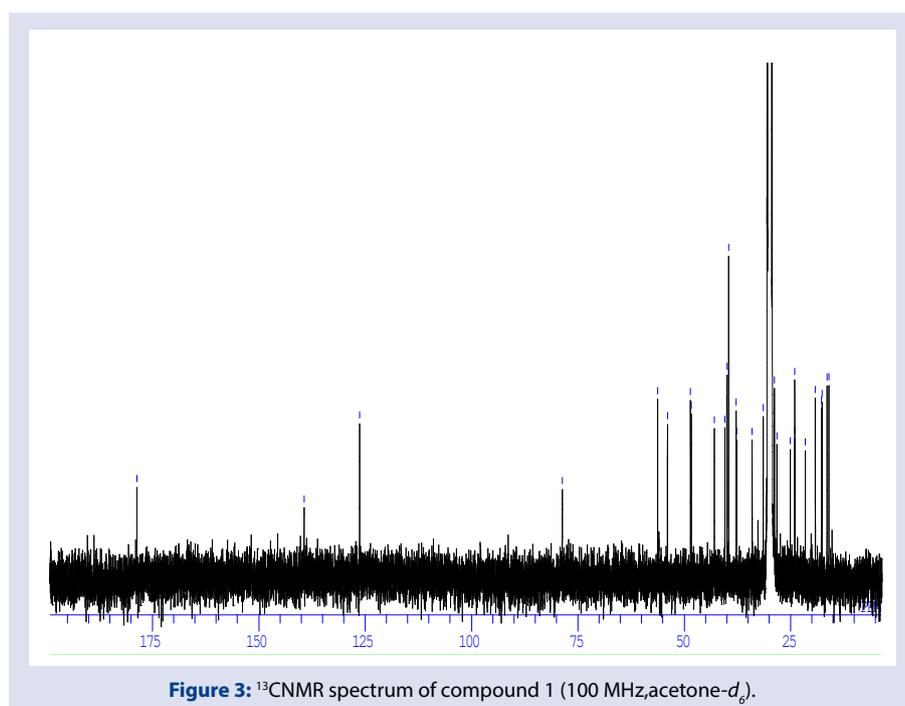
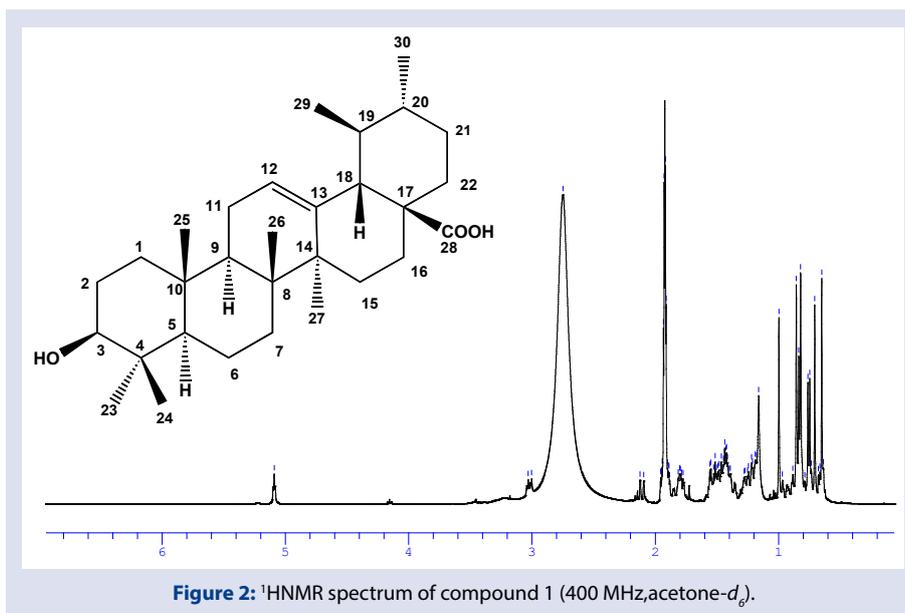


Figure 1: Cumulative dose-response curve to noradrenaline in aortic strips of rats made hypertensive by DOCA (hypertensive control rats) and hypertensive rats treated with PGE (400 mg/kg). P < 0.05 compared to hypertensive control group.



On the basis of spectroscopic information, compound **1** was identified as 3 β -hydroxyurs-12-en 28oic acid (ursolic acid).

Compound **2** was isolated as yellow needles and it showed blue color with FeCl_3 on TLC. It confirmed by ^1H and ^{13}C NMR data (Table 7) to be quercetin. The ^1H and ^{13}C NMR spectra of compound **2** (Figure 4 and 5) showed signals with good agreement with those previously reported.

DISCUSSION

This study reveals that in experimentally induced hypertensive models, PGE has a major antihypertensive effect. DOCA-induced hypertension is due to the sodium and water retention. The altered membrane permeability in the DOCA salt-treated hypertensive models has also been shown to cause an abnormal cation turnover. Such irregular loss of cation leads to vasoconstriction and eventually to an elevated B.P. In DOCA salt-treated hypertensive rats, the enhanced vascular sensitivity

to noradrenaline is also attributed to enhance mobilization of Ca into the vascular smooth muscle.¹⁹ Changes in voltage operating calcium channels or calcium permeability may be the main reasons for maintaining hypertension in the salt-treated hypertensive model DOCA.²⁰ Current *in vivo* and *in vitro* studies have shown that DOCA salt-treated hypertensive models have improved vascular reactivity to noradrenaline and adrenaline.

Both 10(-7) M PE and 10(-6) M ACh significantly increased K (+) uptake in endothelium-intact aorta versus control (121 % PE, 117 % ACh).²¹ Reducing VAD vascular reactivity in DOCA salt-treated hypertensive rats indicates that the sensitivity of the adrenoceptor to either adrenaline or adrenaline is altered. Focusing on the hypertension mechanism in the DOCA salt-treated hypertensive models, it is indicated that the antihypertensive effects of PGE may led to its alteration in cation transport across the cell membrane. It was reported

Table 5: The nonpolar paraffin oil fraction of the peels of *P. granatum* induced a significant decrease in the blood pressure of hypertensive rats as compared to hypertensive control rats. The polar aqueous fractions of peels of *P. granatum* showed no change in the blood pressure of hypertensive rats (Figure 1).

Treatment	Hypertensive rats (systolic BP \pm SEM)			
	Days			
	0	7	14	21
Normal control	110.50 \pm 3.64	110.83 \pm 3.38	90.50 \pm 0.81	112.30 \pm 1.89
Hypertensive control	145.16 \pm 0.79	144.83 \pm 1.47	155.40 \pm 1.41	140.11 \pm 1.63
Polar aqueous fraction (10 mg/kg po)	143.50 \pm 1.31	146.40 \pm 1.89	141.20 \pm 0.89	136.90 \pm 1.29
Nonpolar paraffin oil fraction (10 mg/kg po)	146.10 \pm 1.38	136.20 \pm 0.83	128.40 \pm 1.12	123.26 \pm 0.89

Table 6: ^1H NMR and ^{13}C NMR data of compound 1.

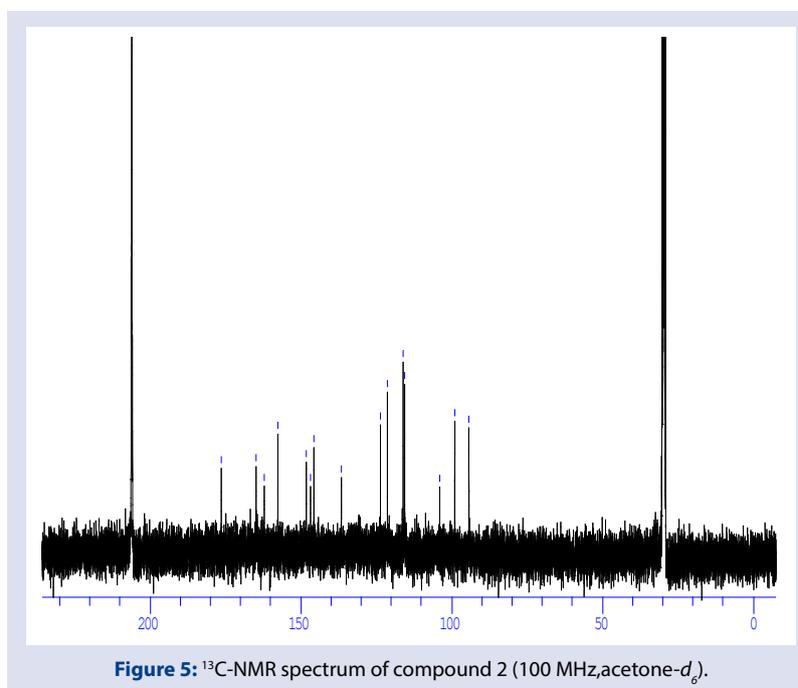
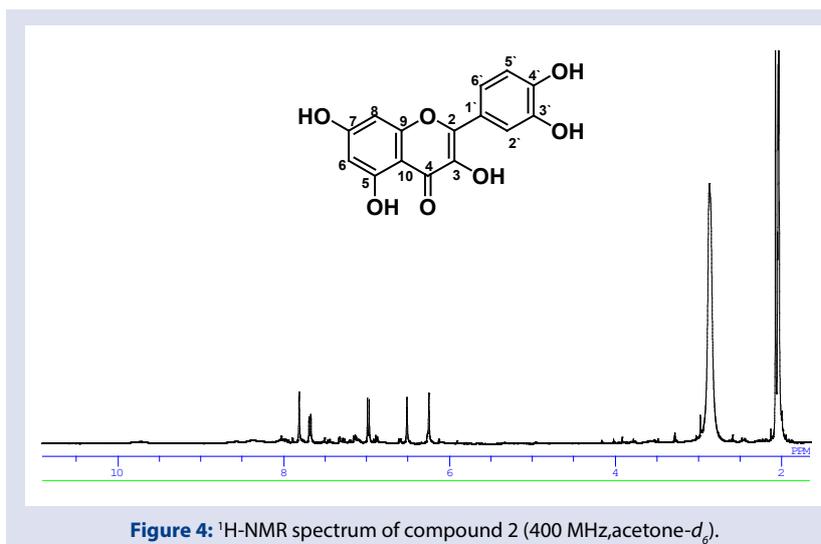
position	Δc	δ_{H} (integral, mult., J Hz)
1	39.45	1.50 (2H, m)
2	28.23	1.41 (2H, m)
3	78.55	3.00 (1 H, dd, J = 4.8, 10.8 Hz)
4	32.74	-
5	56.14	0.66 (1H, d, J = 5.6 Hz)
6	19.02	1.41 (2H, m)
7	33.92	1.80 (1H _b , m) - 1.28 (1H _a , m)
8	40.34	-
9	48.22	1.41 (1H, m)
10	37.71	-
11	23.89	1.80 (2H, m)
12	126.19	5.08 (1H, s)
13	139.26	-
14	42.82	-
15	28.69	1.41 (2H, m)
16	24.95	1.50 (2H, m)
17	48.43	-
18	53.83	2.09 (1H, d, J = 12.8 Hz)
19	39.85	1.50 (1H, m)
20	39.80	1.41 (1H, m)
21	31.35	1.41 (2H, m)
22	28.69	1.50 (2H, m)
23	28.75	0.85 (3H, s)
24	16.32	0.84 (3H, s)
25	15.89	0.64 (3H, s)
26	17.50	0.71 (3H, s)
27	23.95	0.99 (3H, s)
28	178.54	-
29	17.65	0.75 (3H, d, J = 6.8 Hz)
30	21.43	0.82 (3H, d, J = 6.0 Hz)

Taken in acetone- d_6 at 400 MHz for ^1H NMR and at 100 MHz for ^{13}C NMR.

Table 7: ^1H NMR and ^{13}C NMR data of compound 2.

Position	Δc	δ_{H} (integral, mult. J Hz)
2	147.94	
3	136.34	
4	176.15	
5	161.91	
6	98.74	6.24 (1H, d, J = 1.6 Hz)
7	164.57	
8	94.05	6.52 (1H, d, J = 1.6 Hz)
9	157.35	
10	103.72	
1'	123.40	
2'	115.82	7.80 (1H, d, J = 2.0 Hz)
3'	145.41	
4'	146.52	
5'	115.36	6.96 (1H, d, J = 8.8 Hz)
6'	121.04	7.67 (1H, dd, J = 8.8, 2.0 Hz)

Taken in acetone- d_6 at 400 MHz for ^1H -NMR and at 100 MHz for ^{13}C -NMR.



that quercetin reduces the raised blood pressure, due to its antioxidant properties.²² A number of proposed mechanisms may be responsible for the observed decrease in blood pressure including decreased oxidative stress, inhibition of angiotensin-converting enzyme activity, enhanced endothelial function, direct action on the smooth vascular muscle, and/or modulation in cell signaling and gene expression. Even if proof exists *in vitro* and *in vivo* to help and refute any possibility, Although there is evidence *in vitro* and *in vivo* to support and disprove every possibility, quercetin is likely to influence multiple targets through a combination of known and yet undiscovered mechanisms.²³ Mechanistically, the protective effect of ursolic acid is indeed by restoring the intracellular redox state and by inducing an antiapoptotic protein expression MCL⁻¹. The striking finding that ursolic acid has anti-apoptotic and antioxidant activity against ER stress-associated myocardial damage indicates that ursolic acid supplementation may be a possible strategy for mitigating the harmful effects of heat stress in cardiomyocytes.²⁴ Despite antihypertensive activity of ursolic acid was not reported this study showed its synergistic action for antihypertensive activity of quercetin compared to previous studies.

CONCLUSION

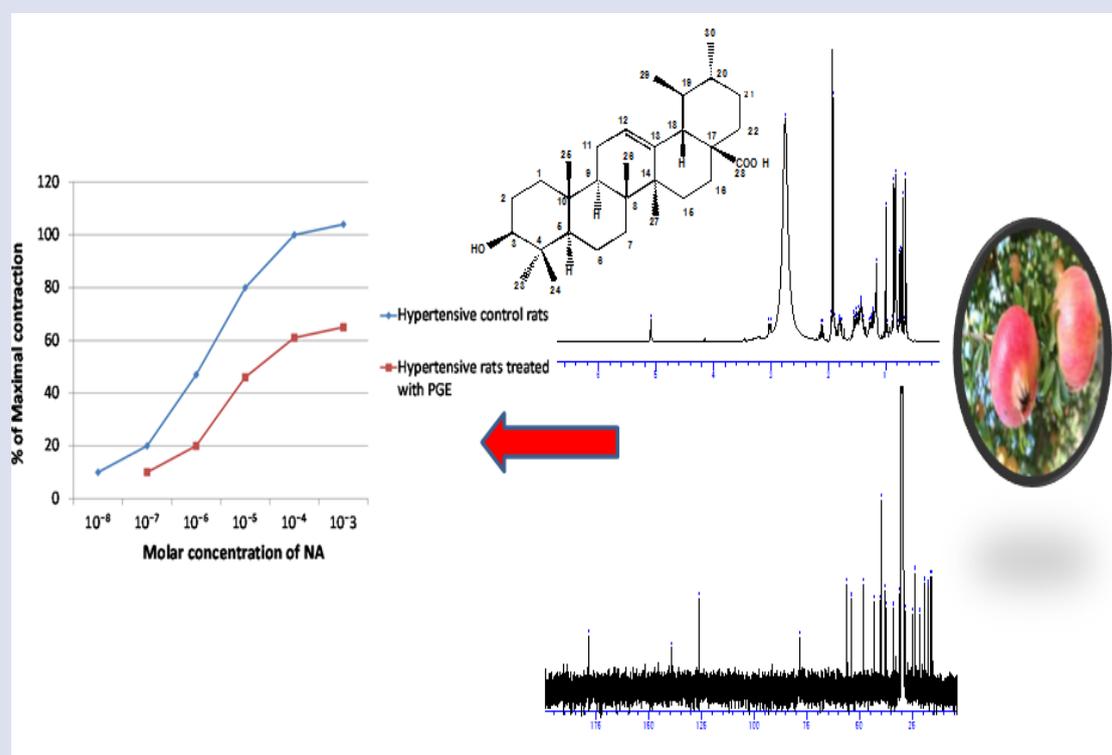
The bioactive mixture (ursolic acid and quercetin) induced a significant decrease in the blood pressure of hypertensive rats as compared to hypertensive control rats at a concentration of 10 mg/kg.

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GRAPHICAL ABSTRACT



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