Chemical Composition and Phytoestrogen Analysis of Iranian Black Pomegranate Juice Concentrate and Seeds

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ABSTRACT

In this study, as preliminary research for the development of natural estrogen supplement the chemical properties of Iranian black pomegranate juice concentrate and seeds were evaluated. Proximate compositions of pomegranate juice concentrate and seeds were as follows; crude lipid 0.4% and 8.2%, moisture 39.9% and 6.6%, crude protein 0.9% and 12.2%, ash 1.4% and 1.7%, and carbohydrate 42.0% and 84.5% respectively. Major amino acids are glutamic acid (1310.0ppm) and aspartic acid (896.2ppm) in juice concentrate, and glycine (611.1ppm) and arginin (401.6ppm) in seeds. Ascorbic acid has the highest concentration of 20.0mg/100g in juice concentrate and 0.23mg/100 in seeds. The compositions of unsaturated fatty acids such as linoleic acid and linolenic acid were higher than those of saturated fatty acids such as stearic palmitic acid. Major minerals were potassium, calcium and sodium, potassium was highest in both juice concentrate and seeds. Vitamins were composed of ascorbic acid (20.0mg/100g), vitamin B_1 (0.12mg/100g) and niacin (0.80mg/100g) in juice concentrate, and only ascorbic acid(0.23mg/100g) in seeds. Organic acids such as citric and L-malic acid were detected only in pomegranate juice concentrate. The contents of total polyphenols were 4.55g/L in juice concentrate and 3.5mg/100g in seeds, respectively. Phytoestrogens detected in pomegranate juice concentrate and seeds were daidzein, quercetin, genistein and 17 β-estradiol.

Key Words : Phytoestrogen, pomegranate, chemical properties

INTRODUCTION

The supplementation of estrogen becomes necessary for women who experience postmenopausal symptom due to the radical decrease of estrogen. The reduction of estrogen is known to cause the atrophy of urinary and hot flashes, rigor(Morishige et al, 2001; Cauley et al, 2001). Synthetic hormone supplementation effectively

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diminishes the risk factors of postmenopausal symptom, however, it grows the possibility of breast cancer and uterine cancer(Clavel-Chapelon et al, 2000; Pike et al, 2000). Therefore, in food and drug industry, a great effort has been undergoing to find the plants that contain the alternative natural hormones acted as biologically active material and non-endocrine disrupter.

Genistin, daidzin, daidzein, genistein, coumestrol are famous for the alternative natural products acted as estrogen. The plants such as soy, red clover, moghate root, olive seed, pinus pinia and pomegranate seeds are known to contain those alternative natural products(Herrmann et al, 1981; Moneam et al, 1988).

Pomegranate is self-generated in west-south of Asia and north-west of India centered at Iran and currently spreaded at subtropical and tropical region. It is known to be cordial, anthelmintic and has shown the good effect on a diarrhea, dysentery, gastralgia, leucorrhea and the prevention of hypertension and arteriosclerosis (Namsandang, 1989; Ann, 1989). Only small portion of pomegranate is produced domestically and it has low abundance of sugar and other components, which is used merely as fruit, tea and drink. A majority of pomegranate is imported from Japan, America and Iran and many research has been performed in those countries for the study of the chemical components, biological effect and intake efficacy(Kaplan et al, 2001; Amakura et al, 2000; De Pascual-Teresa et al, 2000). Several studies were published domestically including the research of anticarcinogenic properties(Shim, 2001), the control of intestinal microflora and evaluation(Han, 1995) and the colorant effect of pomegranate peel(Cho, 2000). In this study, chemical and phytoestrogen compositions of Iranian black pomegranate juice concentrate and seeds were analyzed for the development the alternative natural hormones and health foods.

MATERIALS AND METHODS

Materials

Iranian black pomegranate juice concentrate (including seeds), seeds, soy-isoflavon extract and arrowroot-isoflavon extract were supported from Hanilyanghaeng (Ansung, Korea).

Proximate analysis

Moisture, protein, fat and ash percentage of pomegranate juice concentrate were determinated according to AOAC(1995).

Amino acid analysis

Sample (1.0g or 1mL) was acid-hydrolyzed by 20 mL of 6-N HCl using test tube for hydrolysis at 105℃ for 24 hrs. The product was filtered using 0.2 ㎛ filter paper, then analyzed by amino acid analyzer using ninhydrin method. Amino acid analyzer (Model 6300, Beckman Inc., Fullerton, CA, USA) at detection wavelengths of 460 nm and 530 nm was employed using a sodium column (4.6 mm × 15 cm).

Mineral analysis

Sample pretreatment was performed by hydrolysis with conc. HNO_3, and they were analyzed by inductively coupled plasma-atomic emission spectrophotometer (Spectro flame Modula E, Fitchburg, MA, USA). Table 1 shows the detailed conditions for mineral analysis.

Vitamin analysis

Vitamin C was further analyzed after the rapid extraction with 5% methaphosphoric acid, then by HPLC using μ-Bondapak C_{18} (4.6 mm I.D. × 250 mm) with 100% H_2O solvent at the speed of 1 mL/min (270 nm detection). Other water-soluble vitamins were extracted with water/methanol(1:1) solvent, then analyzed by HPLC using μ-Bondapak C_{18} (4.6 mm I.D.

Table 1. Operating conditions of ICP for mineral analysis.

Power	1.2 Kw for aqueous	
Nebulizer pressure	3.5 bar for meinhard type C	
Aerosol flow rate	0.3 L/min	
Sheath gas flow	0.3 L/min	
Cooling gas	12 L/min	
Wavelength (nm)	Fe	275.574
	Cu	324.754
	K	766.491
	Zn	213.856
	Mn	257.610
	P	178.290
	Na	588.995
	Ca	373.690

× 250 mm). The gradient elution was applied to 100% H_2O by 60% methanol for 20 min at the speed of 1 mL/min (270 nm detection).

Fatty acid analysis

Lipid was extracted by Bligh and Byer method (1959), then n-hexane layer was treated to produce methyl ester form. The product was dried with Na_2SO_4, then analyzed by gas chromatography (GC). GC (Hewlett Packard 6890A, Palo Alto, CA, USA) was performed using FFAP column and other conditions are as follows: injection port; 260℃, detection port; 270℃, oven temperature; initially 180℃, then finally 220℃ at the speed of 2℃/min; helium as the eluent, injection; 0.5μL, split ratio; 50:1, FID detector.

Sugars and organic acids analysis

Glucose, fructose and sucrose were determined by HPLC (Shisheido Model, SI-1, Japan) using a refractive index (RTP-6A) detector, on a carbohydrate analysis column (4.6 mm I.D. × 25 cm, Waters, Milford, MA, USA). The eluent and flow rate were 75% acetonitrile (Sigma, St. Louis, MO, USA) and 0.8mL/min. Sample preparation and chromatographic procedure were conducted as described in AOAC(1995) and total sugar was calculated by summation of individual sugar.

Organic acids were performed using HPLC (Waters 510, Waters, USA) with UV 215nm(Waters 996, Waters USA) on a 30 cm column(Supelcogel C-610H, 7.8 × 30 mm) after preconditioning with Sep-pak C18 cartridges(0.45 μm). The eluent and flow rate were 0.1% phosphoric acid and 0.5 mL/min.

Polyphenols determination

Polyphenols were determined following the method described by Price and Butler(1977). A 60 mg portion of fruit juice was shaken manually for 60 s with 3 mL of methanol in a test tube. The mixture was filtered and the tube was quickly rinsed with 3 mL of methanol. The filtrate was mixed with 50 mL water and analyzed within an hour. To 1 mL of filtrate, 3 mL of 0.1M $FeCl_3$ in 0.1 N HCl and 3 mL of 0.008 M K4 $Fe(CN)_6$ were added. The absorbance of the color, developed after 30 min at 30℃, was read at 720 nm. A standard curve was prepared, expressing the result as catechin equivalent, i.e. amount of catechin (mg/mL) which gave a colour

intensity equivalent to that given by polyphenols after correction for the blank.

Analysis of phytoestrogen and estrogen

Phytoestrogens employed in this experiment were flavone, hexaestrol, DES, enterodiol, equol, Chrysin, enterolactone, β-zearalanol, α-zearalanol, zearalanone, 17-β-estradiol, Biochanin-A, catechin, β-zearalenol, α-zearalenol, daidzein, genistein, kaempferol, apigeninm, and quercetin. Phytogens were sstrone, 17-β-estradiol, 2-hydroxyestrone, 2-hydroxyestradiol, 6-α -dehydroestrone, 6-α -hydroxyestradiol, 4-methoxyestradiol, estriol, 16-epiestriol, 16,17-epistriol, 16-α-hydroxyestrone, 17-epiestriol, 6-ketoestriol, 2-methoxyestriol, 6-hydroxyestriol, and 16-ketoestradiol. Internal standard was d4-17β-estradiol. All chemicals were pruchased from Sigma. Standard solution was prepared by dissolving 0.01g in methanol at the concentration of 1,000 ppm. It was stored at refrigerator before use. MSTFA(N-methyl-N-trimethylsilyl-trifluoroacetamide) and trimethylchlorosilane (TMCS) were used for derivatization as a mixed solution of MSTRA:TMCS=100:1(v/v). Serdolit AD-2 resin(100-200 micron) was purchased from Serve (German).

Sample pretreatment and analysis followed Chung's method(Choi, 2001). Briefly, sample(25mg) was extracted with methanol and 10㎍/mL of d_4-17β-estradiol as the internal standard was added. Then, it was hydrolyzed at 55℃ for 3 hrs. Diethylether (5mL) was added to this solution for extraction, then evaporated. MSTRA:TMCS=100:1(v/v) (50㎕) was added to organic layer to generate TMS ester. Derivatized ester (2㎕) was injected to GC/MS for analysis.

Hewlett-Packard 5890 PLUS Gas Chromatography and a directly interfaced 5970 mass selective detector (MSD) were employed. The column was Ultra-2 (length; 25mm, i.d.; 0.20 mm, film thickness 0.33 ㎛) with the temperature gradient of 180℃ to 260℃ at the speed of 20℃/min, 6 min stay, 275℃ at the speed of 2 ℃/min, 8 min stay, 300℃ at the speed of 15℃/min, and final 10 min stay. Injection temperature was 300℃, detector temperature 300℃, helium eluent at the speed of 0.85mL/min with the split mode(ratio 1:12). The ionization energy was 70 eV and selected ion monitoring(SIM) was used.

RESULTS AND DISCUSSION

Approximate composition

Moisture, protein, fat and ash percentage of pomegranate juice concentrate are shown in Table 2. The overall composition of approximate compositions of seeds contained higher than that of juice concentrate. Protein contents of the juice concentrate and seeds were 0.9% and 12.2%, respectively. Similar values for the juice were reported by Morton(1987), however, El Memr et al. (1992) reported 13.2% protein in seeds that is higher than our value. Ash contents of juice concentrate and seeds were 1.4% and 1.7%

Table 2. Proximate compositions in pomegranate juice concentrate and seeds (% of total weight).

Compositions	Pomegranate juice concentrate	Pomegranate seeds
Moisture	39.3	6.6
Lipid	0.4	8.2
Protein	0.9	12.2
Ash	1.4	1.7
Carbohydrate	42.0	84.5

Table 3. Amino acids in pomegranate juice concentrate and seeds (ppm).

Composition	Pomegranate juice concentrate	Pomegranate seed
Aspartic acid	896.2	248.7
Threonine	88.7	117.5
Serine	459.5	198.6
Glutamic acid	1310.2	-
Proline	174.0	156.2
Glycine	106.5	611.1
Alanine	270.3	213.6
Cystein	7.6	-
Valine	97.5	175.3
Methionine	92.7	42.8
Isoleucine	52.7	150.7
Leucine	64.8	275.9
Tyrosine	59.4	61.4
Phenylalanine	57.5	139.8
Histidine	158.3	-
Trypropan	74.5	-
Lysine	82.1	56.7
Arginine	187.7	401.6

respectively, and these values were close to those reported by Morton (1987).

Amino acids and minerals

Table 3 shows the content of amino acids in pomegranate juice concentrate and seeds. Major amino acids are glutamic acid (1310.0ppm) and aspartic acid (896.2ppm) in juice concentrate, and glycine (611.1ppm) and arginin (401.6ppm) in seeds.

Table 4 shows the minerals in pomegranate juice concentrate and seeds. The amounts of potassium, calcium and sodium, potassium was highest in both juice concentrate and seeds. It is obvious that potassium is the most abundant element in fruit, followed by sodium and calcium. The content of other elements except iron in the juice and seeds were similar to the paper reported by Chauhan et al (1991).

Sugars and organic acids

The sugars contents in pomegranate seeds were glucose, 6.6g/100g and sucrose, 7.7g/100g, respectively. Total sugars were determined as 150g/L in pomegranate juice concentrate.

Organic acids were detected only in pomegranate juice concentrate. Organic acids such as citric, L-malic, tartaric, oxalic and succinic acids were individually detected and quantitated. Citric acid was predominant with a 4.4 g/L. L-malic acid was the second most abundant, with a range of 0.5g/L. Other organic acids detected were tartaric(0.3g/L), oxalic(0.05g/L) and succinic acids(0.4g/L).

Vitamins and fatty acids

Table 5 shows the composition of water-soluble vitamins from pomegranate juice concentrate at the

Table 4. Minerals in pomegranate juice concentrate and seeds (mg/100g).

Composition	Pomegranate juice concentrate	Pomegranate seed
K	255.0	243.0
Ca	8.0	65.3
Na	1.0	97.5
P	15.0	7.49
Fe	664.0	1.88
Mg	0.59	11.9
Cr	0.02	-
Zn	0.2	1.26
Cu	0.1	0.04

Table 5. Vitamins in pomegranate juice concentrate and seeds (mg/100g).

Composition	Pomegranate juice concentrate	Pomegranate seed
Thiamin (Vit B1)	0.12	-
Riboflavin (Vit B2)	0.02	-
Ascorbic acid (Vit C)	20.00	0.23
Niacin	0.80	-
Vitamin B_6	0.02	-

Table 6. Fatty acids in pomegranate juice concentrate and seeds(Peak area %).

Composition	Pomegranate juice concentrate	Pomegranate seed
Palmitic (16:0)	8.3	8.4
Stearic (18:0)	9.4	8.1
Oleic (18:1)	6.8	6.8
Linoleic (18:2)	12.9	13.8
Linolenic (18:3)	47.0	60.0
Others	15.6	2.9
Saturated	17.7	16.5
Unsaturated	66.7	84.6

level of mg/100g. Fat soluble vitamins were not found, and only five different water-soluble vitamins were observed in samples. Ascorbic acid has the highest concentration of 20.0mg/100g in juice concentrate and 0.23mg/100 in seeds, and relatively low concentrations of vitamin B_1 (0.12mg/100g), vitamin B_2 (0.02mg/100g), niacin (0.80mg/100g) vitamin B_6 (0.02mg/100g) in juice concentrate were observed.

Table 6 shows the composition of fatty acids from pomegranate juice concentrate and seeds. Five different

Table 7. Phytoestrogens in pomegranate juice concentrate, seeds and other source (ppm).

Compositions	Pomegranate juice concentrate	Pomegranate seeds	Soybean isoflavon concentrate	Arrowroot isoflavon concentrate
Equol	-	-	-	0.63
Biochanine-a	-	-	0.66	0.65
Catechin	1.48	3.1	-	-
Daidzein	23.72	24.20	745.46	51.15
Genistein	0.29	2.65	3.48	0.31
Apigenin	-	-	35.33	-
Quercetin	9.75	29.54	-	-
17β-estradiol	0.15	-	-	-
2,3-di-MeO-estradiol	0.04	-	-	-

fatty acids were found with the majority of linoleic acid and linolenic acid. The composition of unsaturated fatty acid composition were higher than those of saturated fatty acid in both samples.

Total polyphenols and phytoestrogens

Total polyphenols were 4.55g/L in juice concentrate and 3.5mg/100g in seeds, respectively.

Phytoestrogens are plant compounds with estrogen-like biological activity. The use of certain plants in traditional medicine and folklore may be ascribed to their estrogenic properties. For example, the pomegranate is associated with fertility(Price, 1985), and the Thai vine and Pueraria Mirifica are used as a rejuvenant and aphrodisiac(Bradbury, 1954; Fuhrmann, 1986. About 20 different phytoestrogens and 16 estrogens in isoflavon concentrate from arrowroot, soy and pemegranate seeds were analyzed in this experiment.

As shown in Table 7, daidzein has the highest concentration of 51.75 ppm from arrowroot extract. Equal (0.63 ppm), biochanine-A (0.65 ppm), and genistein (0.15 ppm) were also observed. Daizein from soy isoflavon extract has 15 times higher concentration (745.46 ppm) than that from arrowroot. Apigenin (35.33ppm), genistein (3.48ppm), biochanine-A (0.66ppm) were also found from soybean isoflavon. About six phytoestrogens were observed from pomegranate extract. They were daidzein (23.72ppm), quercetin (9,75ppm), catechin (1.48ppm), genistein (0.29ppm) 2,3-di-MeO-estradiol (0.04ppm), and 17β-estradiol (0.15ppm). In pariticular, 17β-estradiol was detected at pomegranate extract, but not at soy and arrowroot isoflavon.

In our study, phytoestrgens from pomegranates were confirmed and compared with the extracts from soybean and arrowroot isoflavon. The procedure for the extraction of high concentration of phytoestrogen from pomegranate and the animal test with rat for *in vivo* activity are under study.

ACKNOWLEDGMENT

This work was supported by Small and Medium business administration, and Hankyong national university as Consortium project, 2002-2003.

REFERENCE

Amakura, Y., Okada, M., Tsuji, S. and Tonogai, Y. 2000. High-performance liquid chromatographic

determination with photodiode array detection of ellagic acid in fresh and processed fruits. J. Chromatogr A. 27;896(1-2):87~93.

Ann, D.K. 1989. Hankukbonchodogam. Kyohacksa, Seoul.

AOAC. 1995. Official methods of analysis. Association of official analytical chemists, Washington DC.

Bligh, E.G. and Dyer, W.J. 1959. A rapid method of total lipid extraction and purification, J. Bio. Physiol. 37: 911.

Bradbury, R.B. and White, D.E. 1954 Estrogens and related substances in plants. Vitamins Horm. 12:207~233.

Cauley, J.A., Zmuda, J.M., Ensrud, K.E., Bauer, D.C. and Ettinger, B. 2001. Timing of estrogen replacement therapy for optimal osteoporosis prevention. J Clin Endocrinol Metab. 86(12):5700~5705.

Chauhan, K.S., Pundir, J.P.S. and Erbas, S. 1992. Extraction and composition of pomegranate juice. Fluessiges-Obst. 59(2):335~340.

Cho, E.K. 2000. Dye of textile using Pomegranage peel, Chonnam national Univ., MS Thesis.

Choi, M.H., Yoo, Y.S. and Chung, B.C. 2001. Biochemical roles of testosterone and epitesto-sterone to 5 alpha-reductase as indicators of male-pattern baldness. J Invest Dermatol. 116(1):57~61.

Christie, W.W. 1982. The preparation of methyl and other esters of fatty acids. In Lipid Analysis, 2nd ed. Pergamon press. New York p.52.

Clavel-Chapelon, F. and Hill, C. 2000. Hormone replacement therapy in menopause and risk of breast cancer. Presse Med. 21;29(31):1688~1693.

De Pascual-Teresa, S., Santos-Buelga, C. and Rivas-Gonzalo, J.C. 2000. Quantitative analysis of flavan-3-ols in Spanish foodstuffs and beverages. J. Agric Food Chem., 48(11): 5331~5337.

El-Nemr, S.E., Ismail, I.A. and Ragab, M. 1992. The chemical composition of juice and seeds of pomegranate fruite. Fluessingesobst. 59(11). Fruit processing 2(11):162~164

Fuhrmann, H. 1986. Germany in the middle ages. Cambridge medieval textbooks. Cambridge: Cambridge University Press.

Han, B.J. 1995. Screenign biomaterials for the control of ntestinal microflora and evaluation of akebia, pomegranate and chicory. Hanyang Univ., Ph.D Thesis.

Herrmann, K. 1981. Review on chemical composition and constituents of some important exotic fruits. Z Lebensm Unters Forsch. 173(1):47~60.

Kaplan, M., Hayek, T., Raz, A., Coleman, R., Dornfeld, L., Vaya, J. and Aviram, M. 2001. Pomegranate juice supplementation to atherosclerotic mice reduces macrophage lipid peroxidation cellular cholesterol accumulation and development of atherosclerosis. J. Nutr. 131(8):2082~2089.

Moneam, N.M., Sharaky, A.S. and Badreldin, M.M. 1988. Oestrogen content of pomegranate seeds. J. Chromatogr. 22;438(2):438~42.

Morishige, K., Matsumoto, K., Ohmichi, M., Nishio, Y., Adachi, K., Hayakawa, J., Nukui, K., Tasaka, K., Kurachi, H. and Murata, Y. 2001. Clinical features affecting the results of estrogen replacement therapy on bone density in Japanese postmenopausal women. Gynecol Obstet Invest. 52(4): 223~226.

Morton, J.F. 1987. Pomegranate. In J. F. Morton (Ed), Fruits of warm climate. Miami. FL. pp352~355.

Namsandang. 1989. Color Natural Medicinal plant Encyclopedia. Seoul.

Pike, M.C. and Ross, R.K. 2000. Effect of hormone replacement therapy on breast cancer risk: estrogen versus estrogen plus progestin. J. Natl. Cancer Inst. 6:92(23):1951~1952.

Price, K.R. and Fenwick, G.R. 1985. Naturally occurring oestrogens in foods. Food Addit Contam. 2:73~106.

Price, M.L. and Butler, L.G. 1977. Rapid visual

estimatin and spectrometric determination of tanin content in sorghum grain. Journal of Agricultural and Food Chemistry. 25:1268~1273.

Shim, S.M., Choi, S.W. and Bae, S.J. 2001. Effects of Punica granatum L. fractions on quinone reductase induction and growth inhibition on several cancer cells. Journal of the Korean society of food science and nutrition. 30(1):80~85.

(Received Mar. 5, 2003)
(Accepted Apr. 20, 2003)