International Journal of Chemistry and Pharmaceutical Sciences

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Research Article **Open Access**

Antitumor Activity (*In-vitro*) of Extracellular Polysaccharide Produced by Ropy *Lactobacillus delbrueckii* ssp. *bulgaricus* Isolated from Tradisional Fermented Milk

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A B S T R A C T

An extracellular polysaccharide (EPS) was purified by ethanol precipitation from culture supernatant of Ropy strains of *Lactobacillus delbrueckii* ssp. *bulgaricus*, a lactic acid bacteria that was isolated from commercial yoghurt. The EPS was produced under aerobic conditions in a medium Skim Milk Reconstitution (SMR) 10% (w/v). This EPS have studied to potential health – promoting effect as antitumor activity. The objective of this study was to determine of potential of the EPS on proliferation of tumor cell in vitro. EPS with different concentration (0.01; 0.02; 0.05; and 0.001 mg/ml) was examined in vitro for antitumor activity on Leukemia K-562 and Hela cell. Result of these studies had showed that EPS in doses 50 µg/ml can inhibited of 45.4 % of tumor leukemia and 59.2% for tumor human cervix (Hela cell).

Keywords: antitumor activity, *Lactobacillus bulgaricus*, antiproliferation, exopolysaccharide (EPS).

A R T I C L E I N F O

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Article History: Received 15 March 2016, **Accepted** 25 April 2016, **Available Online** 27 May 2016

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Manuscript ID: IJCPS2950

PAPER-QR CODE

Citation: Ratmawati Malaka, *et al.* Antitumor Activity(*In-vitro*)of Extracellular Polysaccharide Produced by Ropy *Lactobacillus delbrueckii* ssp. *bulgaricus* Isolated from Tradisional Fermented Milk. *Int. J. Chem, Pharm, Sci.*, 2016, 4(5): 263-266.

1. Introduction

Microbial exopolysaccharides often show clearly identified properties that form the basis for wide range of applications in food as industrial thickeners, gels and stabilizers (Malaka, 1997; Malaka 2005), pharmaceutical, petroleum,

and other industries. Polysaccharides with have potential as antitumor against certain allogenic tumors have been isolated from diverse sources including higher plants, fungi, lichen, bacteria and yeast. The nature of their antitumor action is not entirely clear, but the polysaccharides from botanical sources cannot be shown to exert any direct action on tumor cell. Their antitumor action must therefore be considered to be dependent on the reaction of the host or cell (Sasaki *et al*., 1987).

Kitazawa *et al.* (1990) have been shown that Lactococcus lactis subsp. cremoris isolated from Scandinavian ropy sour milk "viili" would protect the growth of S-180 tumor in ICR mice. A later study (Kitazawa, Yamauchi and Itoh, 1992) showed a significant increase of the B-cell dependent mitogenic activity induced by the slime material products from L. lactic subsp. cremoris KVS 20. The yoghurt starter *Lb. delbrueckii* subsp. *bulgaricus* OLL 1073R-1, which produces an EPS, has been reported to exert a host-mediated antitumor activity (Maduri and Prabhakar, 2014). By in the long time ago in the Eastmidle Asia was recommended that fermented milk have beneficial health effect and recovery from many diseases (Tamime and Robinson, 1985). Futhermore, this effect was known that because of microorganism are contains by lactic acid bacteria in fermented milk. So that the lactic acid bacteria produced polysaccharide excreted extra cell (finally known as exopolysaccharide EPS) where have beneficial function as medicine or food addictive (Cerning, 1990; Malaka, 1997 and Malaka, 2005). There is much hypothesis that need confirmed about the tumor activity of LAB such as *Lactobacillus bulgaricus* include it production EPS are (1) direct inhibition to procarsinogens by binding, blockading, or remove the carsinogen: (2) reduce bacteria produce enzyme that convert procarsinogens to carsinogens; (3) decrease intestinal pH cause of reduce the microbial activity and bile salt; reduce the transit time of pathogen bacteria thorough intestinal so reduce the toxin as procarsinogen; (4) activated the host immune system (Salminen and Wright, 1993; Fooks *et al*., 1999).

The name exopolysahharides (EPS) as proposed by Sutherland in 1972 provides a general term for all these form of bacterial polysaccharides found outside the cell wall. Based on the composition of EPS, the polymer separated into two major groups that homopolysaccharides (for example dextran and mutan) and heteropolysahharides (EPS of *L. bulgaricus*). There have been reported that bacterial bacterial EPS was generally used as food addictive to increase the food quality, antihipertention, antivirus, cosmetic, or anti AID'S. One of the bacterial EPS that have produced by commercial scale are "Curdlan" is EPS from *Alcaligenes faecalis* var *myxogenes* (Malaka, 1997). According the Yamanaka (1992) showed that EPS LAB have antitumor effect on tumor growth in mice. Mice by intravenous implantation with Melanoma B-16 tumor cell high metastation 5.1 x 10^5 cells and treatment with EPS show the inhibited the metastases of tumor cell to pulmonary system 29.4%. Treatments by EPS per oral have the effect for increase the antibody immune system. Studied about antitumor activity by milk fermented culture starter such as L. bulgaricus not focus to its EPS production but still focus to LAB self (Cerning, 1990). However, researchers suggested that the role important to antitumor activity are polysaccharide as metabolite product by LAB (Sasaki et al., 1978; Kelkar et al., 1988; Kitazawa et al., 1993; Shiraguchi et al., 1994). The researches not yet isolated EPS before examined to animal laboratory. The objective of this study was to determine of EPS extracted from L. bulgaricus in reconstituted skim milk medium on the ability to inhibited proliferation of Leukemia and Hela cell line in vitro.

2. Experimental

Propagation and medium of *Lactobacillus bulgaricus*

Lactobacillus bulgaricus was isolated from commercial ropy yoghurt and routinely propagated in Reconstituted Skim Milk (RSM) 2 times a week. The research was conducted in Biotechnology Laboratory of Research Center, Hasanuddin University, Indonesia.

Production and Isolation of Exopolysaccharide (EPS)

Exopolysaccharide produced by *L. bulgaricus* was doing in 10 liter bioreactor used medium according to the modified of Malaka (2004). The medium was used Reconstituted Skim Milk (RSM) 10% + glucose 1% + sodium citric 0.5% where incubated at 30^{o}C for 16 hr with initial pH 6.5. Exopolysaccharide was isolated by Shellhaass Methods (1983) modified that flocculants from cell-free medium was added the isopropyl alcohol and storage in 4^{o}C before centrifugation (4000 x g for 10 min). The crude EPS was freeze dried and storage in -18^{o}C before use in experiment.

Tumor cells

Leukemia K-562 and Hela cell tumor were obtain from Laboratory of Tissue Culture, Division of Clinical Pathology, Department of Reproduction and Pathological, Faculty of Veterinary Medicine, Bogor Agriculture University. The tumor cell have been maintained with sterile condition for prevent virus and bacterial contamination by penicillin-streptomycin with doses 100 IU-100 μg/ml administration.

Antiproliferation test in vitro methods

The leukemia K-562 and Hela tumor cells (1 x 10^5 cells) were incubated in a CO_2 incubator at 37oC in a medium containing EPS suspension in various concentration (0; 1; 5; 10; 50 μg/ml). The each treatment repeats for tree times. One milliliter of suspension of line cell K-562 and Hela cell (10^5cells/ml) in 24 tissue culture well plate added with EPS. Each plate was incubated at 37^{o}C, 5% CO_2. The medium was added 20% of calf serum. The cell was harvested if the control cell in optimal growth condition (if the cell was covered 70% of inside of well plate, about 3 – 4 days). The medium was resuspensed with 500 μl of 2 mM EDTA-PBS solution. The cell removed from the well plate by trypsin 0.05%. The total cells were measurement by Haemocytometer Neubauer. The cell was stained with 0.17% of trypan blue and view by light microscopic (100 x magnification) (Yamamoto, 1994; Priosoeryanto *et al*., 1995a, b; 2001; 2002 a, b, c). The growth activity (%) was calculated by the following equation:

$$\% \text{ growth activity} = \frac{\textit{mean of total number the tumor cell treatment}}{\textit{mean of total number the tumor cell control}} \times 100\%$$

3. Result and Discussion

Table 1. shows the effect of EPS of *L. delbrueckii* ssp. *bulgaricus* by different doses on the proliferation of human leukemia cell (LeukemiaK-562). Antitumor activity process on Leukemia K-562 tumor cell at a dose 50 µg/ml) showed significantly inhibited growth cell by 45.4%. It was noteworthy that even 50 µg/ml treatment caused a well defined proliperative inhibition effect.

The result suggested that the leukemia cell decrease the cell number and the size of cell by direct contact of EPS. The phenomena may cause of sytolitic activity of EPS. Generally EPS can give a sytolitic effect in recovery of infection disease and tumor (Zheng *et al.* (2006). Exopolysaccharide as macromolecule with high of weigh molecule mass are a primary effect on inhibition of cell tumor growth. This effect may cause of direct sytosidal action effect on cell, like generally biological mechanism. Macromolecules disturb the cell metabolism by division cell inhibition. The experiment suggested that the tumor cell growth inhibiting activity because of potential inhibition proliferation effect and cell colony formed. Antitumor activity process on Hela tumor cell at a dose 50 µg/ml) showed significantly inhibited growth cell by 59.2 %. The effect of EPS on Hela cell most effective than on Leukemia cell on inhibition of growth of cell.

Several EPS have been shown to posses' immunological activities with potential pharmacological applications as biological response modifier (BMRs). BMRs are agents that alter the normal immune response and whose mechanisms of action include induction of cytokines. Research on pharmacological applications of BMRs has led to development of both immunosuppressive and immunostimulating drugs that are effective in preventing the rejection of transplanted organs, for the treatment of some autoimmune diseases, as cancer immunotherapy. A number a bacterial EPS have been identified as BMRs and have been found to have the ability to stimulate tumor rejection (Ruiz-Bravo *et al.*, 2001).

Although polysaccharides are considered to be T-cell-independent antigens, a number of microbial EPS are immunomodulators, with activities for T cells and macrophages. The possibility of inhibition mechanism on tumor cell was suggested. Polysaccharide A, a component of the capsular complex of *Bacteroides fragilis*, possesses mitogenic activity fot T lymphocytes and the production of interleukin-2 (IL-2) by $CD4^+$ T cells appears to play an essential role in the in vivo (Tzianabos, 2000). A number of fungi and yeast produce β-(1-3)-glucans with imunomodulatory properties. Studies on the mechanisms of immunomodulatory by a soluble derivate of β-(1,3)-glucan have shown that it has the ability to prime granulocytes and macrophages for enhanced cytokine release, reactive nitrogen intermediate production, and bactericidal capacity in response to a secondary stimulus.

Table 1: Effect of EPS of *L. delbrueckii* ssp. *bulgaricus* on Leukemia K-562 (Erythroleukemia cell line)

Treatment of EPS (doses in µg/ml)	Mean of cell (x 10^4)	% growth	% Inhibition
Control (0.0)	69.2	100	0
1	59.2	85.5	14.5
5	56.6	81.8	18.2
10	44.4	64.2	35.8
50	37.8	54.6	45.4

Table 2: Effect of EPS of *L. delbrueckii* ssp. *bulgaricus* on Hela cell (Human Cervix Tumor)

Treatment of EPS (doses in µg/ml)	Mean of cell (x 10^4)	% growth	% Inhibition
Control (0.0)	75.0	100	0
1	64.6	86.1	13.9
5	61.8	82.4	17.6
10	48.6	64.8	35.2
50	30.6	40.8	59.2

4. Conclusion

Exopolysaccharides of *Lactobacillus delbrueckii subsp. Bulgaricus* shows of potensial application as antitumor. Result of these studies had showed that EPS in doses 50 µg/ml can inhibited of 45.4 % of tumor leukemia and 59.2% for tumor human cervix (Hela cell).

5. Acknowledgements

This work was supported by grant HIBAH BERSAING from DIKTI (Directorate General of Higher Education) Jakarta, Indonesia.

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