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ORIGINAL ARTICLE

# Analysis of pomegranate juice components in rat corpora cavernosal relaxation

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This study evaluated the action of pomegranate juice (PJ) and its five principal phenolic constituents on rat corpus cavernosum smooth muscle (CCSM). Isometric tension studies were performed after precontraction with phenylephrine in CCSM from rats. Relaxant responses to PJ and its constituents ellagic acid (EA), chlorogenic acid, caffeic acid, cumaric acid and rutin were investigated. PJ and EA caused CCSM relaxations (94.1 ± 3.7 and 51.3 ± 9.9%), while others induced limited relaxant responses. EA response was not inhibited by L-N(G)-nitroarginine methyl ester (100 μM) and 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (1 μM). Tetraethylammonium (100 μM) and apamin (10 μM) and nifedipine (10 μM) inhibited EA-induced relaxations at $10^{-3}$ M by 84%, 82% and 78%, respectively. Glibenclamide (10 μM) inhibited EA response (97%, 100 μM). PJ-induced relaxation was not altered by several inhibitors. EA was estimated to be responsible for 13.3% of relaxation caused by PJ. Our study demonstrated that PJ and EA-induced marked relaxations in CCSM. The opening of Ca(2 +)-activated K+ channels and the inhibition of Ca(2 +)-channels regulate the relaxation by EA, but not PJ. EA has a minor contribution to the marked relaxation obtained by PJ, suggesting the presence of other PJ constituents, which induce nitric oxide-independent corporal relaxation. Further studies are needed to examine the potential of PJ in combination with a PDE5 inhibitor in ED.

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## INTRODUCTION

Normal penile erection is the result of a complex interaction between vascular, hormonal, neurologic and psychological factors.[1] ED is a common condition, which is identified by persistent or intermittent failure to achieve and/or maintain penile erection for satisfactory sexual performance.[2] Various options are available for the treatment of ED, such as oral, topical or intracorporal injection agents, vacuum constriction devices and penile prostheses.[3] In addition, many different herbal products have been investigated for the treatment of ED including Red Korea ginseng, maca, yohimbine, damiana and tribulus terrestris, Red Korea ginseng being the only promising one.[4–8]

Pomegranate, fruit from the tree Punica granatum contains significantly higher levels of antioxidant polyphenolic substances than more commonly consumed herbal liquids such as wine, green tea and apple juice.[9,10] The principal polyphenols in pomegranate juice (PJ) include the ellagitannins, which account for 92% of the antioxidant activity.[11] Punicalagins are the major ellagitannins in the whole fruit and can be hydrolyzed to ellagic acid (EA).

In the past decade, numerous studies on the antioxidant, anticarcinogenic and anti-inflammatory properties of pomegranate constituents have been focused on treatment and prevention of cancer,[12,13] cardiovascular,[14,15] metabolic,[14,16] inflammatory,[17,18] neurodegenerative diseases,[19,20] and so on.

Vascular risk factors, including hypercholesterolemia, atherosclerosis, hypertension and diabetes mellitus, can affect the neurovascular mechanisms of normal erection.[21,22] It is recognized that these diseases cause oxidative stress leading injury in tissues because of accumulation of reactive oxygen species. In earlier data by Azadzoi *et al.*[23,] it was demonstrated that atherogenic ED in the rabbit model reduced cavernous function, which involved in loss of smooth muscle relaxation, decreased endothelial nitric oxide synthase (NOS) and neuronal NOS and raised oxidative stress products. Interestingly, prolonged enteral PJ administration to this model restored cavernous response to the normal values. In addition, erectile tissue fibrosis was also delayed by the treatment. In this study, PJ had marked free radical scavenging activity.[23] Along with the above-mentioned study, the literature about the actions of the phenolic substances (including PJ) against chronic diseases with prolonged processes, that is, cancer,[12,13] cardiovascular,[14,15] metabolic,[14,16] inflammatory[17,18] and neurodegenerative diseases[19,20] are mostly based on their antioxidant properties. However, to our knowledge there is no data regarding the direct effects of PJ on erectile tissue function.

Mentioning together with its prophylactic and therapeutic effects by decreasing fibrosis[23] and oxidative stress products[24] in the atherogenic ED,[23,24] this study examined the direct potential relaxant effect of PJ and some of its constituents on rat corpus cavernosum smooth muscle (CCSM) function.

## MATERIALS AND METHODS

### Animals

All experiments were conducted in accordance with established guidelines and were approved by the local committee on animal experiments. Experiments were performed on adult male Sprague–Dawley rats (300–320 g, $n = 40$). Animals were housed two per cage on a 12-h light–dark cycle and were fed standard chow and water *ad libitum*.

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### Tissue procurement

After anesthesia with ketamine injection (100 mg $kg^{-1}$, intraperitonally), the penis was removed and placed in a Krebs–Ringer bicarbonate solution (containing: NaCl 118.1, KCl 4.7, $KH_2PO_4$ 1.0, $MgSO_4$ 1.0, $NaHCO_3$ 25.0, $CaCl_2$ 2.5 and glucose 11.1) and the cavernosal tissue was carefully dissected free from the surrounding tunica albuginea and mounted in organ baths.

### Isometric tension measurement in *in vitro* studies

Strips of cavernosal tissue ($1 \times 1 \times 9$ mm) were prepared and mounted under 1 g of resting tension in 20-ml organ bath chambers with one end attached to an electrode holder and one end to a wire connected to a force transducer (FT03 Grass Instruments, Quincy, MA, USA). Strips were maintained in Krebs-bicarbonate solution. Oxygen saturation and pH of 7.4 were maintained by continuous aeration with a mixture of 95% $O_2$ and 5% $CO_2$. After placement in the organ chamber at 37 °C, the tissues were allowed to equilibrate for a minimum of 60 min and the bath solution was replaced every 15 min.

After tissue equilibration at optimal isometric resting tension, the relaxant response elicited only by the PJ after precontraction with phenylephrine (PE; $10^{-5}$ M) were obtained. The same experimental procedures were repeated with EA, chlorogenic acid, caffeic acid, cumaric acid and rutin, which all of the concentrations that used were between $10^{-8}$ and $10^{-3}$ M. To investigate the mechanism(s) involved in PJ and EA-induced relaxations, experiments were repeated in the presence of the NOS inhibitor L-N(G)-nitroarginine methyl ester (L-NAME, 100 μM), the soluble guanylate cyclase inhibitor, 1H-(1,2,4)oxadiazolo[4,3-α]quinozalin-1-one (ODQ, 1 μM), BK(Ca) channel blocker tetraethylammonium (TEA,100 μM), small conductance Ca(2+)-activated K(+) channel blocker apamin (10 μM), ATP-sensitive K(+) (K(ATP)) channel blocker, glibenclamide (10 μM), an L-type Ca(2+)-channel blocker nifedipine (10 μM), PDE5 inhibitor sildenafil (10 μM) and adenylyl cyclase activator forskolin (10 μM). A total of 8–10 tissues for each experiment were used. Incubation period was 20 min for pharmacological agents. PJ concentrate was used in our experiments. The effects of PJ and its constituents were tested in a concentration-dependent manner. MAY Recorded Equipment Computer System (COMMAT, Ankara, Turkey) was used to record the mechanical activities.

### High pressure liquid chromatography analysis procedure for quantifying EA abundance in PJ concentrate

Liquid chromatography with diode array detection analyses were performed using an Agilent Technologies 1200 series (Santa Clara, CA, USA; high pressure liquid chromatography). Chromatographic separations were performed on Eclipse XDB-C18 column (15 cm × 4.6 mm, 5 μm). Acetonitrile and formic acid (40 mM) were used for separation. The flow rate was 1.0 ml $min^{-1}$ and compounds were detected at 254 nm. All the calculations concerning the quantitative analysis were performed with external standardization by measurement of peak areas. EA (2.5 mg) was accurately weighed into a 25 ml volumetric flask and filled up to volume with methanol. The PJ concentrate samples were accurately weighed (10 mg) and dissolved in 10 ml volumetric flask. After keeping 5 min in an ultrasonic bath, the solution was filtered through 0.45 μM Millipore (Billerica, MA, USA) solution and injected to chromatographic system. In high pressure liquid chromatography experiments, we exclusively focused on EA because of its high relaxant response, whereas other PJ constituents had very small relaxation responses.

### Chemicals and solutions

PE, EA, chlorogenic acid, caffeic acid, cumaric acid and rutin, L-NAME, ODQ, TEA, glibenclamide, apamin, nifedipin and forskolin were purchased from Sigma Chemical (St Louis, MO, USA). EA was dissolved in small amounts of dimethylsulphoxide. Then, aqueous solution of EA was prepared by diluting. Sildenafil (Viagra) was obtained from Pfizer (New York, NY, USA). PJ concentrate used was produced by MEPAS Food and Drug Company (Ankara, Turkey).

### Analysis of data

All values are expressed as mean ± s.e.m. A cumulative log concentration–relaxation curve for each compound was constructed, and results from measurement of graphs are expressed as percentage of the inhibition of contraction produced by PE (10 μM) added at the end of each experiment.

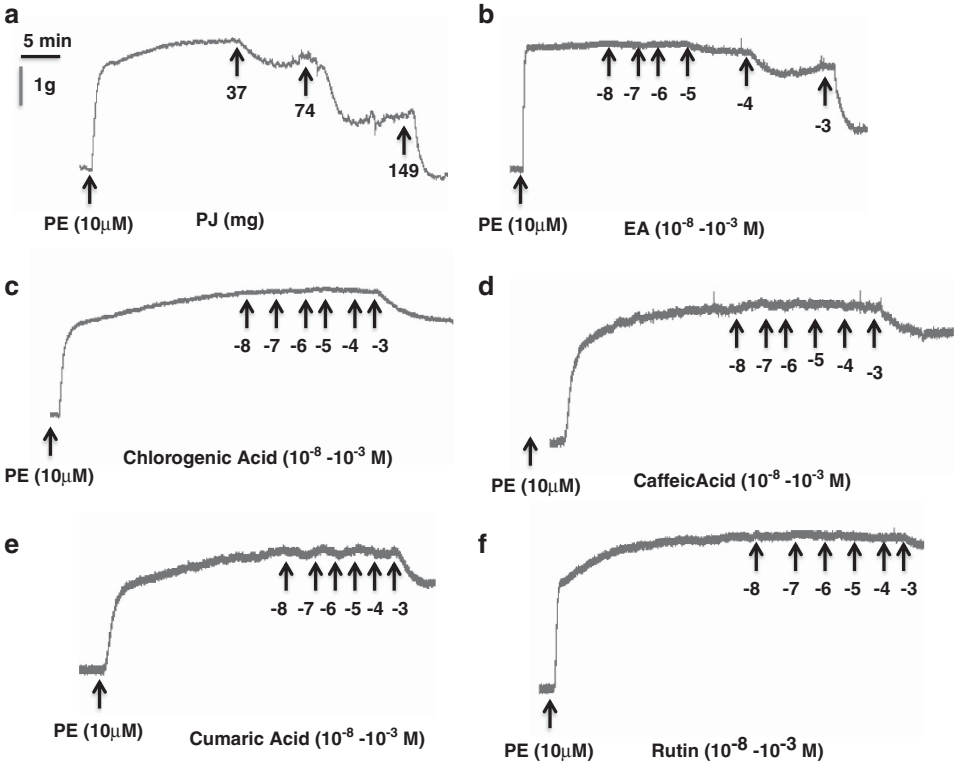


**Figure 1.** Representative traces showing the relaxation effect of pomegranate juice (PJ, **a**), ellagic acid (EA, **b**), chlorogenic acid (**c**), caffeic acid (**d**), cumaric acid (**e**) and rutin (**f**) on phenylephrine (PE)-pre-contracted rat corpus cavernosum.

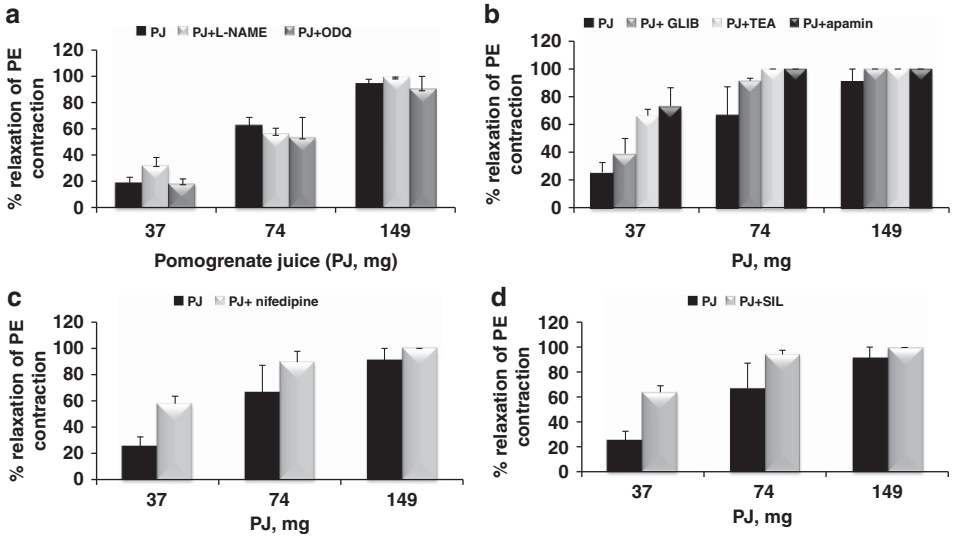


**Figure 2.** Relaxation effects of pomegranate juice (PJ) on phenylephrine (PE; $10^{-5}$ M) pre-contracted rat corpus cavernosum strips. (**a**) Please notice that relaxation responses to PJ were repeated in the presence of L-N(G)-nitroarginine methyl ester (L-NAME; 100 μM) and 1H-(1,2,4)oxadiazolo[4,3-α]quinozalin-1-one (ODQ; 10 mM). (**b**) Glibenclamide (Glib; 10 μM), tetraethylammonium (TEA; 100 μM) and apamin (10 μM) had no inhibitory effect on PJ-induced relaxation. (**c**) Presence of nifedipine (1 μM) did not alter the relaxant response. (**d**) Note the moderate but statistically insignificant potentialization of relaxation by sildenafil (SIL). Results are expressed as percent relaxation according to concentration in logarithmic scale and given as s.e. mean ($n = 8$–10).

Isometric force generation was measured as contraction (g tension per g tissue) in the absence and presence of each of the studied agents. Statistical differences were determined by one-way analysis of variance with repeated-measures followed by Bonferroni *post test* performed using Prism 4 statistical analysis packages for Windows (GraphPad Software, La Jolla, CA, USA). A $P$-value $< 0.05$ was considered to be significant.

## RESULTS

Representative traces of tissue responses to PJ, EA, chlorogenic acid, caffeic acid, cumaric acid and rutin are shown in Figure 1.

### PJ-induced relaxation of PE pre-contracted rat corpus cavernosum (CC) strips, and effects of L-NAME, ODQ, glibenclamide, TEA, apamin, nifedipine, sildenafil and forskolin

The maximal relaxation induced by PJ was 94.1 ± 3.7%. The relaxant responses to PJ were not inhibited by the presence of L-NAME and ODQ (Figure 2a). Similarly, tissue incubations with glibenclamide, TEA and apamin did not suppress the PJ-induced relaxant responses (Figure 2b). Incubation with nifedipine (Figure 2c), sildenafil (Figure 2d) or forskolin (data not shown) did not modify the persistent relaxation responses to PJ in the isolated CC.

### EA-induced relaxation of PE pre-contracted rat CC strips and effects of L-NAME, ODQ, glibenclamide, TEA, apamin, nifedipine, sildenafil and forskolin

EA produced a consistent dose-dependent relaxation between $10^{-8}$ and $10^{-3}$ M, and the maximum relaxation was 51.3 ± 9.9% at $10^{-3}$ M. The relaxant effect of EA was not attenuated in the presence of L-NAME, and ODQ at any EA concentration ($P > 0.05$ for all; Figure 3a). Incubation of the tissues with TEA and apamin antagonized EA-induced relaxation at every concentration, and by 84% and 82% at the highest EA concentration, respectively ($P = 0.033$ and $P = 0.016$ for $10^{-3}$ M; Figure 3b). Glibenclamide significantly suppressed EA-induced relaxant response at concentrations up to $10^{-4}$ M (97%, $P = 0.004$), but not at the highest concentration of EA, $10^{-3}$ M (31%, $P = 0.671$; Figure 3b).

Incubation of the tissues with nifedipine significantly diminished EA-induced relaxation at all concentrations and the inhibition was 78% at $10^{-3}$ M ($P = 0.023$; Figure 3c). On the other hand, sildenafil did not alter EA-induced relaxation response ($P = 0.32$; Figure 3d). Forskolin did not alter EA-induced relaxation of CC strips (data not shown).

### Relaxations induced by chlorogenic acid, caffeic acid, cumaric acid and rutin

No significant dose-dependent relaxations were observed with other studied PJ constituents; chlorogenic acid (30.3 ± 3.99%), caffeic acid (32.6 ± 17.0%), cumaric acid (14.4 ± 8.4%) and rutin (7.24 ± 0.52%; Figure 4). All observed relaxant responses were obtained only at the highest concentration of $10^{-3}$ M, with no marked relaxation between $10^{-8}$ and $10^{-4}$ M concentrations (Figure 4).

### EA content in PJ concentrate

EA content of the PJ extract was expressed as percent weight to weight present using the known weight of 1 ml (1492.2 mg). EA content was found to be 0.5352 ± 0.0166% in PJ concentrate by high pressure liquid chromatography analysis. Weight of 100 μl PJ was calculated to be 149.22 mg. Hence, the applied volumes (25, 50 and 100 μl) in organ baths consisted of 37.3, 74.6 and 149.2 mg of PJ, which included 199.6, 399.2 and 798.5 μg of EA, respectively.

### Estimated contribution of EA content to PJ-induced CCSM relaxation

On the basis of our findings, EA at $10^{-3}$ M induce approximately 53% relaxation. When the calculated amount of EA administered in the organ baths were considered, we could hypothetically estimate that EA content (0.78 mg) in 100 μl PJ would be responsible from 13.3% of total relaxation caused by PJ (94%,Figure 5).

## DISCUSSION

PJ induced dose-dependent relaxation in isolated rat CCSM. Among the constituents included in the study, EA caused the greatest response with 53% relaxation at the highest concentration. The other PJ constituents elicited modest relaxant responses.

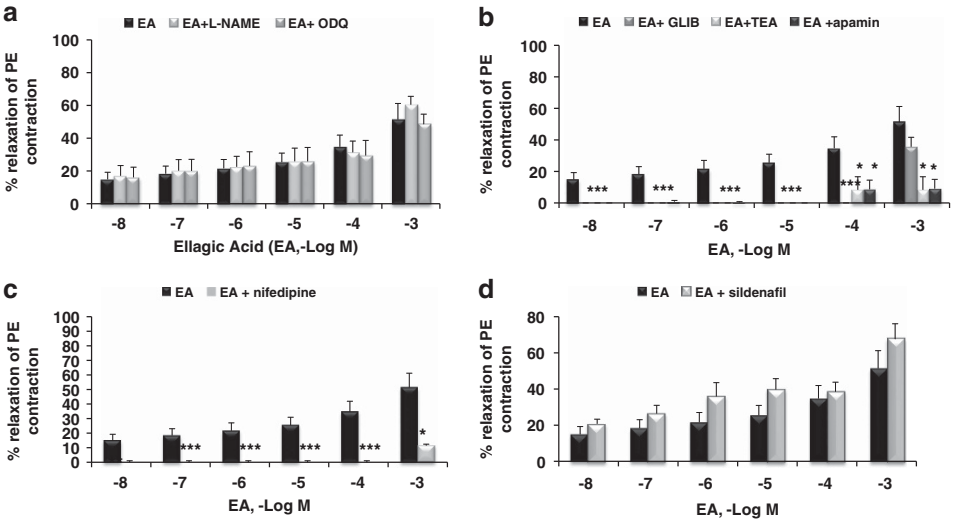


**Figure 3.** Relaxation effects of ellagic acid (EA) on phenylephrine (PE; $10^{-5}$ M) pre-contracted rat corpus cavernosum strips. (**a**) Please notice that relaxation responses to EA were repeated in the presence of L-N(G)-nitroarginine methyl ester (L-NAME; 100 μM) and 1H-(1,2,4)oxadiazolo[4,3-α]quinozalin-1-one (ODQ; 10 mM). (**b**) Tetraethylammonium (TEA; 100 μM) and apamin (10 μM) markedly inhibited EA-induced relaxation at concentrations between $10^{-8}$ and $10^{-3}$ M. Glibenclamide (Glib; 10 μM) had an inhibitory effect up to $10^{-4}$ M concentration but not at $10^{-3}$ M. (**c**) Presence of nifedipine (1 μM) caused marked inhibition of EA-induced relaxation at every EA concentration. (**d**) Note the moderate but statistically insignificant potentialization of the relaxation by sildenafil (SIL). Results are expressed as percent relaxation according to concentration in logarithmic scale and given as s.e. mean ($n = 8$–10, $^{*}P < 0.05$, $^{***}P < 0.01$).

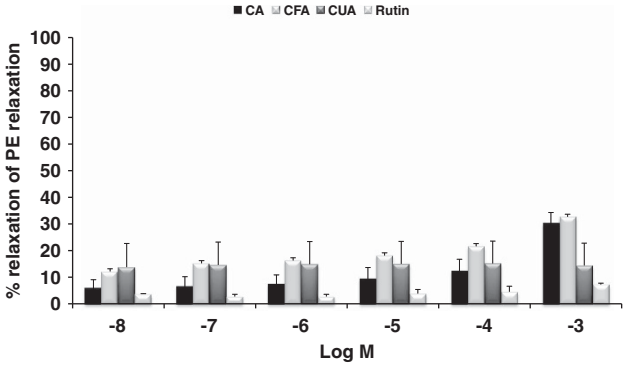


**Figure 4.** Relaxation effects of chlorogenic acid (CA), caffeic acid (CFA), cumaric acid (CUA) and rutin on phenylephrine (PE; $10^{-5}$ M) pre-contracted rat corpus cavernosum strips. Results are expressed as percent relaxation according to concentration in logarithmic scale and given as s.e. mean ($n = 8$–10).

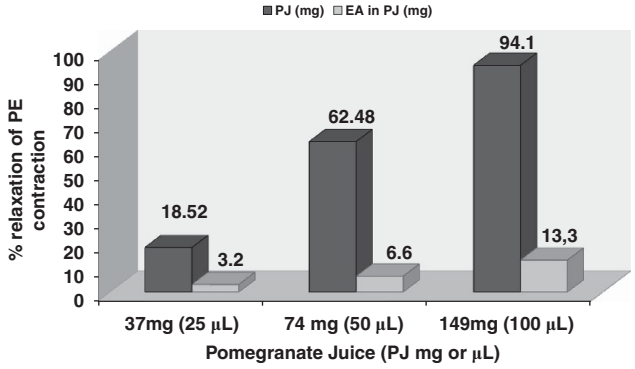


**Figure 5.** The estimated contribution of ellagic acid (EA) to the pomegranate juice (PJ)-induced relaxation of phenylephrine (PE; $10^{-5}$ M) pre-contracted rat corpus cavernosum strips. Note the relatively minor relaxant effect of EA compared with the marked overall relaxation caused by PJ.

The responses to PJ and EA, which induced relaxation in CCSM were not inhibited in the presence of NOS and soluble guanylyl cyclase (sGC) inhibitors, L-NAME and ODQ.

To date, direct effect of PJ on isolated tissues was not studied and published data most often focused on its antioxidant activity.[24] In this study, for the first time, we demonstrate the *in vitro* corporal relaxant activity of PJ, which is not suppressed by NOS inhibitor L-NAME or sGC inhibitor ODQ as well as other studied CCSM relaxation antagonists such as several K+ channel blockers (glibenclamide, TEA, apamin), Ca(2+) channel blocker nifedipine and adenylyl cyclase activator forskolin. Moreover, PDE5i sildenafil did not change the relaxant effect of PJ. At 37 and 74 mg of PJ, PDE5 inhibitor sildenafil (10 μM) had slightly enhanced PJ caused relaxations but both increase were not significant. Perhaps high concentrations (50–100 μM) of sildenafil in the PJ responses can be examined in future experiments. However, we cannot exclude the role of NO-sGC-cyclic guanosine monophosphate pathway in PJ-associated relaxation. The finding may suggest that PJ and its constituent EA act beyond the NOS activity by protecting NO from free radical incursion and increasing its bioavailability.

Azadzoi *et al.*[23,24] demonstrated both prophylactic and therapeutic effects of PJ on arteriogenic ED in animal models in two different investigations. In their recent research, the augmentive effects of pomegranate extract on *in vivo* erectile activity and *in vitro* CCSM relaxation has been shown in a rabbit arteriogenic ED model.[24] Pomegranate extract in the ED group significantly improved mean intracavernosal pressure/mean arterial pressure ratio compared with control group. Similarly, corporal relaxations were also significantly increased in the treated group. Administrated orally, the mechanism of action of PJ on erectile function was indirect and largely through its antioxidant effects in the ischemic penis. In this study, however, we evaluated the direct effects of PJ on CCSM function. We suggest that the *in vivo* and *in vitro* effects of PJ seem appropriate to obtain a beneficial effect.

Previous research indicated that EA is one of the major constituents of pomegranate.[11] In this study, EA caused approximately 51% relaxation in isolated CCSM. To our knowledge, no data exist regarding the direct effects of EA on corporal function. Like PJ itself, the relaxation caused by EA was not suppressed with NOS or sGC inhibitors. However, the relaxant effect of EA was inhibited by pre-treatment with the BKCa(2+) channel blocker (TEA), a small conductance Ca(2+)-activated K(+) channel blocker (apamin) and only partially by the selective ATP-sensitive K(+) channel blocker, glibenclamide. In previous data, it has been indicated that EA alone does not represent pomegranate polyphenols, and it is not as strong as apparently several synergistic chemical relationships that exist within the pomegranate fruit. Although EA alone caused 50% relaxation at the highest concentration, based on our calculations it may be estimated that approximately 13% of the relaxation caused by PJ is dependent on the EA content of PJ. The justification of this difference is not obvious according to our data. EA is one of the small polyphenols hydrolyzed from ellagitannins *in vivo*. Perhaps combined form of EA as in ellagitannins, that is, PJ fails to demonstrate the relaxant effect.

We found that chlorogenic acid-induced CCSM relaxation by 30% at highest concentration ($10^{-3}$ M). However, mild relaxant responses were recorded between $10^{-8}$ and $10^{-4}$ M concentrations. There is no published data about the effects of chlorogenic acid on penile erectile tissue. However, Bankar *et al.*[25] demonstrated relaxation of rat thoracic aorta by Cocos nucifera Linn. Endocarp in DOCA salt-induced hypertensive rats. Cocos nucifera Linn. Endocarp included chlorogenic acid together with vanillic acid and ferulic acid. Although the study does not demonstrate the effect of isolated chlorogenic acid on CCSM *per se*, it may suggest that chlorogenic acid may have a relaxant activity on smooth muscle.

In our study, in corporal smooth muscle, caffeic acid had no effect on PE contracted CCSM up to $10^{-3}$ M concentration, at which only a relaxation of 33% was observed. There are limited information regarding the effects of caffeic acid on smooth muscle. Cicala *et al.*[26] reported caffeic acid-phetenyl ester-induced relaxation in PE and KCl pre-contracted rat aorta. The inhibitor effect of caffeic acid-phetenyl ester on vascular and intestinal tissues was confirmed in other recent investigations.[27,28] On the other hand, Andriambeloson *et al.*[29] failed to demonstrate a relaxant effect of caffeic acid on rat aortic smooth muscle. Taken together, although the published data commonly demonstrate relaxant action of caffeic acid in certain types of smooth muscle, the mechanism is yet to be understood and debate continues.

Cumaric acid caused a minimal relaxation in rat CCSM in our investigation. The maximal observed relaxation was 14% at highest concentration. To our knowledge, there is no published data regarding the effects of cumaric acid on CCSM function. Similarly in Andriambeloson *et al.*'s study, cumaric acid did not cause endothelium-dependent relaxation in rat aortic rings.[29]

Rutin caused extremely minor relaxation (7%) at high concentration in this study. Similar to our findings, rutin induced only about 10% relaxation on electrical field stimulation-induced contractions in isolated male rat bladder.[30] In addition, published data indicated that rutin causes endothelium-dependent relaxation of the vascular smooth muscle.[31–33] Moreover, Cimanga *et al.*[34] demonstrated 93% and 86% relaxation of ACh and KCl-contracted ileum from guinea pigs by rutin. Based on these results, we claim that rutin may exert relaxant effects with varying efficacies in different tissues.

## CONCLUSIONS

This study demonstrated that PJ directly acts on rat CCSM, inducing marked relaxation. However, its constituent EA led to moderate cavernosal relaxation in pre-contracted rat CCSM. These responses are not attenuated with NOS or sGC inhibitors, and probably involve mechanism(s) independent from NO/cyclic guanosine monophosphate pathway. It appears that EA influence by activation of Ca(2+)-activated K+ channels and the suppression of the L-type Ca(2+) channels. On the other hand, EA has a relatively minor contribution to the relaxation obtained by PJ, suggesting the presence of other active constituents in PJ, which induce NO-independent relaxation of CCSM. It may be further hypothesized that, it is the possible synergistical action of more than one active PJ constituents that causes such a significant relaxation observed in the results of our study. Further investigation is warranted for the determination of organic and non-organic constituents of PJ responsible for CCSM relaxation and underlying molecular mechanisms.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

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