Cytotoxicity Assay of Ethyl Acetate Extract Shimeji (*Lyophyllum shimeji* (Kawam.) Hongo) and White Oyster Mushroom (*Pleurotus ostreatus* Jacq.) Against HCT-116 Cell Line

N Rossiana[1]*, Madihah[1], A A Nur[1], B Mayawatie[1] and P Andayaningsih[1]

[1]Departement of Biology, Faculty of Mathematics and Life Sciences Universitas Padjadjaran, Jl. Raya Bandung-Sumedang Km. 21 Jatinangor 45363 telp./fax. : 022-7796412

*E-mail: niarossiana@yahoo.com

Abstract. Cancer is a serious of health problem. Anticancer agent discovery is currently directed toward natural resources, including the edible mushrooms. The purpose of this research was to obtain the edible mushroom extract and to evaluate the bioactivity of the extracts in inhibiting the proliferation of colorectal cancer cell line (HCT-116) using MTT-based cytotoxicity assay. This experimental study was performed using 2 factorial Randomized Complete Design, where the first factor was the mushroom species i.e. *Lyophyllum shimeji* and *Pleurotus ostreatus,* while the second factor was concentration of the extract i.e. 0 (control), 1, 10, 100 and 1000 ppm. In this study, the proliferation inhibition percentage of HCT-116 cell line was measured and then analyzed by probit to determine the IC_{50} value. The results showed mushrooms extract were cytotoxic for HCT-116 cell line, but only the concentration factor that influenced the proliferation inhibition of HCT-116 cell line. IC_{50} values from shimeji and white oyster mushrooms extracts were 111.09 and 239.46 ppm, respectively. Thus, shimeji mushroom extract was more effective for proliferation inhibition HCT-116 cell line and potentially developed as natural anti-colorectal cancer in the future' drugs discovery.
Keywords: HCT-116, IC_{50}, *Lyophyllum shimeji*, MTT-based cytotoxicity assay, *Pleurotus ostreatus.*

1. Introduction

Cancer is defined as the rapid, uncontrolled and non-rhythmic abnormal cell growth that can infiltrate and surpress the growth of normal tissue thus affecting the function of living body[1]. According to the *Global Burden Cancer* (GLOBOCAN)[2] and the *International Agency for Research on Cancer* (IARC), in 2012, there are several types of death cases worldwide due to cancer. Additionally, among types of cancer, colorectal cancer, remains on the 3rd place with estimated percentage of cases approximately 17.2% and considered as death cause of nearly 8.4% of human population, after lung and breast cancer.

The remarkably high numbers are associated with unhealthy diet such as the consumption of fast food with high fat content [3]. High fat consumption facilitates the absorption of carcinogenic compounds into the body and slows the transport time to the intestine [4].

Anticancer agents from natural products are able to treat the source of disease by repairing damaged cells, tissues or organs through enhancement of immune system. Indeed, various methods of cancer treatment have been developed recently, for instance by exploring the natural products as anticancer agents [5].

As one of the natural product, wild mushrooms have an extensive potential not only as food source (edible mushrooms), but also as medicinal ingredients (mycomedicine) [6]. Screening of a bioactive compound against the cancer cells needs to be conducted by performing the cytotoxicity test. One of the most advace method is the MTT *based cytotoxicity assay* (3-[4,5-*dimetilthiazol-2yl] -2,5 diferenil tetrazolium bromide)*. This method is used to determine the effectiveness of a cytotoxic agent against cell proliferation inhibition. The IC_{50} value (*Inhibition Concentration* 50%) shows the concentration value resulting in a 50 % inhibition of cell proliferation and indicates the potential toxicity of particular compound to the cells tested [7].

To date, the study of potential anticancer agent from ethyl acetate extracts of Shimeji (*L. shimeji*) and white oyster mushrooms (*P.ostreatus)* against colorectal cancer cell line is less documented. The most commonly used model of colon cancer cells in humans for *in vitro* studies is the HCT-116 cell line [8]. Therefore, it is necessary to test the potential anticancer agent from ethyl acetate extract of two edible mushroom *L. shimeji* and *P.ostreatus* to inhibit the proliferation of HCT-116 cell line.

2. Materials and methods

2.1 Mushrooms preparation and extraction

A total of 500 g of *L. shimeji* and *P.ostreatus* was cleaned and cut into small pieces and then dried at room temperature (30°C). Mushrooms were then macerated for 3x24 h using ethyl acetate solvent then filtered and evaporated with a rotary *rotary* evaporator until the extract is obtained in the paste form.

2.2 Cytotoxicity test using MTT assay

Cytotoxicity test of two mushrooms extracts with MTT assay was performed in a plate which consists of 96 wells. Briefly, a total of 10^4 cells of HCT-116 was inoculated into each well and a 200 µL of RPMI (Roswell Park Memorial Institute) medium was added, then incubated in a 5% CO_2 incubator for 24 hours. Afterwards, when the incubation was done, a mixed of 200 µL fresh RPMI medium dissolved with shimeji and white oyster mushrooms extracts was added with a series of final concentration: 0 (control), 1, 10, 100 and 1000 ppm. The culture was then reincubated in 5% CO_2 incubator for 48 h. After incubation, 100 µL of medium was taken from each well and 10 µL of MTT *kit* stock solution was added. The culture was incubated for 2 – 4 h. Afterwards, MTT *kit* and medium were removed and DMSO (Dimethyl sulfoxide) was added (100 µl) as a stop reaction. The absorbance value of the cell suspension was observed at 450 nm by using the Multiwell plate reader.

2.3 Data and analysis

The inhibition of HCT-116 cell line population was calculated using formula from Freshney [7], as follows :

$$\%\text{Inhibition} = \frac{\text{Average absorbance of sample}}{\text{Average absorbance of control}} \times 100\,\%$$

Afterwards, probit analysis was performed to determine IC_{50} value. A series of concentration from 0 to 1000 ppm was converted into log 10, whereas the percentage of inhibition was converted into probit form. The IC_{50} value was determined from the equation of two points where 50% of the inhibition is situated in between the two concentrations. The result of the equation was then converted into an antilogical form.

The probit constant as follows:

$y = ax + b$

$a = \frac{Y_2 - Y_1}{X_2 - X_1}$

$b = Y_1 - aX_1$ or $Y_2 - aX_2$

Given the fact that probit 50 is equal to 5 (y = 5), therefore:

IC_{50} or $x = \frac{y - b}{a}$

where :
y : probit 50% of inhibition
x : concentration of 50% of inhibition
b : probit from the percentage of inhibition
Y_1 : probit percentage of inhibition on concentration 1
Y_2 : probit percentage of inhibition on concentration 2
X_1 : log concentration 1
X_2 : log concentration 2
: The probit equation of two levels of concentration

In this study, analyses of variance (ANOVA) with two factors were performed using JMP Pro 11. Normality and homoscedasticity were examined by Kolmogorov-Smirnov and Levene test, respectively. Differences in percentage of inhibition (I%) between different groups of mushrooms species was analyzed. Additionally, Tukey-HSD multiple comparison tests were used when differences were observed to determine which means were significantly different.

3. Results and discussion

Results of ethyl acetate extract test of shimeji *(L. shimeji)* and white oyster mushrooms (*P. ostreatus)* against HCT-116 cell line is provided in table 1. In this study, percentage of inhibition at 0 ppm as the positive control did not show any inhibition process of HCT-116 cell line proliferation. In contrast, the exposure of extracts on HCT-116 from 1 to 1000 ppm showed that the percentage of inhibition of this cell line was positively correlated to the concentration of mushrooms extract.

Table 1. Average percentage of inhibition of HCT-116 cell lines

Concentrations (ppm)	I%	
	L. shimeji	*P. ostreatus*
0 (control)	0	0
1	29.13±0.19	37.76±0.18
10	28.9±0.19	37.73±0.14
100	48.38±0.2	43.07±0.14
1000	79.93±0.18	61.22±0.07

Note : Data was presented in form of $\bar{x}$ ± Strandard Deviation

Our result showed that the average percentage of inhibition of *L. shimeji* against HCT-116 ranged from 28.9 – 79.93 %, while *P. ostreatus* ranged from 37.73 – 61.22 %. These results indicate that both shimeji and white oyster mushroom extracts have the potential to inhibit the proliferation of HCT-116. The mean percentage of inhibition from shimeji and white oyster mushrooms against the HCT-116 was tend to be similar at range of concentration between 1 – 10 ppm and increased at higher range of concentration (100 – 1000 ppm).

The cytotoxic activity from both shimeji and white oyster mushrooms is allegedly due to the presence of bioactive compounds, for instance β-glucans which preventative in enhancing immunity, acts as antitumor and inhibiting the formation of cancer cells (carcinogenesis) as well as inhibiting the proliferation of cancer cells [9]. Indeed, β-glucan compound is a type of polysaccharide with a D-glucose monomer which can be bonded via β-(1,3) and β-(1,6) glucoside bonds with high molecular weight, and belongs to homopolysaccharide compound, which is the polysaccharide that composed of one type of sugar molecule [10]. The β-glucan compounds are hypothesized to have several bioactivities including as anticancer, anti-cholesterol and anti-inflammatory. Moreover, β-glucan compound is contained in bacterial cell wall, fungi and higher plants. This compound has received a safe recommendation from the Food and Drug Administration (FDA) for human consumption [11].

The mechanism of action of β-glucans can actually stimulate non-specific immune systems (naturally) and demonstrate anticancer activity in various ways. For instance, by modulating the immune mechanisms in the body, including by activating effector cells such as macrophages, lymphocytic T cells and natural killer (NK) cells, in order to secretes cytokines such as TNF-α, IFN-γ, IL-1β and other compounds that inhibit the proliferation of cancer cells as well as inducing apoptosis and differentiation of tumor cells. Initially, β-glucans that were studied and explored was mostly from medicinal mushrooms such as *Ganoderma lucidum, Tremetes versicolor, Cordyceps sinensis, etc*. Nonetheless, recently, application of β-glucans from the edible mushrooms has received great attention, due to their safety and long-term use [12].

Confirmation of observation of MTT-based assay on samples that experienced proliferative inhibition can be verified via discoloration after addition of dimethyl sulfoxide (DMSO). As in control (0 ppm), wells that containing HCT-116 culture have a thick purple color after 48 h of incubation. The intensity of the dark purple color is due to the large number of cells that can actively metabolize in producing mitochondrial reductase enzymes, which can react with the methylthiazol tetrazolium salt (MTT) to form purple formazan crystals. This result is consistent with the result from [8] that the intensity of purple color formed on MTT assay is proportional to the number of living cell. Thus, a higher number of living cells resulting excessive intensity of purple color [13].

At the wells containing HCT-116 cell lines with 1-1000 ppm of mushroom extracts, decreased in color intensity occurs after the addition of DMSO. Degradation in color intensity is due to the breaking of formazan crystals into pale purple or even transparent. Additionally, reduction in color intensity is also due to the decrease in proliferation of cancer cells when they are exposed to mushroom extracts. It is suspected that mushroom extracts can decrease the number of cells, hence they are not able to produce mitochondrial reductase enzyme. This condition prevents the formation of formazan crystals that produce purple color, as consequence of the death of cells due to the presence of mushroom extracts.

In this study, IC_{50} of the ethyl acetate extract from shimeji mushrooms against HCT-116 cell line was 111.09 ppm. On the other hand, IC_{50} of the extract from the white oyster mushroom was significantly higher to that of shimeji mushroom (239.46 ppm, figure 1). Based on these results, the extracts from both mushrooms species have potential as a source of anticancer agents with concentration less than 1000 ppm. This confirms the statement from [7] that a substance potentially possesses cytotoxic properties against cancer cells when the IC_{50} value is less than 1000 ppm, whereas non-toxic when it's more than1000 ppm.

The ANOVA results demonstrated that the concentration factor (C) contributed to the significant different of treatment against the proliferation inhibition of HCT-116 cell line as response (table 2). In contrast, mushrooms species factor (S) did not give any significant difference to the treatment. Furthermore, the interaction factors between species and concentration (SC) displayed no interaction occurs. Unobservable interaction between species and concentration factors was perhaps due to the similarity in bioactive properties of the two mushrooms, since both types come from the similar class (Basidiomycetes). Furthermore, both mushrooms were extracted using similar solvent, which is ethyl acetate.

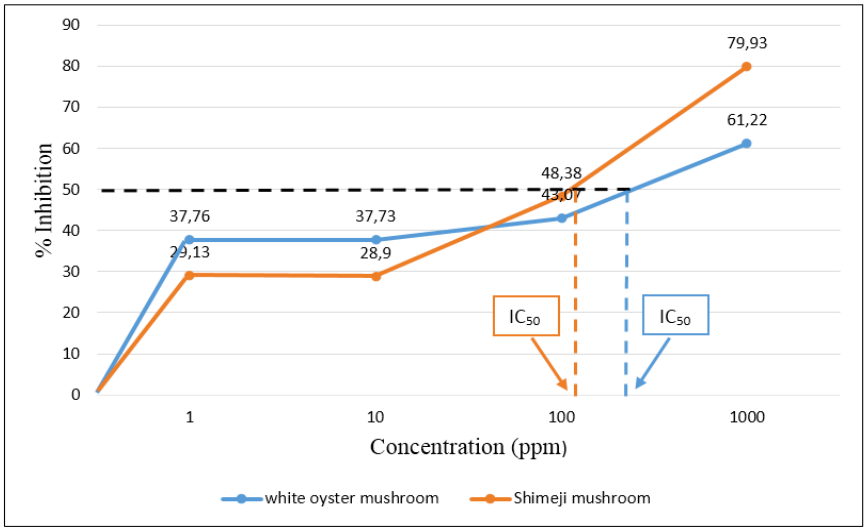

Figure 1. Percentage of inhibition (I%) HCT-116 cell line with IC_{50} value

Table 2. ANOVA two factors of cytotoxicity test from two types of mushrooms extract against the proliferation of HCT-116 cell line

Source	df	SS	MS	$F_{calculated}$	F_{table}	P value
Mushroom species (S)	1	0.011	0.011	0.414	4.35	0.527
Concentration (C)	4	1.964	0.491	17.815*	2.87*	0.000
Interaction (SC)	4	0.121	0.030	1.101	2.87	0.383
Error	20	0.551	0.028			
Total	30	17.668				

Note :
(*) : Significant difference is observed with 5% of confidence level

4. Conclusion

This study revealed that the IC_{50} values of ethyl acetate extracts of shimeji and white oyster mushroom in inhibiting the growth of HCT-116 cell line were 111.09 and 239.46 ppm, respectively. In addition, the ethyl acetate extract of shimeji mushroom is considered to be more effective in inhibiting the HCT-116 cell line proliferation and needs to be investigated further, especially as an anti-colorectal cancer agent.

5. Recommendation

However, further study on cytotoxicity from each mushroom extracts at optimum concentration needs to be performed on several cancer cells. Secondly, isolation of bioactive compounds in mushroom extracts is highly suggested in order to determine the biologically active compounds that are potentially to be developed as anticancer agent.

Acknowledgements

The authors wish to thank to the Academic Leadership Research Grant from the University of Padjadjaran, granted to Dr. Nia Rossiana.

References

[1] Bellizzi K M 2012 *Cancer and Aging Handbook : Research and Practice* (New Jersey, John Willey & Sons, Inc) pp 35-37
[2] Global Burden Cancer (GLOBOCAN) 2012 Estimated Cancer Incidence, Mortality, Prevalence and Disability adjusted life years (DALYs) Worldwide in 2008. IARC Cancer Base No. 11
[3] Winarto E P, Ivone J and Saanin S N J 2009 *JKM* **8**(2) 138-143
[4] Sabiston D C 1995 *Buku Ajar Bedah* (Jakarta: EGC)
[5] Harvey A L 2008 *Natural Product in Drug Discovery* (Glasgow: Elsevier) 894-901
[6] Hendritomo H K 2010 *Jamur Konsumsi Berkhasiat Obat* (Yogyakarta: Lily Publisher)
[7] Freshney R I 2010 *Culture of Animal Cells 10th Edition* (New Jersey: John Willey & Sons,Inc) 373-376
[8] Ahn E H and Schroeder J J 2002 *Exp Biol Med* **227** 345-353
[9] Tjokrokusumo D 2015 *Pros SemNas Biodiv IV Indo.* **1** (6) 1520-1523
[10] Omoregie E S and Sisodia B S 2012 Invitro Antiplasmodial Activity and Cytotoxicity of Leaf Extract of *Jatropha tanjorensis* J.I Eliis and Saroja *Bayero Journal of Pure and Applied Science* **5**(1) 90-97.
[11] Spicer E J, Goldenthal E and Ikada T A 2005 *Toxicolodial Assesment of Curdlan*. Available at http://www.betaxanthin.com/toxicologi research.html
[12] Akramiene D, Kondrotas A, Didziapetriene J, and Kevelaitis E 2007 *Journal of Medicina (Kaunas)* **43** (8) 597-606
[13] Junaedi, S. 2000. *Uji Sitotoksik metode MTT. (*Yogyakarta: Cancer Chemoprevention Research Center Fakultas Farmasi Universitas Gadjah Mada) pp18