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Antiproliferation activity of tuber protein from *Typhonium flagelliforme* (Lodd.) blume on MCF-7 cell line

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Abstract

Typhonium sp. (*keladi tikus*) has been traditionally used as anticancer in Indonesia. This plant produces lectin as an active plant protein in the tuber. *Typhonium flagelliforme* (Lodd.) Blume is widely distributed in the country and no information about the tuber protein of this plant is available. It is necessary to evaluate lectin content of the tuber protein and its antiproliferative effect toward MCF-7 cell line. The tuber protein extracts of *T. flagelliforme* from various locations (Bogor, Singaraja, Merapi Farm, Indmira, Ogan Ilir, Matesih, and Solok) were obtained from 1, 3, 5, and 6 months old plant. The presence of lectin in those extracts was analyzed based on hemagglutination activity, while the toxicity effect was evaluated using brine shrimp lethality test. The extract with the highest lectin level and toxicity was further evaluated against human breast cancer cell line MCF-7 and fibroblast cell line for antiproliferative and cytotoxicity assay, respectively. The results showed that the lectin level of *T. flagelliforme* from all accessions increased during the growing period. The Bogor accession showed the highest lectin content, while the highest toxicity effect was found in Solok accession. Those extracts showed antiproliferative effect against MCF-7 cells, and nontoxic for fibroblast cells until 100 ppm. Therefore, the tuber protein extract of *T. flagelliforme* is potential for breast cancer treatment.

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Introduction

Typhonium sp. is a perennial herbaceous plant, known in Indonesia as *keladi tikus*, and is used traditionally to treat cancer. This genus comprises of approximately 40 species and is widely distributed in tropical-subtropical Asia and Australia (Wang and Yang, 1996). Three species has already been used as traditional herbal medicine, namely *T. flaggelliforme*, *T. trilobatum*, and *T. roxburghii*. These species have genetic relationships among each other. *T. trilobatum* has 63%, similarity to *T. roxburghii* whereas *T. flaggelliforme* has 43% similarity to *T. trilobatum* and *T. roxburghii* (Rout, 2006). Another species, *T. divaricatum* has been known to be used in traditional Chinese medicine (Luo *et al.*, 2007).

One of the bioactive constituents for anticancer activity of *T. divaricatum* is lectin (Luo *et al.*, 2007). Lectin is a bioactive plant protein that showed antiproliferative activity (Liu *et al.*, 2009). One anticancer herbal medicine product that was developed based on lectin as bioactive constituent is *Viscum album* extract. The product has been clinically tested and is used in cancer treatment via intravenous injection (Kienle *et al.*, 2011; Committee on Herbal Medicinal Products, 2013). Therefore, *Typhonium* genus as tropical medicinal plant has potential to be developed for protein-based anticancer herbal medicine.

T. flagelliforme is widely distributed in Indonesia but mostly are not cultivated that may cause a variation in the secondary metabolite content among those plants. The developmental stage of plant and the environmental conditions, could affect the metabolism and secondary metabolites content in plant cells (Lambers *et al.*, 2008). We suggested this plant has lectin in the tuber because they belong to the same genus with *T. divaricatum*. However, there is no information about the tuber protein and lectin content from various accessions of *T. flagelliforme* grown in Indonesia. Those information are necessary for the development of herbal medicine from *T. flagelliforme* to assure the safety and efficacy of the product.

The aims of this research are to identify tuber protein content and lectin activity of seven accessions of *T. flagelliforme* in Indonesia during growing period. Moreover, we determined the antiproliferative activity of the tuber protein extract. Therefore, the results of this research could provide to scientific report about tuber protein profile and lectin activity of *T. flagelliforme* in Indonesia. This information can be used for the the development of anticancer herbal medicine from tuber of *T. flagelliforme*.

Materials and methods

Plant material

Seven accessions of fresh tuber *T. flagelliforme* used in this study were obtained from different locations in Indonesia. Those have been authenticated and cultivated in greenhouse at Biotech Centre, Agency for The Assessment and Application of Technology, Indonesia (Table 1). The tubers were harvested from 1, 3, 5, and 6 months old plant and used for this research.

Tuber protein extraction

The fresh tubers from all accessions were weighed before extraction. Afterwards, the tubers were ground and stirred in 0.15 M NaCl with the volume by two fold of the tuber weight. After soaked overnight at 4 °C, the mixture was centrifuged at 5000 rpm for 30 minutes (cold condition). The supernatant was collected as crude protein extract, and the total protein content and agglutination activity were evaluated (Luo *et al.*, 2007).

Furthermore, for toxicity test, 15 g tuber of all accessions was collected from the 6 months old plant. The protein was extracted without involving NaCl with the same procedure as before. The extracts with the highest toxicity value and hemagglutination activity were used for *in vitro* pharmacological evaluation, i.e. viability test of fibroblast (normal cells) for cytotoxicity and antiproliferative assays against human cancer line MCF-7 for anticancer activity.

Total protein assay

The total tuber soluble protein from all crude protein extracts was determined by Bradford method (Bradford, 1976). Bovine serum albumin (BSA) was used as a standard protein. The absorbance at 595 nm was used to estimate the protein concentration.

Hemagglutination assay

The presence of lectin in all crude protein extracts of *T. flagelliforme* were determined by hemagglutination assay in 96-well micro titer U plate with several modifications (Oda and Minami, 1986). Fifty micro liters sheep erythrocytes (2% in phosphate buffer saline pH 7.0) was mixed with crude protein extract (150, 125, 100, 75, 50, 25, 12.5 µL). The results were read after 2 hours incubation at room temperature. Hemagglutination activity of the sample was expressed in units, the inverse of the ratio lowest volume samples with erythrocytes volume exhibiting hemagglutination (Wang *et al.*, 2000).

SDS-PAGE

The protein profiles of all tuber extracts were visualized by protein electrophoresis. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to visualize each crude protein extracts profile (Laemmli, 1970). This method used 12% separating gel and 4% stacking gel. About 17 µL samples in loading dye (1:1) were loaded in each well. Electrophoresis was carried out with mini gel electrophoresis unit (165 V constant for 45 minutes) and the bands were visualized by coomassie brilliant blue (Biorad staining solution). Unstained protein ladder (Fermentas) was used for molecular mass markers.

Toxicity assay

Rapid toxicity test for all crude protein extracts were determined by brine shrimp lethality test (BSLT) (Meyer *et al.*, 1982). The artificial sea water (38 g commercial salt in 1 L distilled water) was used as growth medium brine shrimp eggs. The incubation compartment has dark side and light side. The shrimp eggs were incubated for 24 hours in the dark side compartment. The hatched *Artemia* sp. at the light side were transferred into fresh medium and incubated in room temperature for 24 hours with sufficient illumination. Ten brine shrimp were transferred into a test tube containing 5 mL media with different concentration of crude protein extracts (5, 7.5, 10, 15, 20 ppm) and incubated for 24 hours with illuminated in room temperature. The percent mortality of brine shrimp at each concentration was measured to determine LC_{50} value.

Antiproliferative and cytotoxicity assays

Antiproliferative assay for *in vitro* anticancer evaluation of the crude protein extracts was conducted using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method (Luo *et al.*, 2007). The human breast cancer cell line (MCF-7) was provided by Centre for Medical and Pharmaceutical Technology, Agency for The Assessment and Application of Technology, Indonesia. The cell line was maintained in RPMI 1640 medium with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco) at 37 °C in CO_2 incubator (5% CO_2) for 24 hours. After plated the cell line into 96-well plate ($5x10^3$ each well) with the same incubation condition as before, various concentrations (6.25, 12.5, 25, 50, 100, 200 ppm) of crude protein extracts were added and the mixtures were incubated again with the same condition. Each well was washed with PBS to remove death cells and was added 100 µL MTT (0.5 mg/ml in PBS). Following incubation (4 hours, 37 °C, 5% CO_2), 100 µL SDS 20% was added and the plate was placed in a dark place overnight. The formazan precipitate absorption was read at 570 nm and the results were represented as LC_{50}.

The cytotoxicity was evaluated against normal human fibroblast cells. The cell line was provided by the Centre for Medical and Pharmaceutical Technology, Agency for The Assessment and Application of Technology, Indonesia. The method was similar to the MTT method as described earlier.

Results

Total tuber protein and tuber lectin of *T. flagelliforme*

All accessions of *T. flagelliforme* were grown well in

the greenhouse for 1, 3, 5, and 6 months. The fresh tuber weight and total protein from each accession increased from 1 to 6 months growing period. In 1 month old plant, Ogan Ilir accession showed the lowest tuber weight, about a quarter of the highest value of Merapi Farm accession, as well as the total protein this accession was 0.2 fold than that of the Merapi Farm accession. In 6 month-old plant, Singaraja accession showed the highest tuber weight, about 1.5 fold of the lowest value of Solok accesion. Meanwhile, the Merapi Farm accession showed the highest total protein content in 6 month-old plant, approximately 1.9 fold of the total protein content than the lowest value of the Solok accession. The increase of tuber weight was followed by the increase of total protein content. The tuber weight and the total protein content of Indmira and Solok accession in 6 month-old plant was slightly decreased (Figure 1A and 1B). The increasing of tuber weight is in correlation with the total protein content (r = 0.98). Approximately 0.15 % of the total protein yield was produced in 6 month-old plant (Table 2). These yields were increasing according to the planting time. Each tuber from each accession had no significant differences in total protein yield.

Table 1. Source of *T. flagelliforme* sample collection for this study in Indonesia

Accession	Location
Bogor	West Java
Singaraja	Bali
Merapi Farm	Yogyakarta
Indmira	Yogyakarta
Ogan Ilir	South Sumatera
Matesih	Central Java
Solok	West Sumatera

The presence of lectin in the extracts is detected starting from 1 month old plant. Furthermore, some accession such as Bogor, Ogan Ilir, Matesih, and Solok produced lectin initially in 3 months growing period. The lectin content from each accession were steadily increased from 1 to 6 months growing period and correlated with the total protein content (r = 0.58). It is indicated by the positive results in hemagglutination assay (Figure 1C). The mean value of hemagglutination activity in 6 month-old plant from all accessions was 0.56 and two accessions exhibited the highest activity, namely Bogor and Singaraja.

Table 2. Tuber protein yield of tuber *T. flagelliforme* (Lodd.) Blume during growing periods.

Accession	% Yield of Tuber Protein (w/w)*			
	1 month	3 months	5 months	6 months
Bogor	Nd**	0.15 ± 0.00	0.15 ± 0.02	0.16 ± 0.02
Singaraja	0.09 ± 0.02	0.15 ± 0.01	0.15 ± 0.03	0.17 ± 0.01
Merapi Farm	0.16 ± 0.00	0.19 ± 0.02	0.15 ± 0.00	0.19 ± 0.06
Indmira	0.16 ± 0.03	0.15 ± 0.05	0.16 ± 0.00	0.14 ± 0.01
Ogan Ilir	0.13 ± 0.05	0.15 ± 0.00	0.15 ± 0.00	0.15 ± 0.02
Matesih	0.10 ± 0.02	0.13 ± 0.04	0.15 ± 0.04	0.15 ± 0.00
Solok	0.09 ± 0.00	0.14 ± 0.00	0.17 ± 0.02	0.15 ± 0.00

* Results presented here are average tuber protein yield in average fresh tuber weight

** Nd: not detected.

Tuber protein profiles

The tuber protein profiles of each accession in each growing period were not different (Figure 2). The protein profiles showed the tuber proteins have many proteins less than 30 kDa. The protein with molecular weight more than 30 kDa was detected in 5 to 6 month-old plant.

Toxicity analysis of tuber protein extract

Therefore, the tuber protein extract collected from 6 months old plant of each accession were used in *in vitro* bioactitvity study, e.g. toxicity test, antiproliferative and citotoxicity test. Prior to the toxicity test, we reanalyzed the total protein level and lectin hemagglutination activity of those tuber extracts. Bogor accession showed the highest total protein level as well as hemagglutination activity.

Table 3. Total protein, hemagglutination activity, toxicity value of 15 g tuber 6 months old plants with water sterile extraction.

Accession	Total protein (mg)	Hemagglutination activity (unit)	Toxicity test (LC_{50} in ppm)	% Tuber Protein Yield (w/w)*
Bogor	28.51 ± 0.38	0.66 ± 0.00	19.72 ± 7.47	0.19 ± 0.00
Singaraja	17.43 ± 0.15	0.57 ± 0.15	22.77 ± 4.63	0.12 ± 0.00
Merapi Farm	21.22 ± 0.15	0.52 ± 0.13	19.84 ± 4.69	0.14 ± 0.02
Indmira	24.87 ± 0.75	0.57 ± 0.15	17.29 ± 1.17	0.16 ± 0.00
Ogan Ilir	21.85 ± 0.28	0.61 ± 0.09	16.69 ± 1.24	0.14 ± 0.00
Matesih	21.75 ± 0.10	0.61 ± 0.09	16.36 ± 1.93	0.14 ± 0.00
Solok	21.21 ± 0.05	0.55 ± 0.09	9.93 ± 1.00	0.13 ± 0.00

Data are presented as means ± SD, * Average tuber protein yield in average fresh tuber weight.

Solok accession had the lowest LC_{50} value at 9.93 ppm while that of the Singaraja was the highest LC_{50} value at 22.7 ppm. Furthermore, Bogor accession had the highest total protein level and hemagglutination activity than the other accessions (Table 3). Based on the highest toxicity effect of Solok accession and the lectin content Bogor accessions, these accessions were selected for their antiproliferative and cytotoxic activity analyses.

Antiproliferative and cytotoxicity analysis of tuber protein extract

The results of antiproliferative test with MTT method showed the tuber protein extract of Solok accession has lower LC_{50} value than Bogor. Solok accession LC_{50} was 90.78 ppm and Bogor accession was 130.93 ppm. This data showed the tuber protein extract from Solok is more capable to inhibit MCF-7 cell proliferation (Figure 3). Bogor and Solok accessions extract were also tested against normal cells (fibroblast). This assay was performed to determine the toxic effect of the extract to the non-cancer cells. The fibroblast growth was higher than that of the control when the extract was added. However, in the highest concentration, at 200 ppm, the fibroblast growth was slightly inhibited (Figure 4). These results showed that the extracts may induce fibroblast proliferation with certain concentration until 200 ppm.

Discussion

Many studies on bioactive plant protein have been conducted for medicinal purposes, especially bioactive protein commonly referred to lectins or phytohemagglutinin. In the past, this field of study were more focused on seed lectin such as *Phaseolus vulgaris* (bean), *Pisum sativum* (pea), *Lens culinaris* (lentil), and *Vicia sativa* (vetch). This is reasonable because the first lectin was found in seeds. However, vegetative tissues could be from large plant lectin storage in many plant groups (Van Damme *et al.*, 1995). Mostly, lectin biosynthesis is the same with other proteins. The lectin is synthesized at ribosome attached to the endoplasmic reticulum and transported through Golgi apparatus to the vacuole. This organelle becomes protein storage or protein bodies when it is fully loaded with protein. For

example, in monocot plants such as wheat, this protein bodies have been placed in cereal and the inner periphery of the cells without associated with cell wall at stem or leaves are become uncommon locations for protein bodies in another monocot plants (Rudiger and Gabius, 2001).

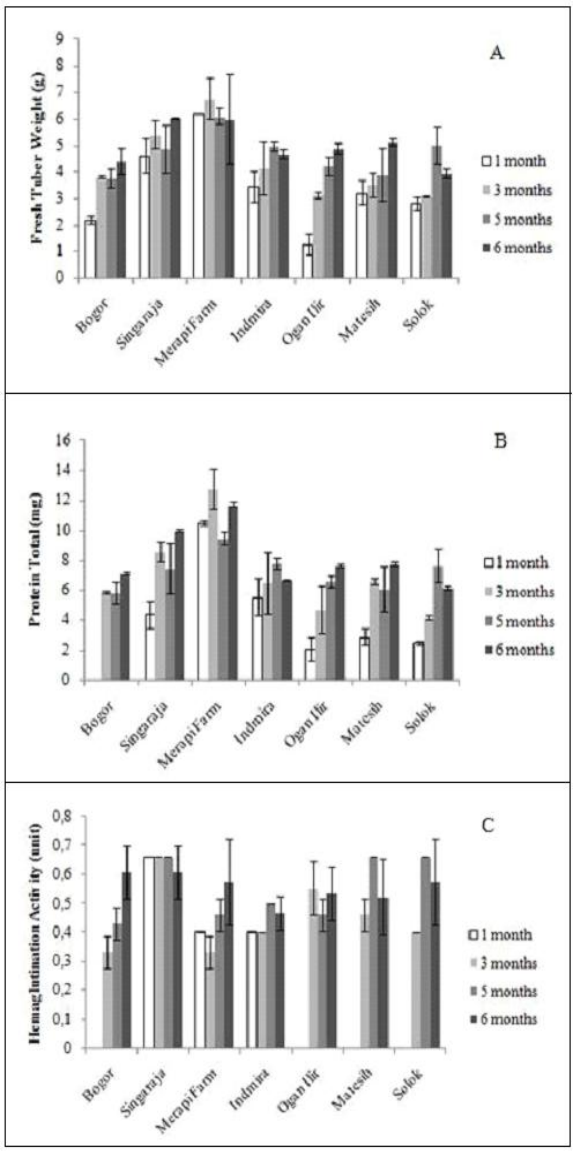

Fig. 1. The growth of tuber weight, total tuber protein, and hemagglutination activity of tuber protein extracts from *T. flagelliforme* (Lodd.) Blume during 6 months growing periods. (A) The weight of fresh tubers, (B) total tuber protein, and (C) tuber protein hemagglutination activity. Data are presented as means ± SD.

Lectin biosynthesis in plants could be induced by stress conditions or pathogen attack. Although both conditions could increase the lectin level, in normal physiological conditions, lectin was found in low content (Van Damme *et al.*, 1998). Growing periods could affect lectin content. For example, lectin level increase in germination process to 34 days after planting in wheat germ (Mishkind *et al.*, 1980). Furthermore, in *Dolichos biflorus*, there was no lectin detected in developing seeds. The increasing lectin level was shown during stem elongation and decreased after completion of elongation (Roberts and Etzler, 1984). In general, two functions of lectin in plant were as protein storage and defense. Specifically on legume, lectin has a physiological role in plant-nitrogen fixing bacteria interaction (Singh and Sarathi, 2012). The specific reaction between lectin legume and surface polysaccharide or lipopolysaccharide in bacteria causes this interaction (Sharon and Lis, 2004).

Based on lectin family, monocot mannose binding lectin (MMBL) is a lectin that can be found in most monocot plants, such as Alliaceae, Amaryllidaceae, Araceae, Bromeliaceae, Liliaceae, and Orchidaceae. This lectin can be specifically interacted with mannose and stored in vegetative tissues such as leaves, flowers, tubers, rhizomes, and roots. The MMBL content varies in each plant, tissue, and developmental stage of the plant. Approximately 0.1-5% of the total protein is lectin. However, in some plants, lectin content can be up to 50% of the total protein, such as in garlic bulb (Van Damme *et al.*, 1998). In *T. flagelliforme* from Araceae, lectin was stored in tuber. Lectin is stored in vacuoles or in other specific organs (tubers and roots). About 12% lectin is stored in the total soluble tuber protein of *T. divaricatum* and could be used as antiviral and antiproliferative human cancer (Luo *et al.*, 2007).

In this study, some accessions showed the small amount lectin in the tuber protein since 1 month after planting which are indicated by the hemagglutination activity. This showed that tuber lectin have been synthesized since young tuber (Figure 1C). This result substantiates lectin function as a protein storage in Araceae and stored in vegetative storage organs (Van Damme *et al.*, 1995). The increasing amount of tuber

lectin in *T. flagelliforme* is in correlation (r = 0.58) with the duration of growing period and total tuber protein level. It shows that lectin production occurred continuously during tuber development. The total protein and lectin level are high in the mature tuber than that of the young tuber in all accessions.

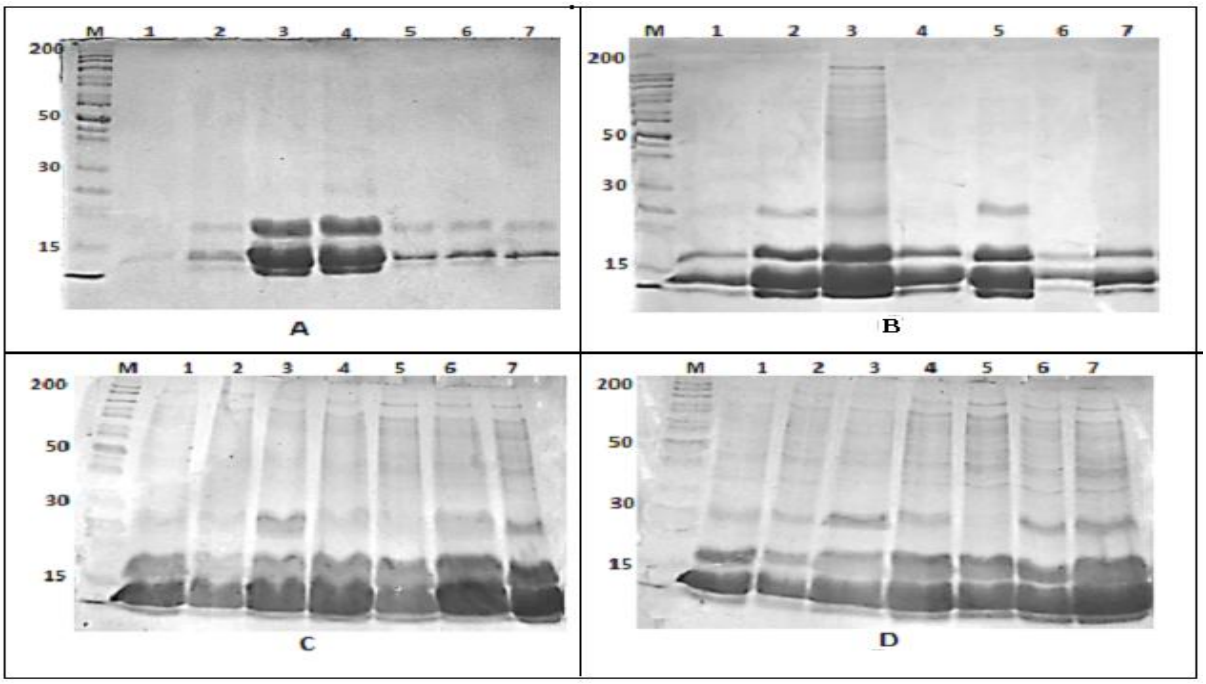

Fig. 2. Tuber protein profile of *Typhonium flagelliforme* (Lodd.) Blume in growing periods. (A) 1 month, (B) 3 months, (C) 5 months, (D) 6 months. M: Markers (kDa), 1: Bogor, 2: Singaraja, 3: Merapi Farm, 4: Indmira, 5: Ogan Ilir, 6: Matesih, 7: Solok.

The protein tuber profiles are the same from 1 to 6 months of planting time and its concentration increasing every month. The tuber also contain many low molecular weight proteins around 10-30 kDa. These proteins detected since 1 month to 6 months planting time on the electrophoresis gel (figure 2). This data supports several early researches concerning lectin from Araceae family and tuber of *Typhonium divaricatum* (L.) Decne were 12-14 kDa on the electrophoresis gel (Luo *et al.*, 2007; (Van Damme *et al.*, 1998).

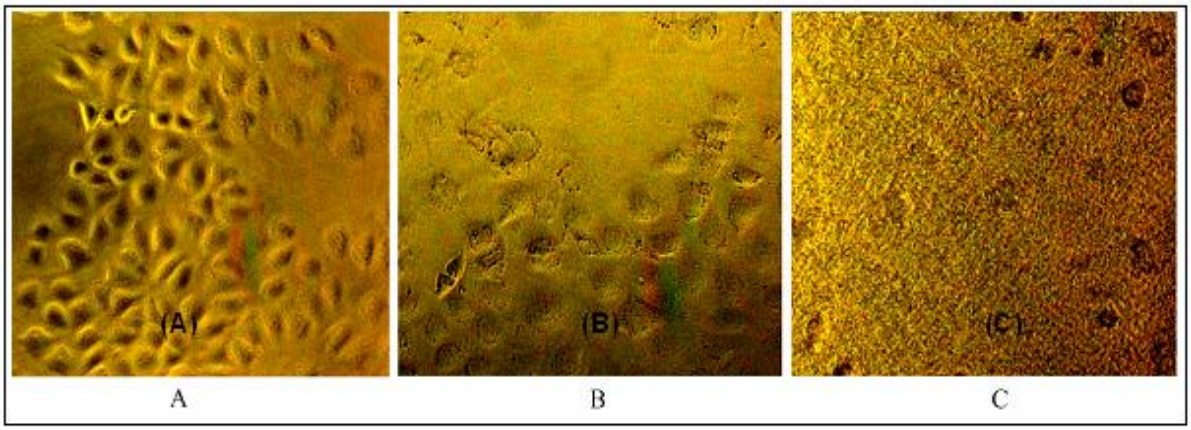

Fig. 3. Inhibition of MCF-7 cells proliferation with the tuber protein extract (40 magnifications). (A) MCF-7 control cells, (B) MCF-7 cells were added 200 ppm of tuber protein extract Bogor accession, (C) MCF-7 cells were added 200 ppm of tuber protein extract Solok accession.

The toxicity value from extract of total protein containing lectin of each accession of *T. flagelliforme* was determined by BSLT method. This method was used for rapid screening toxicity test. The extracts exhibited various toxicity values and the lowest LC_{50} of extract was tested for pharmacological evaluation using MCF-7 human cancer cell line and fibroblast as human normal cells. These extracts successfully inhibited MCF-7 proliferation and non-toxic for fibroblast cells. We assumed the lectin in the extracts affected that condition. It has been know that the lectin may inhibited or stimulated the growth of the cells. This may have occurred by the difference in interacting lectin on cell surface which caused by different sugar composition from glycoprotein on cancer cell surface and on the normal cell (Sharon and Lis, 2004).

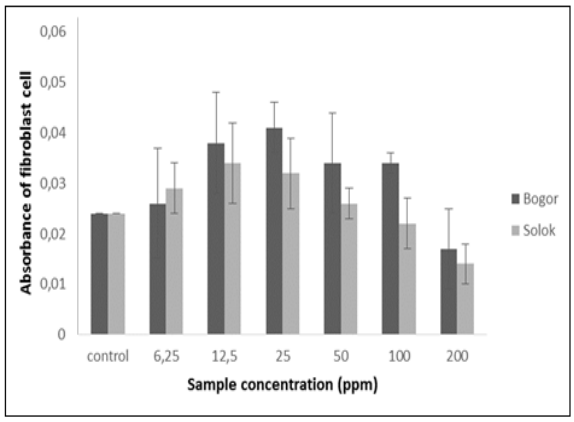

Fig. 4. Fibroblast proliferation with certain concentrations tuber protein extract. Data are presented as means ± SD.

Conclusion

It is concluded that the tuber protein extracts from *T. flagelliforme* contain lectin, detected since the first month after planting and steadily increased during growing periods. The extracts has antiproliferative activity toward cancer cell line, but not toxic in normal cells. Further work is required to purify and to identify the lectin and other bioactive proteins from this species that may very important aspect in developing of protein-based herbal medicine.

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