ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 11, Issue 12, 2018

Online - 2455-3891
Print - 0974-2441

Research Article

ANALYSIS REACTIVITY OF *PUNICA GRANATUM* POLYPHENOLS TO THE OSTEOCALCIN, BONE MORPHOGENETIC PROTEIN-2, AND COLLAGEN TYPE-1

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Received: 11 September 2018, Revised and Accepted: 24 October 2018

ABSTRACT

Objective: *Punica granatum* (PG) contains anthocyanins which are useful as antioxidants, anti-inflammatory, and prevent cancer, while also increasing bone cell proliferation and osteoblasts differentiation in bone remodeling.

Methods: The reactivity of osteocalcin protein markers with PG polyphenol fractionation hydrogels observed using enzyme-linked immunosorbent assay, the degree of reactivity was determined by optical density at 550 nm.

Results: PG butanol fraction has better reactivity compared to the total extract, ethyl, and hexane fraction. Based on the reactivity distribution, bone morphogenetic protein (BMP)-2 and collagen Type-1 had a dominant distribution compared to osteocalcin, but the theses proteins had a strong relation ($r = 0.8$) with probability ($P < 0.05$).

Conclusion: PG butanol fraction had better reactivity to osteocalcin, BMP-2, and collagen Type-1 compared with total extract, hexane, and ethyl fraction. The four PG polyphenol fractionations have dominant reactivity to BMP-2.

Keywords: *Punica granatum*, Reactivity, Osteocalcin, Bone matrix protein-2, Collagen Type-1.

 DOI: http://dx.doi.org/10.22159/ajpcr.2018.v11i12.29683

INTRODUCTION

Pomegranate (*Punica granatum* [PG]) peel contains proanthocyanidin which is a family of flavonoids [1]. This active component acts as an antioxidant, anticancer, and also anti-inflammatory by inhibiting pro-inflammatory cytokines [2]. Moreover, these active components can also inhibit a number of enzymes that play a role in cell differentiation such as cyclooxygenase, lipooxygenase, cytochrome P450, phospholipase A2, ornithine decarboxylase, carbonic anhydrase, 17 beta-hydroxysteroid dehydrogenase, and serine protease [3].

PG peel extract contains various active components that differ depending on the extractor which used. The largest phenolic levels were found in butanol fraction compared to other extractors. The ethanol extract of PG peel produces a number of polyphenols which can trigger the expression of angiogenesis cells in the process of new bone formation [4]. A number of studies have reported that PG polyphenol fractionation has a significant effect on osteoblast cell viability as well as curcumin polyphenol fractionation as an immunostimulator for osteoblast expression in bone remodeling case [5]. The pomegranate peel of the Ganesh variety that extracted with ethanol and methanol was evaluated for the phenol content which contained at those extract. Methanol extract has better immunotolerant potency against pathogens than ethanol extract, while a mixture of ethanol and methanol extract has a very good antioxidant potency with phenol content in it which can act as an antibacterial either antioxidant [6].

Siddiqui *et al.* reported that PG can increase bone cell proliferation and osteoblast differentiation that is characterized by the expression of the runt-related transcription factor 2 (Runx2) gene. This assumption can be used as a reference for osteoporosis medication [7]. Meanwhile, PG ethanol extract can also be used as an anti-osteoporosis drug due to its ability to induce glucocorticoid hormones in osteoporosis mice model [8]. Furthermore, Bahtiar *et al.* reported that the use of PG polyphenol fractionation in concentrations of 50, 100, and 200 mg/kg can significantly prevent bone loss, this is related to an increase in bone calcium, particularly by increasing osteoblast [9], likewise in ovariectomy case, PG can be a stimulus to prevent bone loss [10]. The ability of PG in bone remodeling had been used to be a reference for this study, so the aim of this study was to test the ability of PG that interacts with proteins involved in bone remodeling such as osteocalcin, bone morphogenetic protein (BMP)-2, and collagen Type-1.

MATERIALS AND METHODS

Material

This study has passed ethical clearance from the Dentistry Faculty, North Sumatra University, Medan-Indonesia. This study used PG polyphenol fractionation as the assay material to measure the degree of reactivity from osteocalcin, BMP-2, and collagen Type-1 (Abcam, Cambridge, USA) proteins. The Enzyme-linked Immunosorbent Assay (ELISA) assay will be used to the reactivity analysis PG polyphenol with the bone marker proteins as the indicator of bone remodeling.

Extraction and fractionation of PG

The first stage is the extraction and fractionation of PG as a test material based on methods that had been done by Arma *et al.* [11]. 900 g of fresh PG peel that has been cleaned and peeled off, wind dried for 2×24 h, cut into small pieces and mashed. Subsequently macerated with 96% ethanol (1:10) 9 L for 24 h and stirred in the first 6 h. On the 2nd day, the macerate was filtered (macerate I), the pulp continued maceration with 96% ethanol (1:5) 4.5 L for 24 h. On the 3rd day, the macerate was filtered and merged with the macerate I. Furthermore, the solvent was evaporated so that a thick extract of 1164.4 g was obtained. Fractionation

was then carried out into polyphenols, where the extract was diluted with aquadest and then fractionated with 4×500 mL n-hexane which produced 3.25-g hexane fraction. Fractionation was then also carried out too with ethyl acetate 5×500 mL which obtained 13.9 g ethyl acetate fraction. Then, the least fractionation with 5×500 mL butanol was obtained 44 g butanol fraction.

Determination of total phenol levels was carried out using the Folin–Ciocalteu method, by measuring the indicator of gallic acid. 4 mg gallic acid was dissolved in 10 mL ethanol (400 ppm) with various concentrations (30 ppm, 40 ppm, 50 ppm, 60 ppm, and 70 ppm). The absorbance of the solution was measured with the Folin–Ciocalteu method and carried out as follows: The sample was put into a plate, then 50 ml of Folin reagent 7.5% was added. 50 µl of a sample was put into it and incubated for 8 min, then added 1% NaOH followed by incubation for 1 h. The absorbance of gallic acid expression was measured by spectrophotometry at 730 nm. Determination of total phenolic levels equivalent of gallic acid was carried out using the Folin–Ciocalteu method, with gallic acid as a comparison, assuming that all phenolics contained in the extract or fraction specified total phenolic content were considered as gallic acid or gallic acid equivalents.

Reactivity assay by ELISA

The ELISA technique was adopted from Gani *et al*. [12] to find out the lowest concentration of the PG fraction which still shows reactivity to bone marker proteins (osteocalcin, BMP-2, and collagen Type-1). Then, the concentration of the test material was measured based on optical density (OD) at 655 nm. The OD that obtained was used to get the concentration based on the conversion with the standard formula (OD×7.65) −0.3=µg/ml).

The ELISA test was carried out by entering 200 µl of each PG polyphenol fractionation sample to 96-well plate (triplo) which had been converted to its concentration value and shaken at 200× *g* for 5 min, then incubated at room temperature for 1 h and washed with Tween-20 for 3 times. Furthermore, in each well was added 200 µl of 5% non-fat milk and incubated on a shaker for 1 h at room temperature and washed with Tween-20 for 3 times. After that, each marker protein for osteocalcin, BMP-2, and collagen Type-1 was dissolved in 5% non-fat milk with a ratio of 1:2000, diluted to get a concentration of 100 µg/ml, then put into the well.

In the first well was added 200 µl of each bone marker protein and the next well was 100 µl each, then dilution was carried out leveled 8 times, sequentially (1000, 500, 250, 125, 62.5, 31.25, 18.62, and 7.81) after that incubated on a shaker for 1 h at room temperature and washed with Tween-20 for 3 times. In each well was added 100 µl of anti-protein marker antibody (HRP - rabbit anti-osteocalcin, BMP-2, and collagen Type-1 IgG (H+L) with a ratio of 1:5000 and incubated on a shaker for 1 h at room temperature, then washed with Tween-20 solution for 3 times. Moreover, in each well was added 50 µl of ELISA substrate solution and placed in a dark room for 20 min. The reaction that occurred between PG polyphenol fractionation and bone marker protein was stopped by adding 50 mL of HCl 1 N. The result was read with an ELISA reader based on OD at 550 nm.

Statistical analyses

PG polyphenol fractionation percentage profile which identified by bone marker protein was analyzed using parametric and non-parametric tests with $p<0.05$ as a determinant of statistical significance and correlation ($r=1$) as a determinant of the relationship between the analyzed variables.

RESULTS AND DISCUSSION

The gallic acid of PG polyphenol fractionations has value above equivalent to 150 mg/ml. The butanol fraction has gallic acid equivalent (157.62 mg/ml) more dominant than hexane fraction (113.81 mg/ml), ethyl fraction has 155.90 mg/ml, and extract total (ethanol extract) of PG has 169.61 mg/ml of gallic acid.

Bone formation metabolism consists of the balance of matrix deposition, mineral formation, and reabsorption [13]. The herbs used had been reported to have an effect on the process of new bone formation, specifically inhibiting bone reabsorption. Garlic and parsley have been reported could inhibit bone reabsorption and osteoclast activity which causes an increase in mineral density in ovariectomy cases [14]. PG has 50% active components, where phenolics and flavonoids are the dominant ones and very important as phytochemicals [15]. In addition, there are 40% water, sugar, pectin, and organic acids such as ascorbic acid, either 10% fibers, vitamins (E, C, and K), polysaccharides, and minerals [16].

Based on measurements of total phenolic from extraction and fractionation, PG phenolic levels were found to be quite high, especially in the butanol fractionation. These results proved that ethanol extract from PG peel produces optimal polyphenols in triggering angiogenesis-osteoblast cells by inducing osteocalcin, BMP-2, and collagen type-1. This way gives the possibility of PG can play a role in bone remodeling. PG has a number of active components that contribute to bone repair by inducing bone marker proteins [17]. The results of this study showed that PG polyphenol fractionation has good reactivity to all three bone marker proteins (Figs. 1-3).

ELISA test used a based concentration to measure the lowest concentration titers that still show good reactivity. This study used eight different concentrations (µg/ml) (1000, 500, 250, 125, 62.5, 31.25, 18.62, and 7.81). The results of OD (550 nm) was obtained scale reactivity 1.11> (strong), 1.1–1.10 (medium), and <1.09 (low). From these results, each PG polyphenol fractionation was averaged to obtain a percentage value of bone marker reactivity as shown in Figs. 1-3.

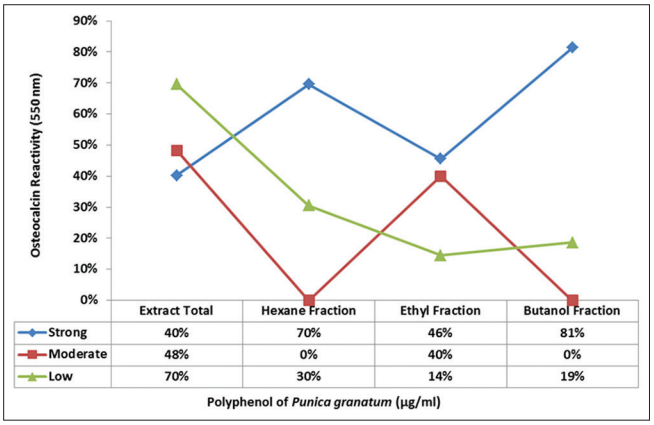

Fig. 1: Reactivity of osteocalcin protein on the *Punica granatum* polyphenol

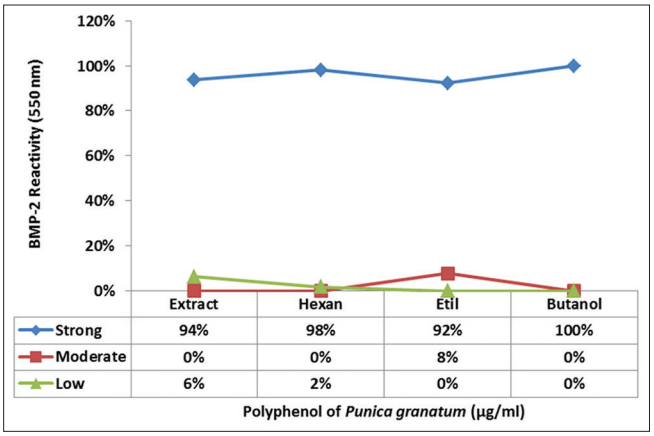

Fig. 2: Reactivity of bone morphogenetic protein-2 protein on the *Punica granatum* polyphenol

PG butanol fraction has a very strong reactivity to osteocalcin, followed by hexane and ethyl fractionation (Fig. 1). PG polyphenols are reported to play a role in the osteogenic activity. Giving PG with concentrations of 10, 100, 1000, and 10,000 µg/ml can increase the number of cartilage nodules in cartilage formation, so it can be recommended that PG can increase bone formation [18]. Osteocalcin protein plays a role in bone calcium formation, with a molecular weight 5.8 KDa and amounts about 10–12% of the total non-collagen protein, this protein is closely related to the bone mineralization phase [19]. Some other bone proteins such as thrombopoietin, glycoprotein acids, and fibronectin are proteins that contain arginine-lysine aspartate acid which has a large affinity for calcium formation, these proteins have the ability to be bound by integrin receptors [20]. Growth factors and cytokines such as transforming growth factor beta (TGF-β), insulin growth factor, interleukin, and BMP are present in small amounts in the bone matrix, where the protein binds bone mineral and the matrix will then release during bone resorption by osteoclasts [21].

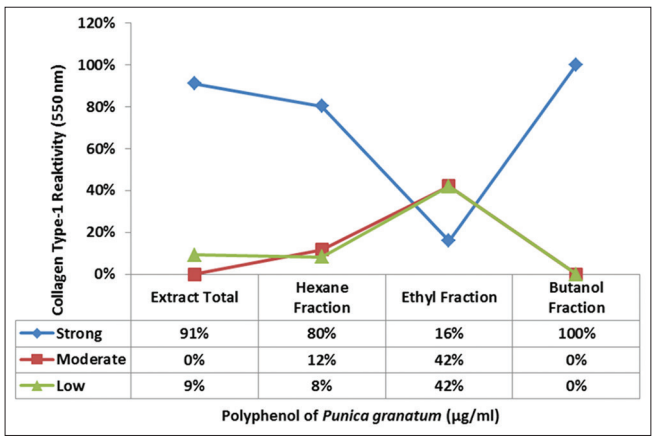

Fig. 3: Reactivity of collagen Type-1 protein on the *Punica granatum* polyphenol

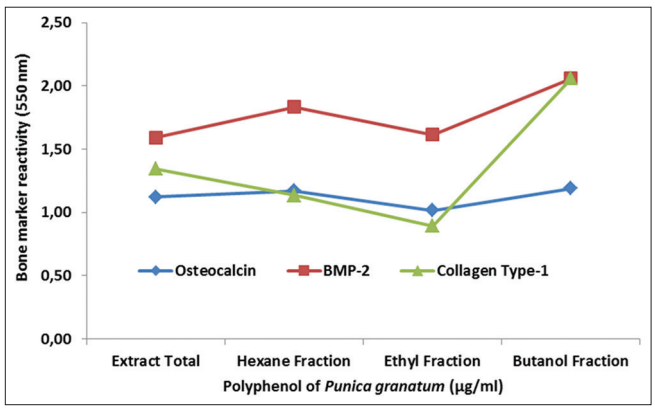

Fig. 4: Distribution and frequency reactivity of bone marker protein (osteocalcin, bone morphogenetic protein-2, and collagen Type-1) to the *Punica granatum* polyphenol

Osteocalcin serum was reported acts as a marker of bone turnover associated with osteoporosis [22]. In general, osteocalcin serum can increase new bone formation [23]. Lansky *et al.* reported that the amount of substance from PG ellagic acid can increase the signal for new bone formation [24]. Wnt/β-catenin from PG is an active component that acts to increase the signal for osteogenesis increased. Specifically, ellagic acid can increase osteopontin and osteocalcin [25]. PG seed oil extract was reported to play a role as a bone biomarker, specifically to detect osteocalcin [26]. This activity linked with the increase of the Runx2 gene ($p<0.001$) which triggers the expression of proteins that involved in new bone formation. This indicated that PG can prevent bone loss by increasing the activity of inflammatory responses and stress oxidative [4].

Fig. 2 shows BMP-2 bone markers having excellent reactivity to PG butanol fractionation, even though all PG polyphenol fractionations had strong reactivity against BMP-2. The proliferation and differentiation of osteoblasts mediated by growth factors such as BMPs, TGF-β, and core-binding factor alpha 1, which are reported as osteogenic receptor targets associated with the Runx2 [27]. This indicated that PG polyphenols fractionations may be able to induce the expression of BMP-2 when bone remodeling occurs by maintaining osteoblast activity and controlling osteoclast [28]. Giving PG with a concentration of 100 µg/ml can increase bone nodule formation which illustrated the occurrence of osteoblast differentiation within 21 days [29], by increasing calcium and osteoblasts as an indicator of BMP-2 expression [30]. Giving PG with this concentration can increase calcium deposits up to 68.85% ($p<0.001$) compared to controls. This ability is a form of an extracellular matrix that can regulate osteogenic tissue expression [31]. The results also showed that PG polyphenol fractionation has the ability to bind the osteocalcin, BMP-2, and collagen Type-1 starting at concentrations of 1000, 500, 250, 125, 62.5, 31.25, 18.62, and 7.81 µg/ml.

The collagen Type-1 has a very good sensitivity to PG butanol fractionation (Fig. 3) and the PG better than effect reactivity to BMP-2 compared the osteocalcin and collagen Type-1 (Fig. 4). In line with other studies using butanol fractionation of *L. ferrugineus* extract also had better results compared to other fractionation in inhibiting phenylephrine at aortic cardiovascular disorders [32]. PG can improve the function of osteoblasts which play an important role in bone remodeling by inducing the expression of bone collagen protein (collagen Type-1) which indicates that PG has anti-osteoporosis ability and can be used as an alternative herb for new bone repair [7]. The results were in line with this study (Table 1) which showed osteocalcin, BMP-2, and collagen Type-1 had significant differences when tested with PG polyphenols fractionations ($p<0.05$). Research using mice model shown that collagen Type-1 can improve implant bone remodeling [33]. In addition, BMP-2 was also involved in maintaining osteoblasts balance to maintain bone integrity [34]. The result from PG polyphenols fractionations which were tested with osteocalcin, BMP-2, and collagen Type-1 markers indicating that PG can be used as an herbs alternative in bone remodeling after endodontic movement.

Meanwhile using ANOVA one-way test as shown in Table 1, BMP-2 had a normal distribution data with significant differences results between all of PG polyphenol fractionations ($p<0.05$) with a strong correlation ($r=0.8$). Furthermore, non-parametric statistics with Mann–Whitney test showed osteocalcin had significant differences between all PG

Table 1: Statistical analysis of bone marker protein in various *Punica granatum* polyphenol

Protein bone marker	Optical density (550 nm)				Statistical descriptive			
	Extract total	Hexane fraction	Ethyl fraction	Butanol fraction	Mean±SD	p	r	Analysis
Osteocalcin	1.12	1.17	1.01	1.189	1.073±0.01	p<0.05	0.6	Non-parametric
BMP-2	1.59	1.83	1.61	2.06	1.148±0.01	p<0.05	0.8	One-way ANOVA
Collagen Type-1	1.34	1.14	0.89	2.06	1.150±0.01	p<0.05	0.8	Non-parametric

P. granatum: Punica granatum, BMP: Bone morphogenetic protein

polyphenol fractionations ($p<0.05$) with Spearman's correlation ($r=0.6$). Other than that, collagen Type-1 also had significant differences between all PG polyphenol fractionations based on the Friedman test ($p<0.05$) with a strong relationship based on Spearman's correlation ($r=0.809$).

CONCLUSION

The PG butanol fractionation had better reactivity to osteocalcin, BMP-2, and collagen Type-1 compared to total extracts, hexane, and ethyl fractions. In general, the four PG polyphenol fractionations have the dominant reactivity to BMP-2.

ACKNOWLEDGMENTS

Gratefulness to the Biota and Pharmacy Laboratory, Andalas University, West Sumatra; Integrated Laboratory, Gadjah Mada University, Yogyakarta; Biology Laboratory, University of North Sumatra, Medan; and the Laboratory of Microbiology and Pathology, Faculty of Veterinary Medicine, Syiah Kuala University, Banda Aceh.

AUTHORS' CONTRIBUTIONS

EZ was carried out the conception and research design including the methods assessment also drafted the manuscript with BAG and TA. DPP was analyzed biological effect of PG. Specifically, BAG has been arranged the manuscript, statistical analysis, and corresponding author. All of the authors were read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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