Punicic Acid Induces Endothelium-Dependent Vasorelaxation in Rat Thoracic Aortic Rings

Parinarik Asit Sıçan Torasik Aort Halkaları Endotel-Bağımlı Damar Gevşemesi Neden Olur

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ABSTRACT

Objective: The aim of the present study was to investigate the possible mechanism by which pomegranate seed oil causes vasodilatation in isolated thoracic aorta rings form the rat.

Material and Methods: The isolated rat arteries were cut into rings and placed in tissue chambers filled with Krebs solution.

Results: Pomegranate seed oil produced a concentration-dependent relaxation in rings with endothelium. However, it didn't cause any relaxant effect in the absence of endothelium. L-NAME and ODQ produced a significant inhibition in the rings with endothelium. Subsequent addition of L-arginine reversed the inhibitory effects of L-NAME. However, the relaxant effect of PSO did not change with tetraethylammonium, 4-aminopyridine and glibenclamide, in rings either with or without endothelium. Additionally, the relaxant effect of PSO was not affected by indomethacin, propranolol, losartan or captopril incubation. In another set of experiments rats were anaesthetised and surgically equipped with intraperitoneal radio-transmitters to record blood pressure in vivo. PSO caused a slight decrease in systolic and diastolic blood pressure along with mildly decreased heart rate.

Conclusion: These comprehensive findings clearly indicate that vasorelaxation is induced by PSO via modulation of nitric oxide-guanylyl cyclase pathway.

Key Words: Pomegranate seed oil, Punicic acid, Nitric oxide, Vasorelaxation, Aortic rings

ÖZ

Amaç: Çalışmanın amacı, nar çekirdeği yağı izole aorta halkalarında, vazodilatasyon sıçan oluşmasına neden olan olası mekanizmayı araştırmaktır.

Gereç ve Yöntemler: İzole sıçan arterler halkalar halinde kesilmiş ve Krebs çözümü ile dolu doku odalarına yerleştirildi.

Bulgular: PSO, endotel ile halkalarda konsantrasyona bağlı bir gevşeme üretti. Ancak, endotel olmadığında, herhangi bir gevşetici etkiye neden olmadı. L-NAME ve ODS, endotel ile halkalarda önemli oranda önlenmesini sağlar. L-arginin sonraki ekleme L-NAME önleyici etkilerini tersine çevirdi. Bununla birlikte, PSO'nun gevşetici etkisi ile ya da endotel olmayan ya da halkalarda, TEA ile 4-AP ve glibenklamid, değişmedi. Buna ek olarak, PSO'nun gevşetici etkisi indometasin, propranolol, losartan ya da kaptopril kuluçkalama ile etkilenmemiştir. Deneyler farelerin başka grup anestezi uygulandı ve cerrahi olarak, in vivo olarak kan basıncını kaydetmek üzere intraperitonal radyo vericisi ile donatılmıştır. PSO hafif birlikte sistolik ve diyastolik kan basıncında hafif bir düşüş kalp hızı azalmış neden.

Sonuç: Bu bulgular kapsamlı açıkça hipertansif hastalarda nitrik oksit-guanil siklaz yolunun modülasyonu yoluyla PSO tarafından uyarılan olduğunu göstermektedir.

Anahtar Sözcükler: Nar çekirdeği yağı, Parinarik asit, Nitrik oksit, Hipertansif hastalarda aort halkaları

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INTRODUCTION

Current findings indicate that punicic acid (PUA), ellagitannins, flavonoids, and anthocyanins may have therapeutically beneficial effects (1). Pomegranate seed oil (PSO) comprises 12-20% of total pomegranate seed weight is characterized by a high content of conjugated linolenic acids, as well as linoleic, oleic, stearic and palmitic acid (2-5).

Conjugated fatty acids such as linoleic and linolenic acid have recently attracted significant attention because of their health benefits in a variety of models of metabolic and chronic inflammatory diseases (6-9). PUA is a conjugated fatty acid which constitutes 64–83% of the PSO and found at high concentrations in the seed of pomegranate, naturally (10-13). It was shown that PUA can suppress colon cancer in rats and reduce skin carcinogenesis in mice (14, 15). On the other hand in clinical studies reduced proliferation, xenograft growth, and invasion of human prostate as well as a remarkable anticancer activity in breast cancer cells have been demonstrated after PUA administration (16-19). PUA was also shown to decrease the liver triacylglycerol accumulation in vivo and to reduce the apolipoprotein B100 secretion and triacylglycerol synthesis in HepG2 cells (20). Furthermore, Irene et al., showed that supplementation with PSO protects against high-fat diet-induced weight gain and fat mass gain in mice (21). Besides PSO intake was reported to decrease weigth gain and type 2 diabetes risk in obese CD-1 mice (22).

Several human studies have also been conducted, most of which have shown benefits of pomegranate products on cardiovascular health in relation to blood pressure, cholesterol, intimae media thickness, and endothelial function. For example, atherosclerotic patients with carotid artery stenosis that consumed pomegranate juice in addition to their regular medication for one year had a significant decrease in intimae-media thickness compared with control patients (23). Elderly, hypertensive subjects that consumed pomegranate juice for two weeks experienced a 36% decrease in serum angiotensin II converting enzyme activity and a 5% decrease in systolic blood pressure, both of which are markers for cardiovascular disease risk (24). Consistent with these findings Filomena de Nigris et al., showed that the pomegranate fruit extract supplementation prevented the rise of total and LDL cholesterol induced by atherogenic diet and reduced mean arterial pressure and heart rate (25). Although the beneficial effects of PSO have been repeatedly demonstrated, there is no evidence for vascular effects of PSO. Therefore, the present study was the first to investigate the mechanisms of vasorelaxation induced by PUA, which is one of the major compounds extracted from PSO in the rat thoracic aorta.

MATERIALS AND METHODS

Animals

10 to12 weeks-old male Wistar rats weighing 250-350 g were used for the present study. Animals were housed in a temperature- and light-controlled room with free access to food and water until the day of experiment. All experiments were reviewed and approved by Animal Research Ethics Committee of the Akdeniz University Faculty of Medicine.

Preparation of Aortic Rings

The animals were killed by decapitation and thoracic aortas were immediately excised and placed in Krebs solutions, cleaned and freed from surrounding connective tissue. The isolated arteries were cut into rings (4-5mm long) and placed in 20-mL tissue chambers filled with Krebs solution of following composition (mM): NaCl, 118; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_47H_2O$, 1.2; $CaCl_2$ $2H_2O$, 2.5; $NaHCO_3$, 25; glucose 11. Tissue baths were maintained at 37° C, pH 7.4, and bubbled with a mixture of 95 % O_2 and 5 % CO_2. Rings were suspended by two parallel stainless steel wires and tension was measured by isometric force transducers (MAY FDT 05, Turkey), connect to a computer-based data acquisition system (MP5, Biopac, Turkey). During the resting periods, the organ bath solution was changed every 15 minutes. Initially, the aortic rings were equilibrated for 60 minutes until a resting tension of 1.0 gr. After the equilibration period, aortic rings were firstly contracted with phenylephrine (PE) (10^{-6} M) to check their contractile responses, and then the tissues were rinsed 3 times with Krebs solution to restore tension to the precontracted level. Optimal tension was selected with which the greatest response to PE (10^{-6} M) could be obtained. We employed denuded aortic rings in some experiments. Endothelium-denuded aortic rings were prepared by turning the rings gently several times on the distal portion of a small forceps. Endothelial integrity was pharmacologically assessed by acetylcholine-induced vasodilatation (1 µM); segments showing no relaxation were considered to be endothelium denuded.

Experimental protocol

The aortic rings were equilibrated for 60 minutes until a resting tension of 1.0 gr. After the equilibration period, aortic rings were firstly contracted with PE (10^{-6} M) to increase tone. Once a stable contraction was achieved, PSO (10^{-8}-10^{-4} M) was added cumulatively on aortic rings with or without endothelium into organ bath. To characterize the mechanisms involved in PSO-induced vasorelaxant effect, the aortic rings were incubated with each inhibitor added to the bath for 30 min before PE was added to increase tone (26).

To characterize the involment of NO, experiments were performed in the presence of ODQ (0.5µM), the guanylyl cyclase inhibitor, added to the bath 5 min prior to the

addition of PE. In another set of experiments, L-NAME (10^{-4} M) added to the bath 5 min before PE. When the PE-precontraction reached a steady state, PSO (10^{-8}-10^{-4} M) was added cumulatively on aortic rings into organ bath. Finally, the rings were incubated with L-arginine (1 mM) for 30 min, and PSO (10^{-8}-10^{-4} M) was added.

To investigate the involvement of the endothelium in PSO induced vasorelaxation and the relevance of this response to prostanoids via the COX pathway, cyclooxygenase (COX) inhibitor indomethacin (10^{-5} M) were used.

Vasorelaxation to PSO was also performed in aortic rings precontracted with PE to assess the role of K^+ channels. These experiments were done in endothelium-denuded aorta to determine any involvement of endothelium-derived relaxing factors in the effects of PSO on activation of K^+ channels. Concentration-response curves were carried out in the presence of tetraethylammonium (TEA) (5 mM), a non-specific K^+ channel inhibitor, 4-aminopyridine (4-AP) (5 mM), a K_v channel inhibitor and glibenclamide (1 µM), a K_{ATP} inhibitor.

Beta-adrenoceptor blocker propranolol (10 µM), angiotensin II receptor antagonist losartan (1 µM) and angiotensin-converting enzyme inhibitor captopril (1 µM) were also tested to evaluate the possible role of different receptors in vasorelaxation to PSO in both endothelium-denuded and intact aortic rings (26).

At the end of experiment, papaverine (10^{-4}M) was added to elicit maximal relaxation and the relaxation to PSO expressed as a percentage of this maximum effect.

Implantation of radio-transmitters (radio-telemetry)

Rats were anaesthetised with a mixture (2 ml/kg, i.p.) of ketamine (90 mg/kg, Alfamine, Alfasan Internatioanl BV, Holland) and xylazin (10 mg/kg, Alfazyne, Alfasan Internatioanl BV) and were surgically equipped with intraperitoneal radio-transmitters with one catheter to record blood pressure (Transmitter model: C50-PXT, Data Sciences International, MN, USA). The catheter was inserted into the abdominal aorta and fixed with tissue adhesive (Vetbond®, 3M, St. Paul, MN, USA) for blood pressure and heart rate measurements. The animals received analgesic (100 mg/kg orally, Acetaminophen, Parcetol, Biokem, Turkey) treatment for 3 days. Thereafter, a 7-day recovery period was allowed before starting any experimental procedures. At the end of the 7-day recovery period, systolic blood pressure, diastolic blood pressure and heart rate were measured by radio-telemetry. Data capture from the telemetry measurements was performed synchronously. The capture periods and intervals were as follows: measurements were taken at pre-administration of PSO and at the end of the 3th and 7th days after its administration. All measurements were taken from each animal in the morning at 10.00 a.m.

Chemicals

All drugs and chemical were purchased from Sigma Chemical Company (St Luis, Missouri). PSO was kindly provided by company of Mavi Deniz (Izmir, Turkey). Indomethacin was dissolved in ethanol. Glibenclamide were dissolved in 20 % ethanol and 20 % DMSO. The remaining drugs were dissolved in Krebs solution. All drugs were prepared on the day of the experiment.

Statistical analysis

Mean responses at each concentration, expressed as mean ± SEM, were compared by analysis of variance (ANOVA) and Student's t-test where appropriate. Relaxation responses to PSO are expressed as percentages of the papaverine-induced vasorelaxation. The concentration of vasorelaxant giving half-maximal relaxation (EC_{50}) was obtained from the concentration-response curve. Maximal responses are expressed as mean± SEM and pD_2 values (-log of EC_{50} values) are expressed as means with % 95 confidence intervals (Cl). P values lower than 0.05 were considered significant. In all experiments, n equals the number of rats from which vessel segment were obtained (26).

RESULTS

Effects of PSO on aortic rings

As shown in Figure 1, PSO (10^{-8}-10^{-4} M) produced a concentration-dependent relaxation in rings with endothelium while it did not induce any relaxant effect in the absence of endothelium. The difference between two conditions was found to be significant (pD_2 value was 6.7±0.04 and maximal relaxation was 78.6%).

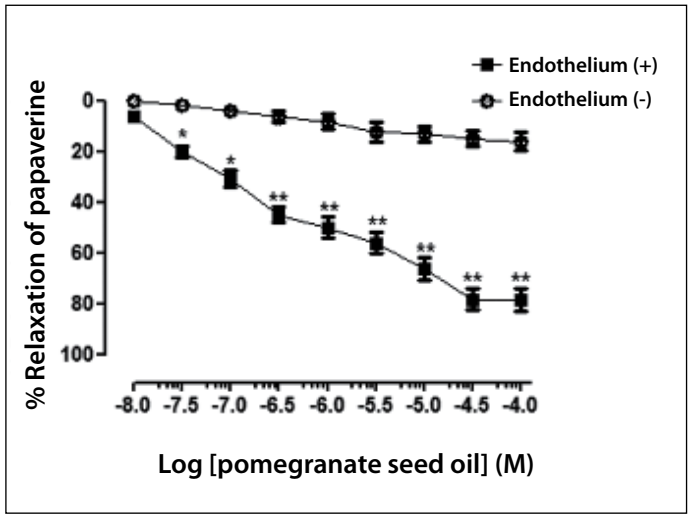

Figure 1: Effect of PSO on aortic rings with or without endothelium precontracted with PE. Each point represents the mean±SEM. [n=10, *P<0.05 and **P<0.001 compared with endothelium (+)].

To delineate the mechanism by which PSO induces vasorelaxation we performed experiments in the presence of pharmacological agents capable of modulating intracellular signals such as ODQ, L-NAME, L-arginine and potassium channel inhibitors. As shown in Figure 2, pre-treatment with the NOS inhibitor L-NAME (10^{-4} M) and ODQ (0.5 µM), the guanylyl cyclase inhibitor produced a significant inhibition of the relaxation curve for PSO in rings with endothelium. Subsequent addition of L-arginine (1 mM), the NOS substrate, reversed the inhibitory effects of L-NAME. The level of relaxation in the presence of L-NAME was significantly decreased from 78.2±4.5 to 13.2±2.1 (n=10, P<0.001), and the level of relaxation in the presence of L-NAME plus L-arginine was significantly increased from 13.2±2.1 to 71.2±4.5 (n=10, P<0.001). However, preincubation with tetraethylammonium (TEA) (5 mM), a non-specific K^+ channel inhibitor, 4-aminopyridine (4-AP) (5 mM), a K_v channel inhibitor and glibenclamide (1 µM), a K_{ATP} inhibitor, did not change the relaxant effect of PSO in aortic rings either with or without endothelium (Figure 3).

Effects of indomethacin, propranolol, losartan, captopril on vasorelexation to PSO:

In this set of experiments indomethacin (10^{-5} M), propranolol (10 µM), losartan(1µM) or captopril (1µM) incubation did not modify the relaxant effect of PSO in aortic rings (data not shown).

Effect of PSO administration on heart rate and blood pressure

Although PSO administration throughout 7 days tended to modify both heart rate and blood pressure of rats, this effect did not attain significance level (Table 1, p>0.05). In vivo administration of PSO caused a slight decrease in systolic and diastolic BP. HR of control rats were also mildly decreased after PSO administration, and statistically significant difference was not found. In addition, PSO-evoked reduction in systolic BP, diastolic BP and HR was not pronounced in the 7th day when compared with 3th day (P>0.05).

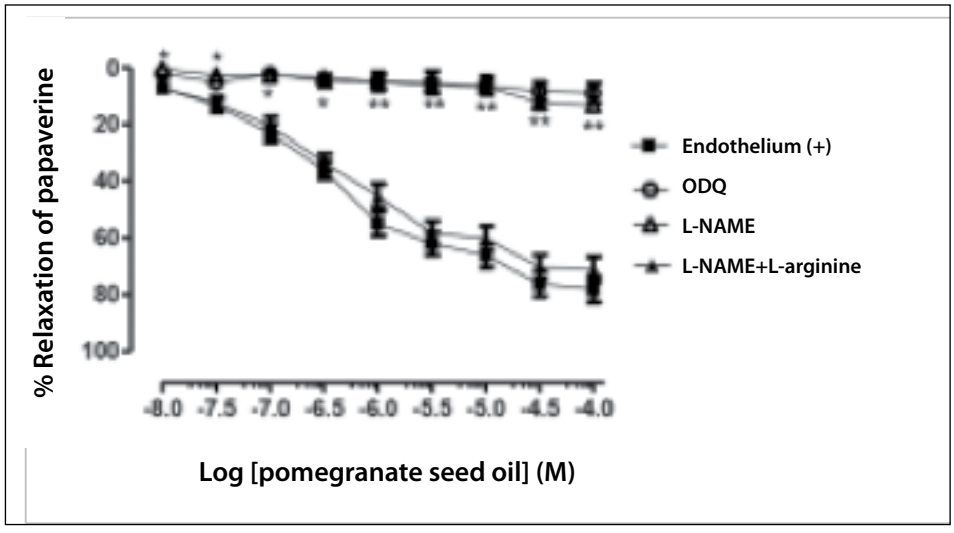

Figure 2: Effect of ODQ (0.5 µM), L-NAME (0.1 mM), and L-NAME+L-arginine (1 mM) on PSO-induced relaxations of aortic rings with endothelium. Each point represents the mean±SEM. [n=10, *P<0.05 and **P<0.001 compared with endothelium (+)].

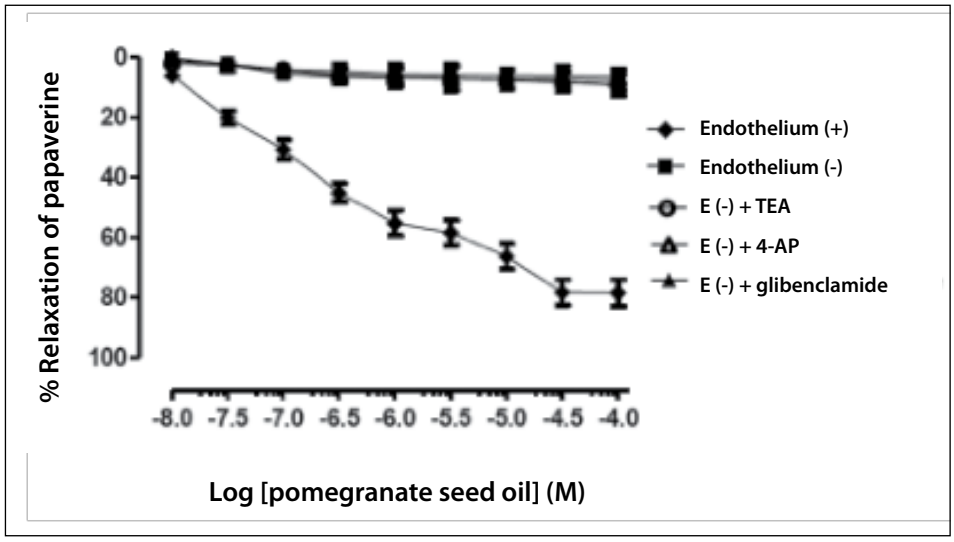

Figure 3: Effect of TEA (5 mM), 4-AP (5 mM), and glibenclamide (1 µM) on PSO-induced relaxations of aortic rings with/without endothelium. Data are represented as mean mean±SEM. (n=10).

Table I: Blood pressure and heart rate values in PSO-administered rats. (Data are expressed as mean ± SEM, n=5).

	Pre-administration	Post-administration	
		3rd day	7th day
Systolic pressure (mmHg)	146 ± 7.9	134 ± 4.1	130 ± 3.6
Diastolic pressure (mmHg)	107 ± 5.5	100 ± 2.1	99.2 ± 2.9
Heart rate (bpm)	355 ± 36	342 ± 16	322 ± 7.0

DISCUSSION

Pomegranate has been used in several systems of medical therapy for a variety of ailments. Over the past decade, significant progress has been made in establishing the pharmacological mechanisms of pomegranate and the individual constituents responsible for them. In our previous study we found that ellagic acid which is also a component of pomegranate can induce endothelium-dependent and independent vasorelaxation, at least in part due to NO and L-type calcium channels (26). In this study the presence of endothelium-derived vasodilator factor NO in PSO-induced vasodilatation was examined. This is the first study showing the vasodilatory effect of PSO in rat thoracic aorta in vitro and describes the underlying cellular mechanism. We found PSO induced vasorelaxation in a concentration-dependent manner and this effect had a rapid time of onset between 2 s and 2 min. The results showed that the vasorelaxation induced by PSO was abolished by removing endothelium, which implies that the action of PSO arises through endothelium-dependent mechanisms in rat aorta. Here it is important to mention that the relatively low concentration of PSO can accomplish acute vascular relaxation, since pD_2 value of PSO was measured 6.70±0.04 in the presence of endothelium. The achievement of significant effect at such a low concentration of PSO is important from pharmacological aspect.

It is well known that NO is a major endothelium-derived relaxing factor that induces relaxation of vascular smooth muscle cells (27). In our study to evaluate whether the relaxant effect of PSO involves NO release, the aortic rings with endothelium were incubated with ODQ the guanylyl cyclase inhibitor and L-NAME (NOS inhibitor). These two substances play a role in NO pathway. The results showed us that PSO dependent vasorelaxation was completely reversed by ODQ and L-NAME, indicating the contribution of NO in PSO-induced responses.

Another possible mechanism that mediates relaxation of vascular smooth muscle is the opening of K^+ channels. To date, various types of K^+ channel have been identified in vascular smooth muscle such as voltage-dependent, Ca^{2+}-activated, ATP-sensitive and inward rectifier K^+ channels (28, 29). Opening of membrane K^+ channels in vascular smooth muscle increases K^+ efflux, which leads to membrane hyperpolarization, closing of voltage-dependent Ca^{+2} channels, and subsequent relaxation (29). In the present study, preincubation of aortic rings with tetraethylammonium (TEA), a non-specific K^+ channel inhibitor, 4-aminopyridine, a K_v channel inhibitor and glibenclamide, a K_{ATP} inhibitor did not modify the relaxant effect of PSO either in the presence or absence of endothelium. These findings clearly suggest that K^+ channels do not play any role in vasorelexant effect of PSO in rat aortic rings. On the other hand, COX inhibitor indomethacin didn't alter the relaxant effect of PSO which suggests that COX inhibition does not take place in endothelium-dependent relaxations induced by PSO.

Our results suggest that the vasorelexant effects of PSO are clearly dependent on endothelium. In previous studies pomegranate extracts have been reported to exhibit hypotensive and anti-diabetic effects (30). Additionally, the administration of 100–300 mg/kg/day for 4 weeks of pomegranate juice extract to diabetic rats treated with angiotensin II decreased mean arterial blood pressure and the biochemical changes induced by diabetes and angiotensin II (31). Therefore angiotensin II receptor and angiotensin-converting enzyme can be suggested to play a role in PSO-induced relaxant effect in aortic rings. However, preincubation with angiotensin II receptor antagonist losartan and angiotensin-converting enzyme inhibitor captopril did not change the relaxant effect of PSO in rings with and without endothelium. Also, beta-adrenoceptor blocker propranolol had not any effect. These results demonstrate that the presence of these types of receptors and angiotensin-converting enzyme is unlikely in the effect of PSO in aortic rings.

In the present study, in vivo administration of PSO caused a slight decrease in systolic and diastolic BP as well as HR which was not statistically significant. Since vasodilatory action of PSO had been defined to be NO dependent *in vitro*, lately we investigated only the short term effect of PSO administration in rats *in vivo*. However, further studies targeting the long term impact of PSO in healthy and hypertensive subjects would be interesting in clinical aspect.

In conclusion, it was clearly shown for the first time that PSO has direct vasorelaxant effects on the rat aortic rings. This PSO induced endothelium-dependent effect is possibly due to NO signalling pathway. Lowering high blood pressure by vasodilatation is very important to prevent high blood

pressure-induced vascular diseases. In clinical trial with hyperlipidaemic individuals, consumption of PSO twice daily for four weeks increased HDL cholesterol while it decreased the total cholesterol significantly (32). These results indicate that there may be some long-term benefits of PSO consumption on plasma lipid profiles that are associated with cardiovascular health. The likely concomitant vasodilatory effect of PSO would potentiate this beneficial effect in cardiovascular diseases. The findings of this study provides evidence that PSO has a vasodilator effect and in future, it may have a potential to be used as a natural product in treatment of clinical disorders such as hypertension and metabolic syndrome.

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REFERENCES

1. Jurenka JS. Therapeutic applications of pomegranate (Punica granatum L.): A review. Altern Med Rev 2008;13:128-44.
2. Lansky EP, Harrison G, Froom P, Jiang WG. Pomegranate (Punica granatum) pure chemicals show possible synergistic inhibition of human PC-3 prostate cancer cell invasion across Matrigel. Invest New Drugs 2005;23:121-2.
3. Fadavi A, Barzegar M, Azizi M. Determination of fatty acids and total lipid content in oilseed of 25 pomegranates varieties grown in Iran. Food Comp Anal 2006;19:676-80.
4. El-Shaarawy M, Nahapetian A. Studies on pomegranate seed oil. Fette Seife Anstrichmitte 1983;85:123-6.
5. Ozgul-Yucel S. Determination of conjugated linolenic acid content of selected oil seeds grown in Turkey. JAOCS 2005;82:893-7.
6. Ha YL, Grimm NK, Pariza MW. Anticarcinogens from fried ground beef: Heat-altered derivatives of linoleic acid. Carcinogenesis 1987;8:1881-7.
7. Tsuzuki T, Tokuyama Y, Igarashi M, Miyazawa T. Tumor growth suppression by alpha-eleostearic acid, a linolenic acid isomer with a conjugated triene system, via lipid peroxidation. Carcinogenesis 2004;25:1417-25.
8. Arao K, Yotsumoto H, Han SY, Nagao K, Yanagita T. The 9cis,11trans,13cis isomer of conjugated linolenic acid reduces apolipoprotein B100 secretion and triacylglycerol synthesis in HepG2 cells. Biosci Biotechnol Biochem 2004;68:2643-5.
9. Yamasaki M, Kitagawa T, Koyanagi N, Chujo H, Maeda H, Kohno-Murase J, et al. Dietary effect of pomegranate seed oil on immune function and lipid metabolism in mice. Nutrition 2006;22:54-9.
10. Sassano G, Sanderson P, Franx J, Groot P, Van Straalen J, Bassaganya-Riera J. Analysis of pomegranate seed oil for the presence of jacaric acid. J Sci Food Agric 2009;89:1046-52.
11. Joh Y, Kim S, Christie W. The structure of the triacyglycerols, containing punicic acid, in the seed oil of Trichosanthes kirilowii. JAOCS 1995;72:1037-42.
12. Kaufman M, Wiesman Z. Pomegranate oil analysis with emphasis on MALDI-TOF/MS triacylglycerol fingerprinting. J Agric Food Chem 2007;55:10405-13.
13. Ahlers N, Dennison A, O'Neill L. Spectroscopic examination of punicic acid. Nature 1954;173:1045-6.
14. Kohno H, Suzuki R, Yasui Y, Hosokawa M, Miyashita K, Tanaka T. Pomegranate seed oil rich in conjugated linolenic acid suppresses chemically induced colon carcinogenesis in rats. Cancer Sci 2004;95:481-6.
15. Hora JJ, Maydew ER, Lansky EP, Dwivedi C. Chemopreventive effects of pomegranate seed oil on skin tumor development in CD1 mice. J Med Food 2003;6:157-61.
16. Albrecht M, Jiang W, Kumi-Diaka J, Lansky EP, Gommersall LM, Patel A, et al. Pomegranate extracts potently suppress proliferation, xenograft growth, and invasion of human prostate cancer cells. J Med Food 2004;7:274-83.
17. Lansky EP, Jiang W, Mo H, Bravo L, Froom P, Yu W, et al. Possible synergistic prostate cancer suppression by anatomically discrete pomegranate fractions. Invest New Drugs 2005;23:11-20.
18. Lansky EP, Newman RA. Punica granatum (pomegranate) and its potential for prevention and treatment of inflammation and cancer. J Ethnopharmacol 2007;109:177-206.
19. Jeune MA, Kumi-Diaka J, Brown J. Anticancer activities of pomegranate extracts and genistein in human breast cancer cells. J Med Food 2005;8:469-75.
20. Arao K, Wang YM, Inoue N, Hirata J, Cha JY, Nagao K, et al. Dietary effect of pomegranate seed oil rich in 9cis, 11trans, 13cis conjugated linolenic acid on lipid metabolism in obese, hyperlipidemic OLETF rats. Lipids Health Dis 2004;3:24.
21. Vroegrijk IO, van Diepen JA, van den Berg S, Westbroek I, Keizer H, Gambelli L, et al. Pomegranate seed oil, a rich source of punicic acid, prevents diet-induced obesity and insulin resistance in mice. Food Chem Toxicol 2011;49:1426-30.
22. McFarlin BK, Strohacker KA, Kueht ML. Pomegranate seed oil consumption during a period of high-fat feeding reduces weight gain and reduces type 2 diabetes risk in CD-1 mice. Br J Nutr 2009;102:54-9.
23. Aviram M, Rosenblat M, Gaitini D, Nitecki S, Hoffman A, Dornfeld L, et al. Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation. Clin Nutr 2004;23:423-33.

24. Aviram M, Dornfeld L. Pomegranate juice consumption inhibits serum angiotensin converting enzyme activity and reduces systolic blood pressure. Atherosclerosis 2001;158:195-8.
25. de Nigris F, Balestrieri ML, Williams-Ignarro S, D'Armiento FP, Fiorito C, Ignarro LJ, et al. The influence of pomegranate fruit extract in comparison to regular pomegranate juice and seed oil on nitric oxide and arterial function in obese Zucker rats. Nitric Oxide 2007;17:50-4.
26. Yılmaz B, Usta C. Ellagic Acid-induced endothelium-dependent and endothelium-independent vasorelaxation in rat thoracic aortic rings and the underlying mechanism. Phytother Res 2013;27:285-9.
27. Vanhoutte PM. Endothelial dysfunction: The first step toward coronary arteriosclerosis. Circ J 2009;73:595-601.
28. Nelson MT, Quayle JM. Physiological roles and properties of potassium channels in arterial smooth muscle. Am J Physiol 1995;268:C799-822.
29. Standen NB, Quayle JM. K+ channel modulation in arterial smooth muscle. Acta Physiol Scand 1998;164:549-57.
30. Takahashi M, Yamashita A, Moriguchi-Goto S, Sugita C, Matsumoto T, Matsuda S, et al. Inhibition of factor XI reduces thrombus formation in rabbit jugular vein under endothelial denudation and/or blood stasis. Thromb Res 2010;125:464-70.
31. Mohan M, Waghulde H, Kasture S. Effect of pomegranate juice on Angiotensin II-induced hypertension in diabetic Wistar rats. Phytother Res 2010;24 Suppl 2:S196-203.
32. Mirmiran P, Fazeli MR, Asghari G, Shafiee A, Azizi F. Effect of pomegranate seed oil on hyperlipidaemic subjects: A double-blind placebo-controlled clinical trial. Br J Nutr 2010;104:402-6.