

Black Seed (*Nigella sativa*) Extract Induce *in vitro* Proliferation and Differentiation of Rat Pancreatic and Bone Cells

(EKSTRAK JINTAN HITAM (*Nigella sativa*) MENGINDUKSI PROLIFERASI DAN DIFERENSIASI SEL PANKREAS DAN SEL TULANG TIKUS SECARA *IN VITRO*)

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ABSTRACT

Black seed (*Nigella sativa*), a medicinal plant, widely used for treating various diseases, including diabetes mellitus and osteoporosis. This study examined the proliferation and differentiation of pancreatic and bone cells of rat cultured *in vitro* in medium supplemented with *N. sativa* extracts (NS). Pancreatic and bone cells were isolated from five days old rat and cultured in Dulbecco modified eagle medium supplemented without NS (0%, as control), and with NS (0.05% and 0.5%, as treatment groups) in 5% CO₂ incubator at 37°C for seven days and observed for cell population doubling time (PDT); proportion and diameter of Langerhans islets, osteoblast, and osteocyte; and proportion of Langerhans islets containing β cell expressing insulin secretion. The pancreatic β cells were observed using dithizone staining, while the bone cells using alizarin red staining. The result showed that supplementation of NS significantly ($p < 0.05$) decreased the PDT of pancreatic and bone cells, increased the proportion and diameter of Langerhans islets, increased the proportion of expression the β cell producing insulin, and increased the diameter of osteoblast. In conclusion, the supplementation of NS in culture medium improved the proliferation and differentiation of pancreatic and bone cells *in vitro*.

Key words: *in vitro* culture; *Nigella sativa*; osteoblast; osteocyte; pancreatic β cell.

ABSTRAK

Jintan hitam (*Nigella sativa*) adalah tanaman obat yang banyak digunakan untuk mengobati berbagai penyakit, termasuk kencing manis (diabetes mellitus) dan osteoporosis. Penelitian ini bertujuan untuk menganalisis kemampuan proliferasi dan diferensiasi sel pankreas dan sel tulang tikus setelah kultur *in vitro* dalam medium yang diberi ekstrak jintan hitam (NS). Sel pankreas dan sel tulang diisolasi dari tikus umur lima hari dan dikultur dalam medium Dulbecco Modified Eagle Medium tanpa diberi NS (0% sebagai kontrol), dan diberi NS (0,05% dan 0,5%, sebagai perlakuan). Sel-sel diinkubasi di dalam inkubator CO₂ 5% pada suhu 37°C selama tujuh hari. Pengamatan meliputi PDT (*population doubling time*); proporsi dan diameter pulau Langerhans, sel osteoblas, dan sel osteosit; serta proporsi pulau Langerhans yang mengekspresikan sel β penghasil insulin. Sel β pankreas diamati setelah diwarnai dengan dithizone, sementara itu sel-sel tulang setelah diwarnai dengan alizarin red. Hasil penelitian menunjukkan bahwa pemberian NS secara nyata mempersingkat PDT sel pankreas dan sel tulang, meningkatkan proporsi dan diameter pulau Langerhans, meningkatkan proporsi ekspresi sel β penghasil insulin, serta meningkatkan diameter sel osteoblas. Dapat disimpulkan bahwa pemberian NS dalam medium kultur mampu meningkatkan proliferasi dan diferensiasi sel pankreas dan sel tulang *in vitro*.

Kata-kata kunci: kultur *in vitro*; *Nigella sativa*; sel β pankreas; sel osteoblas; sel osteosit

INTRODUCTION

Diabetes mellitus (DM) is known as one of the most degenerative disease suffered by people around the world. Presumably, in the end of 2030, there will be 300 million people suffering the DM (Wild *et al.*, 2004). There are two types of DM which in the type 1 caused by the lower level of insulin due to the degeneration of pancreatic β cell, while the type 2 caused by the insulin insensitivity as a manifestation of obesity and dietary changes (ADA, 2013). Insulin plays primary role in glucose metabolism by converting the blood glucose into the glycogen. The insulin replacement therapy can be used to treat the DM, along with the pancreas transplantation. Long-term insulin therapy share several health complication such as retinopathy, nephropathy, neuropathy, and cardiovascular disease (DCCTRG, 1993). The transplantation has several limitation related with the risk of preoperative factors, stress related to the transplant procedure, and the hyperglycemia-inducing medications to maintain a healthy graft (Marvin and Morton, 2009).

Another degenerative disease is osteoporosis. Osteoporosis is a disease commonly occurs in adult individual, especially menopause women, marked by low density and alteration of micro-structural bone material (WHO, 2003). Bone density determines the incidence of the osteoporosis in the future where individuals especially women with higher bone density, will less suffer the disease in the post-menopause period (Compston *et al.*, 1993).

Black seeds (*Nigella sativa*; NS) is one of herbal medicine with several pharmacological properties, such as antiparasitic (Abdulelah and Zainal-Abidin, 2007), antitumor (Mbarek *et al.*, 2007), anticancer (Randhawa and Alghamdi, 2011), hepatoprotector (Kanter *et al.*, 2005a), gastroprotector (Kanter *et al.*, 2005b), antifungal (Al-Jabre *et al.*, 2003), antibacterial (Bourgou *et al.*, 2010), and immuno-stimulant (Meral *et al.*, 2004). Several scientific reports have also supported the NS properties as an antidiabetic agent by promoting normal metabolism of glucose in diabetic rat (Hawsawi *et al.*, 2001; El Dakhakhny *et al.*, 2002) and recovering the damage of pancreatic β cell, as well as enhancing the insulin secretion (Kanter *et al.*, 2003). The other pharmacological activities shared by the herbal are related with the estrogenic effect (Parhizkar *et al.*, 2011) and its ability in

reducing the clinical symptoms of the osteoporosis in ovariectomized rats (Seif, 2014).

The NS is able to regulate cell growth and differentiation of monocyte and monocyte-derived macrophages (Mat *et al.*, 2011), as well as modulating amino acid release in cultured neurons *in vitro* (El-Naggar *et al.*, 2010). Among all pharmacological properties, the *in vitro* mechanism of the NS in inducing proliferation and differentiation the pancreatic and bone cells to our knowledge has not reported yet. This study evaluated the NS ability on inducing the proliferation and differentiation of pancreatic and bone cell of rats *in vitro* as a potential herbal of antidiabetic and antiosteoporosis.

RESEARCH METHODS

Extract Preparation. The NS extract was prepared by dissolving the NS powder in hot water ($\pm 90^{\circ}\text{C}$), and homogenized when the temperature of mixture was cooled down. The homogenized solution was then filtered (pore diameter=0.22 μm), and the filtrate was stored in -4°C for future use.

Animals. The husbandry and all procedures related with this study were approved by the Ethic Committee of Animal Use and Care, Faculty of Veterinary Medicine, Bogor Agricultural University, under license number 012/KEH/SKE/X/2014. Nine of five-days-old Sprague Dawley rats (*Rattus norvegicus*) were anaesthetized with ketamine-xylazine cocktail 0.1 mL/100g rat wt. (contains 91 mg/kg Ketamine 9.1 mg/kg Xylazine) intra peritoneal injection. After the animals were fully anesthetized, the euthanasia was then continued by cervical dislocation. The anesthesia and euthanasia procedures were performed by trained staff and ensured by the attending veterinarian. The necropsy was then continued to collect pancreas and bone tissues with an aseptic surgery apparatus.

Cell Isolation. The pancreatic β cells were isolated by modifying the established methods of Zhang *et al.* (2004) and Johansson *et al.* (2006). Briefly, the pancreatic tissue was prepared and freed from blood vessel and connective tissue and then washed in *Dulbecco phosphate buffered saline* (DPBS, Gibco) solution containing *neonatal calf bovine serum* (NCBS, Gibco) 0.1%. The tissue were then incised into small sized, approximately 1 mm^3 , and dissociated furthermore by DPBS with

collagenase (Sigma) 0.1% and then incubated in 37 °C for 15-30 minutes. The incision were meant to increase the surface area to reduce the connective tissue surrounding the Langerhans' islet therefore, the collagenase will be more effective in dissociating the pancreatic cells. The suspension of pancreatic cells was purified by diluted with DPBS and centrifuged for 10 minutes at 200 g, three times, and with *Dulbecco modified eagle medium* (DMEM, Gibco) for 10 minutes at 200 g two times, respectively.

Bone cells were isolated from femoral, tibia and fibula bones followed the methods of Djuwita *et al.* (2012a). The marrow was cleaned by flushing, and bones were cut up into small size (approximately 1 mm³) and suspended in DPBS. The bone cells suspension were then purified by diluted and centrifuged as previously described.

Cell Culture. The cells were cultured by modifying the methods of Djuwita *et al.* (2012a,b). Briefly, the cell suspension were cultured in a petri dish layered by gelatin 0.1% and 2 mL DMEM containing NaHCO₃ 1%, non-essential amino acids (NEAA, Sigma) 1%, gentamycin 50 µg/mL, NCBS10%, and insulin transferrin selenium (ITS, 5 µg/mL insulin, 10 µg/mL transferin, and 5 µg/mL selenium; Sigma). The final concentrations of the cell were 5 x 10⁴ cell/mL and 1 x 10⁴ cell/mL for the pancreatic cells and bone cells, respectively. The cells were cultured in two separated dishes – one with cover glass for cell's staining and the other one without the cover glass for quantify the population doubling time (PDT). The cells were incubated in 5% CO₂ incubator at 37°C for seven days. The culture medium were subjected to be changed every two days, and after the medium renewed, the 0.05 % and 0.5% of NS extract then were added into the culture. In pancreatic cell's culture, the cells were induced by 1 µL of 26 mM glucose at the day six of culture—a day before cell staining at day seven.

Cell Staining. The staining of pancreatic β cells with dithizone (DTZ) staining was conducted at day seven together with quantify the percentage and diameter of the Langerhans islet. The DTZ staining aimed to observe the insulin production, and the procedure followed Shiroi *et al.* (2002). Briefly, stock solution of DTZ was prepared by dissolving 50 mg of DTZ in 5 mL of dimethyl sulfoxide, and the solution was then filtered with 0.22 µm pores filter. The final solution for staining was made by dissolving 1 µL of above stock solution in 1 mL of DPBS containing 1.25 µL/mL gentamycin. The

cultured cell was washed with DPBS and then stained by incubating in the final DTZ solution for 15 minute at 37°C. After the incubation, the cells were washed with DPBS and then followed by observation of the pancreatic β cells in Langerhans islet. Insulin secreted Langerhans' islet will colorized as red, while it has no insulin production it will be not colorized (Shiroi *et al.*, 2002).

The quantification number and diameter of bone cells were determined by morphological observation after alizarin red staining (Kiernan, 1990). At day seven, the bone cells were fixated in glutaraldehyde 2.5% for 48 hours at 4°C. The cells were then washed with DPBS pH 4.2 and stained with alizarin red 2% at 37°C for two hours. After two times washing with DPBS, the cells were observed using light microscope.

Evaluation. The evaluation parameters are population doubling time (PDT), percentage and diameter of Langerhans' islet and bone cells, and percentage of insulin producing pancreatic β cells.

The proliferation rate was quantified by the value of PDT – defined by the time to double the cells' population from the initial to the final concentration (Davis, 2011). The initial cells population was counted at the day incubation, and the final cells population was then at day seven after incubation. At day seven, after staining, the medium will be discharged and followed by washing with DPBS and dissociation with 0.1% collagenase in DPBS at 37°C for 15-30 minutes. During the dissociation, re-pipetting were done to accelerate cell separation and followed by a centrifugation at 200 g to ensure the process of separation. The population of cell was then quantified in Neubeur haemocytometer, and the PDT quantification followed Davis (2011) with formula described below:

$$PDT \text{ (days)} = \frac{1}{\frac{(\text{Log } \sum \text{ final cells population} - \text{Log } \sum \text{ initial cells' population}) \times 3.32}{\text{Culture duration (days)}}$$

The differentiation rate was evaluated from the proportion and diameter of Langerhans islet, osteoblast and osteocyte, as well as the expression of insulin producing pancreatic β cells. Quantification of Langerhans islet and bone cells were conducted up to 100 cells observed under light microscope, duplo.

The diameter of Langerhans islet and bone cells were measured in 10 cells/islets for each

group/treatment. Especially for measurement of diameter of Langerhans islet, when the islet observed in the symmetrical shape, the longest diameter will be taken, and in the unsymmetrical shape will be measured from the average of the longest and the shortest diameter. The percentage of β cells expressing the insulin production in Langerhans' islet were quantify from 100 cells/islets.

Data Analysis. All of the data were collected from three repetition of cultures and statistically analyzed with analysis of variance (ANOVA), and followed by Duncan test to determine the significance between the groups with 95% of confidence level.

RESULTS AND DISCUSSION

The extract of NS were significantly increased the proliferation of pancreatic and bone cells, indicated by the lowering PDT value along with the increasing NS' dose (Table 1). The PDT value of the pancreatic cells with 0.05 and 0.5% NS were significantly lower ($P < 0.05$) compared with the control group, while the significance were also found in the bone cells with 0.5% NS, but not with 0.05% NS. The PDT value of bone cells was also shorter (two days) compared with the pancreatic cells (four days) in the same culture and NS treatment, which inferred the proliferation of bone cells was generally faster than the pancreatic cells.

The PDT defined as the time to reproduce the cell population twice than the initial population (Pellegrini *et al.*, 2008; Davis, 2011), and the proliferation rate is indicated by the proportion between the amount of the cell at the initial and the final stage. The PDT value is negatively correlated with the proliferation rate, described as the smaller value of the PDT, then the faster proliferation it become. The culture with NS application produced smaller PDT value, which indicated the increasing proliferation activity, both in pancreas and bone cell's culture. Our results support earlier study where the PDT of pancreatic cells valued between 96 hours/four days (Aptea *et al.*, 1998), and the bone cells valued between two-four days (Binderman *et al.*, 1974).

The NS extract enhanced the differentiation of pancreatic cells by increasing the proportion and diameter of Langerhans islet (Table 2, Figure1). Both Langerhans islet proportion and diameter were higher ($P < 0.05$) in the 0.05 and

Table 1 Population doubling time of pancreatic and bone cells after *in vitro* culture in medium supplemented by *Nigella sativa* extract

Treatments	Population Doubling Time (days)	
	Pancreatic Cells	Bone Cells
Control	4.66 ± 0.24 ^a	2.25 ± 0.28 ^a
NS 0.05%	3.98 ± 0.20 ^b	2.21 ± 0.05 ^{ab}
NS 0.5%	3.66 ± 0.21 ^c	1.98 ± 0.11 ^b

NS= *Nigella sativa* extract, ^{a,b} within column significantly different at $P < 0.05$.

0.5% NS group, compared with the control group, as well as the percentage of Langerhans islet with insulin producing pancreatic β cells ($P < 0.05$).

Pancreas is an organ which has a limited proliferation ability (Soria *et al.*, 2001), but still has an ability to proliferate and differentiate *in vitro* (Demeterco *et al.*, 2000). The ability is supported by presence of the duct cells which act as the progenitor or stem cells, and furthermore functioned in neogenesis and development of new Langerhans islet (Bonner *et al.*, 1993; Rosenberg, 1998; Zhang *et al.*, 2004).

The insulin staining will describe the differentiation of pancreatic β cells in the culture. The pancreatic β cells which detected in the Langerhans islet will contain zinc (Zn) inside the cytoplasm. The DTZ is regarded as a specific staining for detection of the pancreatic β cells by the ability to bind the Zn (*zinc-binding substance*) on insulin. The higher colorization from pink to dark red colors, indicated the level of Zn itself, therefore difference in the intensity of above reddish-colorization reflect the difference of the concentration of the Zn inside the β cells in the Langerhans islet. Furthermore, the color intensity indirectly correlates the cells activity in producing the insulin. The Zn in the pancreatic β cells is a function to bind the insulin in the dimer and hexamer formation, which furthermore ease the storage of the insulin inside the secretory vesicles of the pancreatic β cells (Chausmer, 1998; Garnuszek *et al.*, 2000).

The increased expression of insulin by the pancreatic β cells which treated by the NS extract, can be influenced by the chemical compound inside the NS, namely thymoquinone (Ahmad *et al.*, 2013). The thymoquinone shares a pharmacological antidiabetic properties, tested

Table 2. Proportion, diameter and expression of insulin secretion of Langerhans islets after *in vitro* culture in medium supplemented by *Nigella sativa* extract

Treatments	Langerhans Islets		
	Proportion (%)	Diameter (µm)	Expression of insulin secretion (%)
Control	6.00 ± 1.80 ^a	66.11 ± 2.09 ^a	4.67 ± 0.57 ^a
NS 0.05%	14.33 ± 3.01 ^b	101.66 ± 0.83 ^b	9.67 ± 2.08 ^b
NS 0.5%	18.67 ± 1.25 ^c	142.77 ± 1.73 ^c	17.33 ± 3.21 ^c

NS= *Nigella sativa* extract, ^{a,b} within column significantly different at P < 0.05.

Table 3. Proportion and diameter of bone cells after *in vitro* culture in medium supplemented by *Nigella sativa* extract

Treatments	Osteoblast		Osteocytes	
	Proportion (%)	Diameter (µm)	Proportion (%)	Diameter (µm)
Control	83.00 ± 4.58 ^a	29.96 ± 0.66 ^a	17.00 ± 4.58 ^a	17.43 ± 0.95 ^a
NS 0.05%	82.00 ± 5.56 ^a	34.00 ± 3.77 ^{ab}	18.00 ± 5.56 ^a	18.50 ± 0.90 ^a
NS 0.5%	79.67 ± 4.16 ^a	35.63 ± 2.77 ^b	20.30 ± 4.16 ^a	18.93 ± 1.13 ^a

NS= *Nigella sativa* extract, ^{a,b} within column significantly different at P < 0.05.

by the ability to reduce the glucose level in rats and it level could reach 30-48% after a purified NS oil treatment (El-Dakhakhny *et al.*, 2002). Beside of reducing the glucose level, the *in vivo* application of the thymoquinone also functioned in regenerating the pancreatic β cells, therefore the insulin secretion can be normalized (Kanter *et al.*, 2003). Antidiabetic properties of the NS is also predicted by it antioxidant amount which effectively reduce the free-radicals (Sultan *et al.*, 2014), decrease the lipid peroxidation and serum nitric oxide, as well as increasing antioxidant enzyme activity (Kanter *et al.*, 2004).

The Zn level also shares the antidiabetic role of the NS (Ahmad *et al.*, 2013) by enhancing the synthesis, storage and secretion of insulin and glucagon. In the other hand, the Zn also functioned in the cell differentiation (Beyersmann and Haase, 2001), intracellular and cells' signaling (Fukada *et al.*, 2011), as well as functioned as a precursor of more than 300 enzymes (McCall *et al.*, 2000).

The measurement of diameter and morphological observation of bone cells showed that the size of osteocyte was smaller and the red colorization was markedly increased

compared with the osteoblast (Table 3, Figure 2). The NS increased the differentiation of osteoblast marked by the increasing size of the osteoblast diameter compared with control group (Table 3). The osteoblast diameter was higher (P<0.05) in 0.5% NS group compared with the control group, while it was not in the 0.05% NS group. In the other hand, proportion of osteoblast and osteocyte, as well as the diameter of osteocyte were relatively not differed between all NS groups.

Osteoblast are cells shaped as cubical or columnar during the active period and turned to be flat during the inactive stage (Kierszenbaum, 2002). An osteoblast which actively synthesise bone matrix, will have a greater size of nuclei, golgi apparatus (GA) and numerous endoplasmic reticulum (RE). The osteoblast itself produce collagen type I and other extracellular matrix (Manolagas, 2000). Osteoid-osteocyte is shape of transitional cell between osteoblast to osteocyte, and this type of cell is responsible in mineralization process. Mineralized osteoid-osteocyte will further differentiate into osteocyte and this process reduced their availability up to 70% (Palumbo,

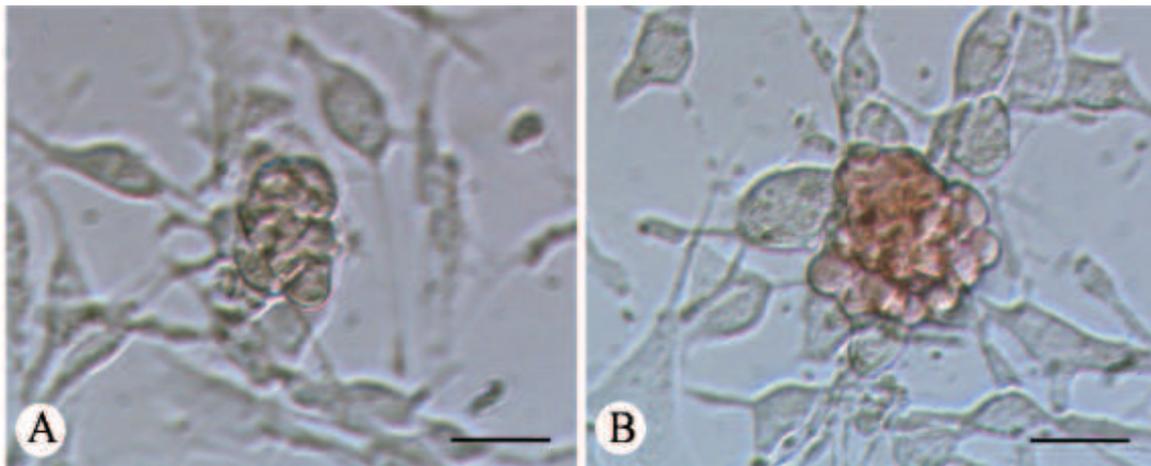


Figure 1. Development of Langerhans islets *in vitro*. A. Langerhans islet with negative α cell expressing insulin secretion, B. Langerhans islet with positive (red color) α cell expressing insulin secretion; dithizone staining, bar = 30 μ m.

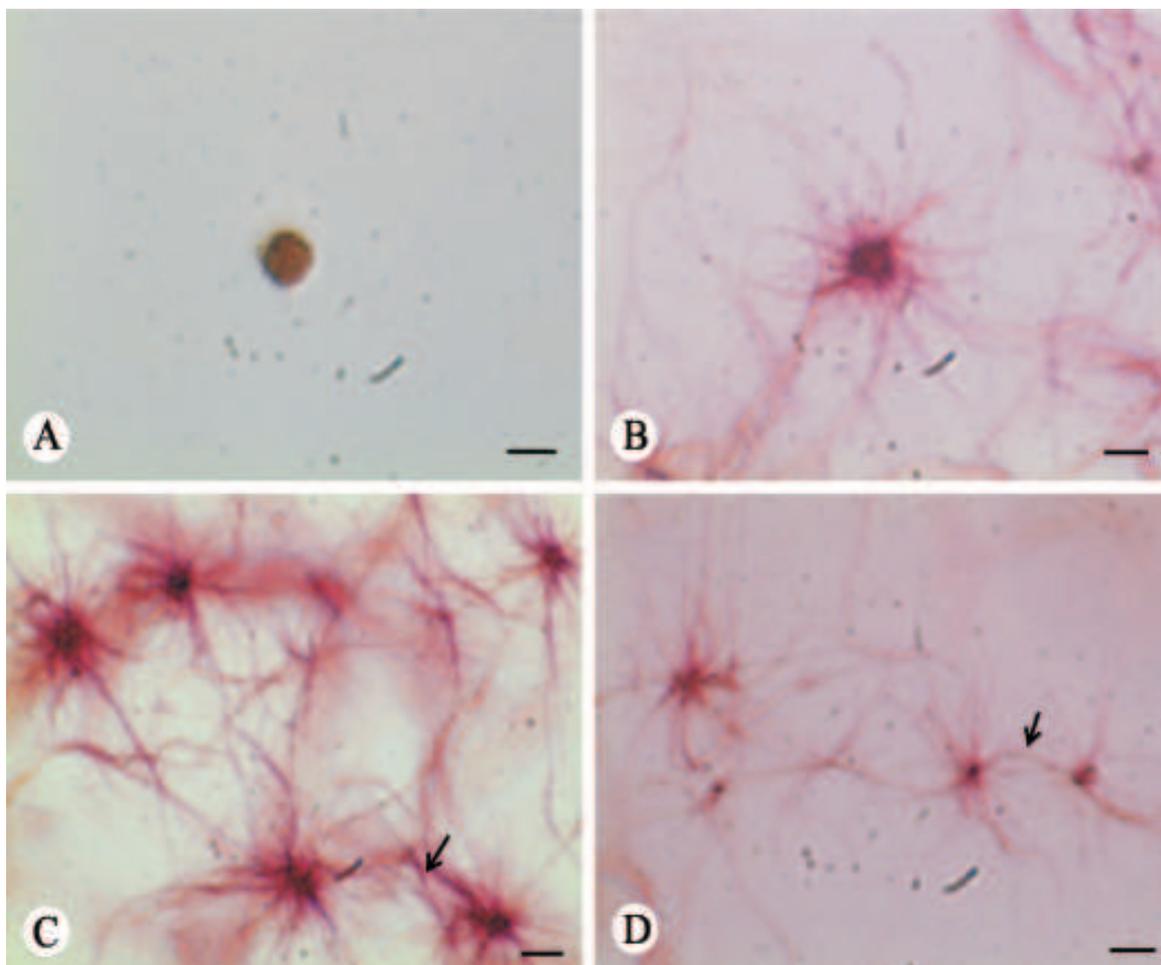


Figure 2. Morphology of osteoblast and osteocyte *in vitro* culture of rat bone cells, A. Osteoblast, B. Osteoid osteocyte, C. Mineralizing osteocyte, D. Mature osteocyte; arrow indicates canaliculi, alizarin red staining, bar = 40 μ m.

1986). Osteocyte is a mature stage of the bone cell with less GA and rough ER, and abundant of lysosome and protruded cytoplasm (Stevenson and Marsh, 1992). The number of ER will decreased in older osteocyte accompanied by the reducing size of GA. The osteocyte has dendritic shape, termed as canalicule which connect one osteocyte to the other, and morphological characteristic of canalicule is important to ensure the transportation of nutrient and other biochemical signals (Stevenson and Marsh, 1992; Bonewald, 2011). On the other hand, the differential morphology and size of the bone cell express the differentiation and development processes of the cells.

Our results showed that the NS extract was effectively induce the differentiation of osteoblast to osteocyte which supported by the positive correlation of the extract concentration and the proportion of osteocyte. The result were similar to the previously report where the diameter of osteoblast is range 20-30 μm , while it is 9-20 μm in osteocyte (Kogianni and Noble, 2007). The finding support the NS extract efficacy in inducing the osteoblast differentiation, marked by the morphological changes and the reduced size of the cells.

Highly amount of bioactive compounds in the NS, such as caroten, α -caroten, tocoferol, fatty acid, and sterol, are responsible in modulating the cells activity (Nergiz and Ötles, 1993). The phytoestrogen content of the NS showed estrogenic activity that carried out using the yeast two- hybrid assay system expressing ER β and ER α (El-Halawany *et al.*, 2011). The phytoestrogen bind the ER intracellularly and activate the transcription process (Kuiper *et al.*, 1998). The unsaturated fatty acid in the NS is found to be higher than the saturated form, one of which is linoleic acid reached 55.6% of the total fatty acid content (Nickavar *et al.*, 2003). The linoleic acid is corelated with the estrogenic activity marked by the ability in inducing the cornification of the vaginal cells in ovariectomized rats (Parhizkar *et al.*, 2011; Parhizkar and Latiff, 2013).

The other important compounds are the phytosterol - alfasterol and stigmasterol - (Ahmad *et al.*, 2013) which share a close proximity to cholesterol compound (Katan *et al.*, 2003) and these properties will be beneficial as a precursor compound of reproductive hormone, such as estrogen (Payne and Hales, 2004). The estrogen receptor in osteogenic cells are functioned in

osteogenesis which explained by the positive correlation between higher dose of estrogen and the higher rate of osteogenesis through the binding of estrogen receptor and stimulation of the cell proliferation (Ohashi *et al.*, 1991).

CONCLUSION

It was concluded that the NS has potential pharmacological activities as an anti-diabetic and anti-osteoporosis *in vitro*. The NS application in pancreatic cells culture significantly increased the proliferation of the Langerhans islet and the insulin producing pancreatic β cells. The extract also increased the proliferation of the bone cells, as well as the differentiation of the osteoblast into osteocyte.

SUGGESTION

It is needed further research to examine which active compound from NS that induce *in vitro* proliferation and differentiation of pancreatic and bone cells.

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