

## SHORT COMMUNICATION

# Bioactive Proteins from *Benincasa hispida* (Thunb.) Cogn

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The research was carried out to isolate and characterize of bioactive proteins from plant parts of *Benincasa hispida* (Thunb.) Cogn and to analyse of the toxicity and cytotoxicity of the proteins. The proteins were extracted with phosphate buffer saline, then they were precipitated using 80% saturated ammonium sulphate, continued with the dialysis using pH 7 phosphate buffer. The dialysate was fractionated through gel filtration chromatography and characterized using SDS-PAGE. The toxicity of the proteins was analyzed through brine shrimp lethality test (BSLT), followed with cytotoxicity test using HeLa and K-562 cancer cell lines. Three bioactive protein fractions were isolated from the fruits, the seeds and roots. The lowest yield of proteins was 0.021% from the fruit, then 0.051% from the seed, while the highest was 0.54% from the root. All proteins were toxic on BSLT with  $LC_{50}$  within the range of 24-39  $\mu$ g. Characterization of proteins using SDS-PAGE indicated the molecular mass of those proteins were approximately 17-29 kDa. The cytotoxicity test of the root protein showed that the protein could inhibit proliferation of HeLa cell up to 28.50% and K-562 cell up to 36.60% compared to that of non treated cell.

Key words: *Benincasa hispida* (Thunb.) Cogn, bioactive protein, cytotoxicity

## INTRODUCTION

*Benincasa hispida* (Thunb.) Cogn, known as bligo in Indonesian, is best suited to the moderately dry areas of the lowland tropics and relatively drought-tolerant. Being an annual and monoecious, this plant is propagated by seed, with the flower is insect pollinated (Rifai & Reyes 1994). In Indonesia, bligo is commonly found at home garden, grown on trellises or trained over the roof. It is also cultivated as a cash crop in the field without support.

Bligo plant is used as food, while all parts of the fruit can be used as medicine. The fruit juice shows significant activity against the symptoms of morphine withdrawal and also suppresses symptoms of opioid withdrawal in mice (Grover *et al.* 2000). The fruit extract produces healing effect on experimentally induced ulcers in rats (Grover *et al.* 2001). It has been reported that the fruit contains certain active compound which have antioxidant effects, which could inhibit gastric mucosal injury by scavenging free radicals and repressing production of superoxide dismutase on rats (Shetty *et al.* 2008).

The methanol extract of the fruit is useful as anti-obesity agent, due to its ability to significantly reduce the cumulative food intake (Kumar & Vimalatani 2004). The alcoholic extract of the fruit can significantly reduce the blood glucose level to a normal level on alloxan induced diabetic rat and this extract

is equipotent with the standard Tolbutamide drug (Battu *et al.* 2007). In addition to above peculiarities, several bioactive proteins also being found on this plant. The protein including the osmotin like protein, a member of pathogenesis related protein (Shih *et al.* 2001b), and other two proteins from the seed, a chitinase that has lysozyme activity (Shih *et al.* 2001a), and a serine proteinase inhibitor that inhibit biologically important molecule such as trypsin, human leucocyte elastase, and cathepsin G (Atiwetin *et al.* 2006).

The research was carried out to isolate and characterize other bioactive proteins from plant parts of *Benincasa hispida*, including fruits, seeds, and roots, which have toxic effect on brine shrimp nauplii and cytotoxic activity on cancer cell. The protein toxicity was analyzed through brine shrimp lethality test (BSLT) and followed by a cytotoxicity using cancer cell line.

## MATERIALS AND METHODS

**Plant Propagation.** Seeds were sown in polybags inside the greenhouse. After germination and they were large enough, the seedlings were planted into individual pots with a rich compost and transferred to the experimental field of IPB Baranangsiang. The plant growth was observed, including time of germination, time of flowering and time of fruit harvesting.

**Extraction of Proteins.** The protein extraction was performed in accordance with the procedure of Di Toppi *et al.*

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(1996) with some modifications. Fruits, seeds and roots were washed with tap water and grounded in cold phosphate buffered saline (PBS), consist of 0.14 mM NaCl in 5 mM pH 7 Na phosphate buffer, homogenized on the same buffer with the ratio of 1:4 (w/v). Each homogenate was stirred overnight at  $\pm 4$  °C, filtered and the supernatant was centrifuged at 10,000 g for 25 minutes. The protein was precipitated by 80% saturated ammonium sulfate, then the extracted protein was dialyzed with 5 mM pH 7 Na phosphate buffer using cellophane. After the insoluble material were removed by centrifugation, the dialysates were lyophilized.

**Gel Filtration Chromatography.** Protein fractionation using gel filtration chromatography also followed the procedure of Di Toppi *et al.* (1996). Crude proteins were diluted on 5 mM pH 7 Na phosphate buffer, subjected to gel filtration on Sephadex G-75, equilibrated and eluted in the same buffer. The excluded proteins were detected from their absorbance at 280 nm wavelength, then the same fractions were pooled and lyophilized.

**Polyacrylamide Gel Electrophoresis.** Proteins were incubated within 1% sodium dodecyl sulfate (SDS) and 1% 2-mercaptoethanol at 50 °C for 2 hours. Electrophoresis of the proteins was done on a polyacrylamide slab consisting of 13% separating gel and 5% stacking gel in the presence of 0.1% SDS. The gel was stained with Coomassie brilliant blue (R-250) and the molecular mass retention time was calculated and calibrated with the low molecular weight (LMW) protein standards. The standards were lysozyme (14 kDa), tyrosine (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (45 kDa), and albumin (66 kDa).

**Brine Shrimp Lethality Test (BSLT).** A water system was set up with temperatures between 28 and 30 °C, 30-35 ppm salinity, pH range of 8-9 and a strong aeration under a continuous light regime. Approximately one teaspoon of brine shrimp eggs was added into the system. After approximately 48 hours of hatching, the phototropic naupliis were collected with a pipette from the lightened side and concentrated in a small beaker applied for testing. Different concentrations (1000, 100 & 10  $\mu\text{g/ml}$ ) of the proteins were prepared. These protein samples were taken into separated test tubes. Twenty brine shrimps were transferred to each test tube using micro pipettes. After 24 hours, the test tubes containing different concentrations of protein samples were observed and the number of survived nauplii in each test tube was calculated. From this result, the percent lethality of nauplii was calculated with each concentration represents each sample. The value of  $\text{LC}_{50}$  was calculated using a Finny computer program.

**Cytotoxicity Assay on Cancer Cell Lines.** HeLa (human cervic carcinoma) cells were maintained as monolayer cultures in RPMI 1640 medium supplemented with antibiotics penicilline-streptomycin and 10% fetal bovine serum in a humidified incubator containing 5%  $\text{CO}_2$  at 37 °C. Subcultures were obtained by trypsin treatment of confluent cultures. The K-562 (human erythro leukemia) cell lines were grown in suspension in the same medium. The cells were plated in 100  $\mu\text{l}$  medium in 96 microwell plates at a density of  $10^5$  cells/ml for HeLa and  $5 \times 10^4$  cells/ml for K-562, and the plates were

placed in a 37 °C, 5%  $\text{CO}_2$  incubator. One day later the cell culture medium was added with 100  $\mu\text{l}$ /well medium containing the indicated concentrations of the protein in triplicate. After 3 days of treatment, the cells were harvested. We compared the different concentration of each protein in inhibiting the growth of both cell lines, using the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] dye uptake method. Each cell was added with 10  $\mu\text{l}$  of 0.5 mg/ml of MTT and maintained at 37 °C in 5%  $\text{CO}_2$  incubator for 4 hours to allow MTT to be converted to formazon crystals by reacting it with metabolically active cells. The reaction was stopped by adding acidic isopropanol ((34  $\mu\text{l}$  HCl in 10 ml isopropanol) and the cell viability was measured at 570 nm using a plate reader (Wang *et al.* 2000).

## RESULTS

**Plant Propagation.** Seed germination was completed within 2 weeks after sowing. The plant grew was well enough, with the flowering stage was started about 9-10 weeks after planting, while the fruit was harvested after 16-20 weeks after planting. The fruit sizes were ranging from 35-50 cm x 15-25 cm. Materials used as source of protein were mature fruits and their seeds, and also the roots from harvested plant.

**Extraction of Proteins.** The result of protein extraction was described by protein yield, that is the ratio between the proteins which resulted from the extraction process and the extracted materials. The protein yield was considerably higher in the root than in the seed, with lowest was from the fruit (Table 1). The root protein was 24 times higher than seed protein, and 3.5 times than the fruit protein.

**Gel Filtration Chromatography.** Protein fractionation using gel filtration chromatography could separate proteins according to their size or molecular weight. Using Sephadex G-75 as a matrix results in porosities that could be passed by protein with molecular mass about 3-80 kDa. Smaller molecule diffuses further into the pore of the beads and moves through the bead more slowly, while larger molecule enters less or may be not at all and thus moves through the beads more quickly (Janson & Ryden 1998). The chromatography elution profile (Figure 1) showed the fractionation seed and fruit proteins, with both of them could be separated into four peaks, while the root protein could only be separated into three peaks. All protein fractions were analyzed for their toxicities through BSLT and the best fraction was elected according to the high yield and toxicity (data not shown). The three elected protein fractions were resulted from the fruit, the seed and the root.

Table 1. Protein yields and their fractions from fruit, seed and root of *Benincasa hispida* (Thunb.) Cogn also their activities on BSLT

Plant parts	Before fractionation		After fractionation		
	Yield (% fresh weight)	$\text{LC}_{50}$ ( $\mu\text{g/ml}$ )	Protein fractions	Yields (% fresh weight)	$\text{LC}_{50}$ ( $\mu\text{g/ml}$ )
Fruit	0.05	44	Fruit	0.021	39
Seed	0.35	41	Seed	0.051	24
Root	1.22	50	Root	0.540	36

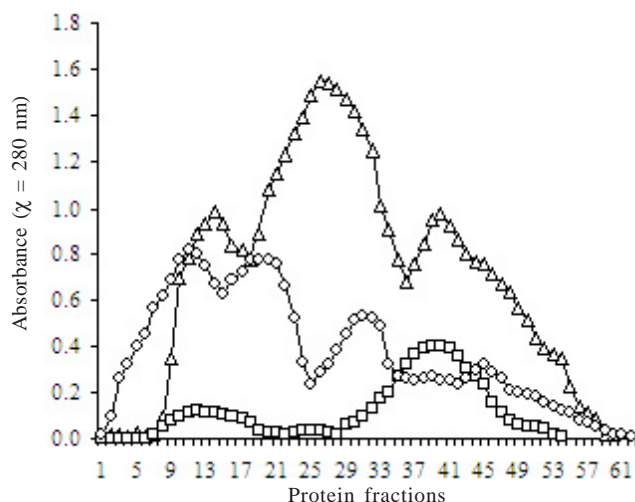


Figure 1. Chromatography elution profile of the root, fruit and seed proteins of *Benincasa hispida* (Thunb.) Cogn on fractionation using gel filtration chromatography.  $\Delta$ : root,  $\square$ : fruit,  $\circ$ : seed.

**Polyacrylamide Gel Electrophoresis.** SDS-PAGE pattern of each protein fraction was visualized by Coomassie brilliant blue staining, showed on Figure 2. All of the protein fractions showed more than one sub unit molecular mass. The molecular mass of fruit protein fraction was 28.5 kDa, the seed protein fractions were 17 and 29 kDa, and the root protein fraction was 27 kDa.

**Brine Shrimp Lethality Test (BSLT).** The  $LC_{50}$  of the protein is the concentration which may kill or inactivate 50% of the test subject.  $LC_{50}$  is inversely proportional to the toxicity of a compound, means that the lower the  $LC_{50}$ , the higher the toxicity. As evidence from the Table 1, each of the sample tested was toxic within 24 hours in different concentrations. The rate of lethality or mortality increased as concentration increased. The percent mortality of  $LC_{50}$  was calculated using a Finny computer program. The result indicates that before fractionation, the seed protein showed the most toxic protein against the brine shrimp naupliis, followed by the fruit and the root protein. After fractionation, the seed protein was still considered as the most active, but the root protein was more active than the fruit protein. In the control all of the naupliis were alive after 24 hours. So it could be said that, the toxicity found in the experiment may be representative for the toxic property of the protein.

**Cytotoxicity Assay on Cancer Cell Line.** The correlation between concentration of root protein fraction and their cytotoxic effect on HeLa and K-562 cells was investigated with MTT assay. The treated cells, with protein concentration ranging from 10-50  $\mu\text{g/ml}$ , showed that the root protein fraction was effective in inhibiting the growth of both two cell lines tested for 72 hours. The cytotoxicity of root protein fraction was carried out in the range of 5.03-28.54% growth of the HeLa cell lines and 5.31-36.6% growth of the K-562 cell lines. The inhibitory effect of the fraction was dose-dependent (Figure 3). The protein addition showed different mortality rate at different concentrations and was increased along with the increasing concentration of the protein.

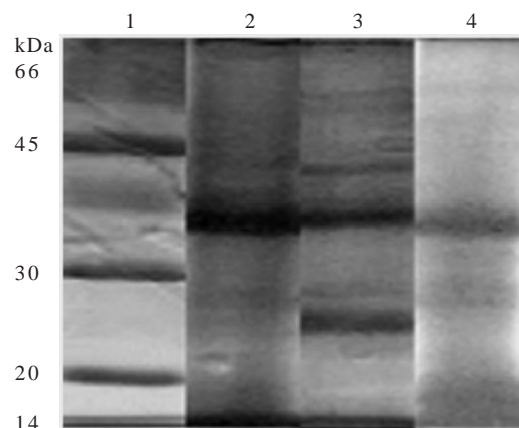


Figure 2. SDS-PAGE of protein fractions from plant part of *Benincasa hispida* (Thunb.) Cogn: 1: protein standard LMW, 2: fruit protein fraction (28.5 kDa), 3: Seed protein fraction (17 & 29 kDa), 4: root protein fraction (27 kDa).

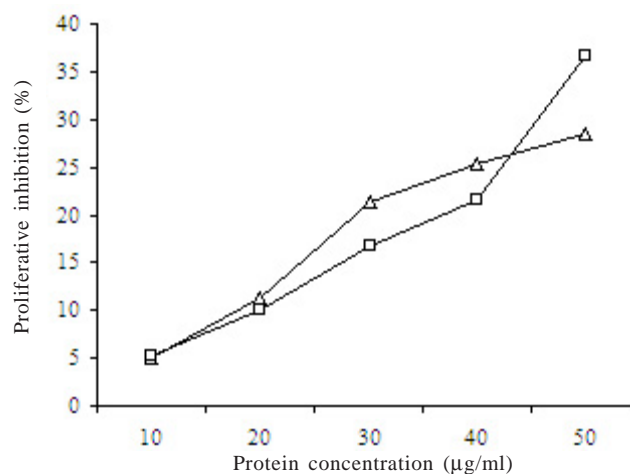


Figure 3. Comparison of the *in vitro* cytotoxicity of the root protein fraction on cancer cell lines HeLa ( $\Delta$ ) and K-562 ( $\square$ ) using MTT method.

## DISCUSSION

Bligo grew well on the experimental field of IPB Baranangsiang as in other fields, therefore the sample could represent other bligo plant in general. According to Rifai and Reyes (1994), seed germination is usually completed within 1-2 weeks, followed by flowering within 7-11 weeks after planting and then the fruit needs about 1-2 months from anthesis until it reaches full maturity.

Comparing all proteins, the lowest yield was 0.05% resulted from fruit protein caused by high water content up to 96% from the edible parts (Rifai & Reyes 1994)., The highest yield was resulted from root protein, approximately 3.62% from fresh weight extracted material. Therefore, fruit is not recommended as the source of protein but still can be useful for another applications, whereas the plant root was suggested for further investigation.

Proteins extracted from bligo plant resulted from this research have different properties compared to several proteins from bligo plant, such as chitinase which has lysozyme activity (Shih *et al.* 2001a), osmotin-like protein, a

pathogenesis related protein from the plant seed which has molecular mass of 28 kDa (Shih *et al.* 2001b), and serine proteinase inhibitor (Atiwetin *et al.* 2006). The isolated protein on this reseach indicated an antiproliferative activity on cancer cell line, although the addition of the protein into the culture cell until 50 µg/ml did not reached the IC<sub>50</sub>. However, protein addition showed different inhibition proliferation rate at different concentrations. The rate increases as the the protein concentration increases.

Another protein showing an antiproliferative activity was protein of 5,422 Da, isolated from the seeds of small scarlet runner beans (*Phaseolus coccineus* 'Minor') designated as phaseococcin. It inhibits proliferation of leukemia cell lines HL60 and L1210 (Ngai & Ng 2005). A protein of 38 kDa from *Casparis spinosa* seeds, inhibits proliferation of hepatoma HepG2 cells, colon cancer HT29 cells and breast cancer MCF-7 cells with an IC<sub>50</sub> of about 1.40 and 60 mM, respectively (Lam & Ng 2008).

Antiproliferative protein was also found on mushroom. For example, a hemagglutinin with a molecular mass of 12 kDa was isolated from the fruiting bodies of the mushroom *Flammulina velutipes*. It inhibits proliferation of leukemia L1210 cells with the IC<sub>50</sub> of 13 µM (Ng & Ngai 2006). Ribosome inactivating protein (RIP) 9,567 -Da was isolated from fresh fruiting bodies of the mushroom *Hypsizigus marmoreus*. The protein was designated as marmorin, inhibits proliferation of hepatoma Hep G2 cells and breast cancer MCF-7 cells, with the IC<sub>50</sub> of 0.15 iM, 5 iM, respectively (Wong *et al.* 2008).

There has not been any previous research discussing the inhibitory effect of *Benincasa hispida* root protein on proliferation of cancer cell line, but other researchers reported the advantage of Benincasa against cancer, for example Huang *et al.* (2004) reported their studies on the antioxidation and inhibition abilities of angiotensin-converting enzyme (ACE) activity of various part of Benincasa by different extraction methods. Higher antioxidant capability of the seed may results from higher total phenolics contents and superoxide dismutase activity. The antioxidation and ACE activity inhibition abilities may provide protective effects against cancers. Then Lee *et al.* (2005) asserted that seed extract of this plant is useful on angiogenesis by decreasing the bFGF-induced endothelial cell proliferation and tube formation in a dose-dependent manner.

It is recommended to conduct a further research to determine the proliferation inhibiting mechanism of the protein. Moreover, this research contributes in increasing the value of Benincasa plant, not only as vegetables, but it could also be more developed as a medicine to treat cancer.

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