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Research Article

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Osteoblast cell proliferation activity of isoflavone aglycones from fermented soybean (*Glycine max* (L) Merill) by *Lactobacillus acidophilus*

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ABSTRACT

This study aims to obtain isoflavones aglycone as genistein form through the process of fermentation of soybean by probiotic bacteria producing ß-glucosidase namely Lactobacillus acidophilus. Isoflavone aglycone obtained then testing the activity of osteoblast cell proliferation in vitro by MTT method that will be read by ELISA. Fermented in the extraction with ethyl acetate. Liquid extract was evaporated with a rotary evaporator and lyophilized by freeze dryer. Extract 3 mg in 10 ml of 80% methanol in UFLC analyzed its genistein levels. Comparators are used genistein standard ≥ 98% at concentrations of 5, 10, 15, 20, 25 ppm. The analysis of three replicate showed average levels of genistein 3.46%. The test results showed cell percent proliferation was highest at 0.5% extract is equal to 143.93%. Statistical analysis by ANOVA showed that H0 rejected, which indicates that there is the effect of the addition of fermented extract on cell proliferation of osteoblasts.

Keywords: isoflavones, genistein, osteoblasts, ß-glucosidase, proliferation

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INTRODUCTION

Isoflavones are a class of flavonoids found in many types of beans, including soy isoflavone glycosides that contain abundant. Isoflavones consists of four forms, namely the aglycone, glycosides, malonil glycosides, and acetyl glycosides, which each have 3 types of isomers. Aglycone consists of genistein, daidzein, and glisitein. Glycosides consisting of genistin, daidzin, and glisitin. Malonil glycoside comprised of 6 "-O-malonilgenistin, 6" -O-malonildaidzin, and 6 "-O-malonilglisitin. Acetyl glycosides consists of 6 "-O-asetilgenistin, 6" -O-asetildaidzin, and 6 "-O-asetilglisitin [1]. In general, most types of isoflavones found in soy are genistein and daidzein [2].

Soy isoflavones in the aglycone form of genistein and daidzein as having estrogenic activity, antifungal, and anticancer [3]. Estrogenic activity of isoflavone based on the effects of estrogen receptor agonists (ER) β-estrogen particularly on the membrane of osteoblasts. Isoflavones are lipophilic so it can penetrate the cell membrane and bind to estrogen receptors subsequently initiate the transcription process. This process then leads to gene expression of alkaline phosphate and osteocalcin (bone formation factor), osteoprotegerin (inhibition of osteoclast activity) and growth factor IGF (proliferation of osteoblasts), which in turn have an effect on the decrease in bone resorption and increase bone formation [4].

Many plant foods contain amounts of the diverse small molecules phytoestrogens that have the potential to improve health. Food phytoestrogens molecules are found predominantly as isoflavones is soybeans (*Glycine max* (L) Merill). This molecules function as antioxidants in plants, but in mammalian tissues this natural products act as agonists, or partial agonists of estrogen [5].

To obtain the aglycone isoflavones can be done by means of enzymatic by probiotic microorganisms, can also be done through a chemical reaction by using a strong acid such as hydrochloric acid or acetic acid [6]. Probiotic microorganisms, such as *Lactobacillus* and *Bifidobacterium* has ß-glucosidase enzyme that plays an important role in endogenous hydrolyze isoflavone during fermentation [7]. Genistein is used as a marker in the determination of the aglycone isoflavone content in the extract because the measure is the most dominant [2].

Research on the use of soy isoflavone aglycone fermented still minimal, though testing the effects of soy isoflavone aglycone fermented on osteoblast cell proliferation has been done yet tested fermented soy derived from traditional foods that have not been clearly about the probiotic microorganisms [8].

Based on these data, it will be done on soybean fermentation process using L. acidophilus bacteria to obtain the extract, and then determine how the content of isoflavone aglycone in the extracts using genistein as marker, then the extracts tested in cell proliferation activity of osteoblasts.

EXPERIMENTAL SECTION

2.1 Chemical Materials

Standard genistein G6649 was purchased from Sigma Aldrich Chemie GmbH with purity ≥98%, medium MRSB (Man Rogosa Sharpe Broth), medium agar, ethyl acetate (80%), acetonitrile (*Merck*®), diethyl ether, methanol (*Merck*®), ethanol 70%, potassium penicillin G (*Merck*®), streptomycin sulphate (*Merck*®), $NaHCO_3$, aqua destillata, PBS (Phosphate Buffer Saline), FBS (Fetal Bovine Serum), HCl-isopropanol, dispase, DMSO (Dimethyl Sulfoxide), trypan blue, soybean (*Glycine max* (L) Merill), aquadest steril, colagenase, medium α-MEM (alpha Minimum Essential Medium) (*Sigma*®), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), NaF (Natrium Fluoride), calcitonin 100 ui, *sterile water for irrigation*, trypsin 0,5%, EDTA 0,04%, osteoblast cell.

2.2 Bacterial Preparation

The bacteria *L. acidophilus* were cultured at medium MRSA, which is medium consist of 2.75 g MRSB and 1.25 g medium agar, then homogenized with 50 ml sterile aquadest.

2.3 Sample Preparation

Soybean 250 g was blended with 2000 ml hot water (70-80^{o}C), and the result was filtrated to omit residual. 50 ml filtrate of soybean added 2% of glucose, pH 6±0.2 was checked, pasteurization while 30 minutes at temperatures 70-80^{o}C, and then inoculated with *L. acidophilus*, fermented as long as 24 hours at 37^{o}C to make starter of probiotic. Starter of probiotic inoculated into 450 ml filtrate of soybean which has pasteurization, and then fermented during 24 hours, check of pH at 4-5 [7].

2.4 Extraction Procedure

Fermented of soybean as much as 500 ml was extracted with 500 ml ethyl acetate, the resulting mixture was heated at 30-40^{o}C for 1 hour and stirred constantly. Then the liquid extract was separated from insoluble fraction. The liquid extract then evaporated by rotary evaporator [9].

2.5 Extract Analysis

Extract was analysis using Ultra Fast Liquid Chromatography (UFLC) reversed phase C_{18} column (Shimadzu). Genistein standard diluted with methanol: water (8:2) with concentration 25 ppm, 20 ppm, 15 ppm, 10 ppm and 5 ppm. Extract as much as 3 mg diluted in 10 ml methanol: water (8:2). Therefore each of concentration and the extract performed 2 ml in UFLC to analysis at 255 nm. The sample injection volume was 10 µL. The mobile phase was acetonitrile : water (8:2) and temperature of the column was maintained at 40^{o}C [6]. Qualitative analysis of isoflavone in extract was made by comparing the chromatogram profile with those of pure standards. Quantitative analysis was done by using linear regression ($y = a + bx$). The result of extract analysis was plotting in to equation to determine the concentration of isoflavone in the extract.

2.6 Extract Preparation for Treatment

Extract as much as 45 mg diluted in DMSO, homogenized in 1 ml medium α-MEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), antibiotics (12.5 mg potassium penicillin G and 7.5 mg streptomycin sulfate) and 0,5625 g $NaHCO_3$ and homogenized by vortex. As much as 660 µL was pipette and homogenized in 3 ml medium α-MEM. This mixture was filtrated by filter 0,22 µm, and the result has a concentration 1 %, and then diluted to made concentration 0.5%, 0.25% and 0.125% b/v [10].

2.7 Cell Culture

The osteoblast cell was maintained in sterile minimum essential medium (α-MEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), antibiotics (12.5 mg potassium penicillin G and 7.5 mg streptomycin sulfate) and 0,5625 g $NaHCO_3$, those supplements were homogenized in 250 ml α-MEM. Cell cultures were grown in flask at 37 ^oC in a humidified atmosphere with 5% CO_2 and subculture every 2-3 days [6,10].

2.8 Cell Treatment

Base on [10] with some modified; Well plates-96 (Iwaki) fill up 200µL cell and its medium except for cell control and medium control. Each of well plates-96 added 200µL calcitonin and natrium flouride (NaF) as positive control (without FBS added), 200µL cell control (just cell and its medium added), 200µL medium (just medium added) and 200µL soybean extract (1%, 0.5%, 0.25%, 0.125%) without FBS added. Then incubated as long as 48 hour at 37 ^oC in a humidified atmosphere with 5% CO_2.

2.9 MTT Cell Proliferation Assay

Base on [6] with some modified; Cell proliferation was estimated according to the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, and Cell Proliferation Kit (MTT) (*Roche*) was used. All medium in well plates-96 was cleaned out, then added 50 µL MTT (5 mg /5 ml in phosfate buffer saline (PBS)) and incubated as long as 4 hours. MTT in well plates-96 was cleaned out and filled up 200 µL 0.01N HCl-isopropanol then homogenized. Cell proliferation was analyzed with ELISA reader at the wavelength 515 nm.

2.10 Statistical Analysis

Every assay was repeated independently at least three times. All values are expressed as the mean ± standard deviation of three independent experiments. Difference between experimental groups was assessed by analysis of variance (ANOVA). Base on analysis result then be made discussion and conclusion.

RESULTS AND DISCUSSION

Isoflavone aglycone could be found by fermented the filtrate of soybean, because enzyme fermented from probiotic bacteria like a *L. acidophilus* will be solving glycoside binding. Soybean fermented result was extracted with ethyl acetate which modified using magnetic stirrer by warming at least 70^oC. We found extract in weight 475 mg.

Pure standard genistein was used as isoflavone aglycone marker to identification and calculating amount of genistein in extract soybean fermented. The result of linear regression based on analysis result (Table 1), we found value of a, b and r respectively : 201385, 96232.12 and 0.964. The extract soybean fermented contain genistein (Table 2) its show that the process of fermentation was successful to cut off glycoside binding of isoflavon.

Table 1. Genistein concentration and area from UFLC calculation

Sample	Concentration	Area (Y)
Genistein standard	25 ppm	2438419
	20 ppm	2195711
	15 ppm	1752267
	10 ppm	1414931
	5 ppm	423006

Table 2. Weight extract and level of genistein.

Weight extract (g)	Vol (ml)	Area (Y)	Level of genistein (µg/mg)	Avr (µg/mg)	% b/b
0,0037		1435714	34.64		
0,0039	10	1531230	35.41	34.69	3.46
0,0035		1347638	34.03		

Activity of cell proliferation could we see at the result of calculating proliferation cell. Extract in well plates-96 was measured of its absorbance by ELISA reader at wavelength 515 nm, then the % proliferation calculated by equation below, and the result of calculating (Table 3) show that the highest percent proliferation at the 0.5% of extract.

$$\% \text{ Proliferation} = \frac{\text{Abs sample} - \text{Abs medium}}{\text{Abs cell} - \text{Abs medium}} \times 100\%$$

Standard comparison was used has known could be increased osteoblast cell proliferation, that is NaF and calcitonin which have % viability till 100%, but the 0.5% of extract has a higher percent proliferation than standard. The result show that extract of soybean fermented has a good effect than the standard. Increasingly of osteoblast cell

proliferation, could be increasing bone mass, therefore as a preventive of osteoporosis. Basically isoflavone could be increased bone mass [11].

Table 3. Absorbance of sample from ELISA reader

Treatment	Abs1	Abs2	Abs3	Average	% Viability
Cell	0.077	0.075	0.072	0.074	100
Naf	0.077	0.074	0.073	0.074	100
Calcitonin	0.075	0.074	0.072	0.073	95.45
1%	0.052	0.065	0.06	0.059	28.78
0.5%	0.096	0.071	0.086	0.084	143.93
0.25%	0.091	0.058	0.054	0.067	68.18
0.125%	0.061	0.063	0.072	0.065	57.57
Medium	0.043	0.057	0.056	0.055	0

Statistical analysis by the method of one-way ANOVA showed that the difference in percent proliferation significantly different between treatments, with the rejection of the value of H0. This indicates that there is the effect of the addition of fermented soy extract on cell proliferation of osteoblasts.

CONCLUSION

This research show that the level of genistein as isoflavone aglycone in extract soybean fermented by *L. acidophilus* average 3.46 % (b/b). The result of % proliferation calculation found the highest percentage is 143.93% at the concentration 0.5%.

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