Phytochemical analysis and in vitro cytotoxicity test of black soybean (*Glycine soja* L.) ethanolic extract as a growth inhibitor of the HCT-116 colon carcinoma cell line

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**Abstract.** The incidence of colorectal cancer is the third highest of all cancers in Indonesia and worldwide, with a high mortality rate and varying degrees of therapeutic success. Black soybean extract has potential as an anticancer agent because it has some secondary metabolites, such as flavonoids and alkaloids, that have cytotoxic effects on cancer cells. This study aimed to determine the cytotoxic effect of black soybean ethanolic extract on HCT-116 colorectal cancer cell line. Thin-layer chromatography (TLC), phytochemical screening tests and the MTT assay were performed. TLC revealed that there were six components of different compounds in black soybean ethanolic extract. Phytochemical screening tests identified six classes of chemical compounds, namely, alkaloids, tannins, flavonoids, saponins, triterpenoids and glycosides. MTT assay of black soybean ethanolic extract on HCT-116 cells exhibited a half-maximal inhibitory concentration (IC50) value of 97.56 μg/ml with moderate cytotoxic properties compared with cisplatin, used as a positive control, which had IC50 of 55.51 μg/ml with moderate cytotoxic properties. Based on statistics, there was a significant differences in absorbance values between groups of concentrations (p = 0.001). Black soybean ethanolic extract had a cytotoxic effect on HCT-116 cell growth.

1. **Introduction**

Diseases which are increasing in frequency worldwide have shifted from communicable to non-communicable diseases, one of which is cancer. Cancer is one of the top 10 causes of death in the world [1]. Cancer has caused 8.8 million deaths in 2015 [2]. Colorectal carcinoma is the third most prevalent cancer worldwide and the second highest cause of death in the United States [3]. Based on GLOBOCAN 2012 data, colorectal carcinoma has been ranked as the third most prevalent cancer in Indonesia, with an incidence of 12.8 per 100,000 population [4].

Along with the increasing incidence of colorectal carcinoma, various treatments, ranging from promotive to curative, continue to be developed. Early detection of carcinoma leads to increasing success of the treatment. However, most patients with colorectal carcinoma are identified at advanced and late stages of the disease, with low life expectancy. This is mainly because of the lack of specific early symptoms that are apparent to the patient and the lack of patient’s
knowledge about these early symptoms. Treatments for advanced and late stages of colorectal carcinoma are adjuvant radiotherapy and chemotherapy, which have numerous side effects [5]. The use of various adjuvant therapies in addition to radiotherapy and chemotherapy for treating colorectal carcinoma has recently been increasing.

Various studies have been conducted on the effect of black soybean on growth of various cancer cells. Park et al. suggested that anthocyanin in black soybeans may inhibit cell growth through mutation of APC tumour suppressor gene by decreasing oxidative stress and inflammatory response [6]. Shin et al. concluded that anthocyanin extracted from Vitis coignetiae inhibited HCT-116 colon carcinoma cell line, with an IC$_{50}$ value $<$ 60 µg/ml [7]. Another study showed that black soybean extracts have an IC$_{50}$ value of 3.69 mg/ml against gastric cancer cells [8], whereas similar studies has also shown the effect of black soybean extracts on HT-29 cell growth, suggesting that delphinidin, an anthocyanin with the highest inhibitory potential, can inhibit 30%–66% growth of HT-29 cells at a dose of 50 µM [9].

Based on the description above, we considered it necessary to conduct studies on the cytotoxic effects of black soybean (Glycine soja L.) extracts on other colorectal carcinoma cells. Human colorectal carcinoma cells commonly used for in vitro research are HCT-116 and HT-29. Black soybean extracts have shown a significant inhibitory effect on HT-29 cell growth. However, HCT-116 and HT-29 cells have several differences, such as aggressiveness, differentiated ability and nitrite oxide synthase activity [10]. These differences encouraged our group to investigate cytotoxic effects of black soybean extract on HCT-116 cell growth.

2. Methods
This was an in vitro study on the effect of black soybean crude extract on HCT-116 colorectal carcinoma cells. Black soybeans were obtained from Lombok, West Nusa Tenggara. The black soybeans were ground into a smooth powder using a simple grinder. The dry powder was mixed with acidified ethanol solvent [40% (v/v) ethanol, containing 1% (v/v) HCl] in a glass container for 24 h. The mixture was then filtered using filter paper into a container. The separation was repeated three times to make sure that all extracts from black soybean were pooled into the filtered solution. Later, black soybean ethanol extract was heated to 57°C–60°C in a rotary evaporator to evaporate the ethanol solvent (for approximately 2 h). The final extract was termed as crude extract because no further separation of the extract component was made. Characterisation of the crude extract was conducted in two ways, namely, thin-layer chromatography and phytochemical screening tests.

The HCT-116 colorectal carcinoma cell line was obtained from the Department of Anatomical Pathology, Faculty of Medicine, Universitas Indonesia. The cells were incubated in a culture medium in a CO$_2$ incubator at 37°C and 5% CO$_2$ for 1 week. The cells were harvested and suspended (in DMEM medium) and 10 µL of the cell suspension was mixed with 90 µL blue step. Number of cells in the suspension was then calculated using a haemocytometer under an inverted microscope at 80× magnification. The density of the cell suspension was then adjusted to 10,000 cells/wells in 100 µL of the growth medium, with the cell suspensions being re-incubated in the CO$_2$ incubator [11,12].

Ten milligram of the dried black soybean crude extract was weighed and added to 1 ml dimethyl sulfoxide (DMSO; 10%) and homogenised. The crude extract solution was then added to 950 µL of medium, containing DMEM, FBS (10%) and streptomycin and penicillin (1% each), and mixed till it was homogeneous so that the working solution had a black soybean crude extract concentration of 10,000 µg/mL. Dilutions were then done to eight doses of the soybean extract: 800, 400, 200, 100, 50, 25, 12.5 and 6.25 µg/mL [12]. The cell with its medium in the well was removed from the incubator after reaching 50% confluence or after 24 h. The medium in the well was discharged until only left the well plate with cell attached to the bottom of the plate. Thereafter, the extract concentration was marked in each well with each of three wells per dose (three technical replicates or triplicates). Finally, 100 µL of extract from each dose for the
treatment group or 100 μL medium (without extract) for the control group were inserted into the corresponding cell well, mixed using a micropipette until it was homogeneous and then re-incubated for 48 h [12].

After incubation, 100 μL of MTT (5 mg/mL) was added to each treatment or control well. Then, cells were incubated for 4 h in the CO₂ incubator at 37°C. An inverted microscope was used for detecting formation of formazan crystals, which were dissolved by adding 100 μL DMSO containing 10% stopper. Absorbance at a wavelength of 515 nm was then measured for each well using an ELISA plate reader; the absorbance values were used to calculate the percentage inhibition of HCT-116 cell growth by black soybean crude extract [12]. The percentage of cell life was obtained by:

\[
\text{Percentage of cell life} = \frac{100 \times (\text{OD control medium} - \text{OD subject})}{\text{OD cell control} - \text{OD medium control}}
\]

Then the inhibition in percent was calculated by (100 %-% cell life).

Data were processed using SPSS software version 20. Analysis was performed using the Saphiro–Wilk test to determine normality of data of each group and check for the significance of inter-group data. The Kruskal–Wallis test and post-hoc test with Mann–Whitney were performed to check the significance of each group. P value < 0.05 was considered to be statistically significant.

3. Results
3.1. Thin-layer chromatography
This qualitative analysis showed that there were six components of different compounds in the black soybean crude extract (Figure 1). Retention factor (Rf) for each component of the compound is shown in Table 1.

![Figure 1. Thin-layer chromatography test results of the black soybean extract.](image-url)
Table 1. Retention factor (Rf) value of each component of the compound in the black soybean crude extract.

| Compound | Rf value |
|----------|----------|
| 1        | 0.818    |
| 2        | 0.727    |
| 3        | 0.575    |
| 4        | 0.454    |
| 5        | 0.394    |
| 6        | 0.333    |

3.2. Phytochemical tests
Results of the phytochemical tests conducted on the black soybean (Glycine soja L.) crude extract are presented in Table 2. The crude extract contained alkaloids, flavonoids, tannins, triterpenoids, glycosides and saponins.

Table 2. Phytochemical screening results of the black soybean crude extract.

| Group/metabolite | Black soybean crude extract |
|------------------|-----------------------------|
| Alkaloid         | +                           |
| Flavonoid        | +                           |
| Tannin           | +                           |
| Triterpenoid     | +                           |
| Steroid          | -                           |
| Glycosides       | +                           |
| Saponin          | +                           |

3.3. Comparison of MTT test of black soybean crude extract and cisplatin on HCT-116 cell and IC_{50} value
Absorbance value of cell and medium control groups is shown in Table 3. Based on the regression equation of % inhibition versus log concentration, IC_{50} of the black soybean crude extract on HCT-116 cell growth was 97.56 μg/ml (Figure 2) compared with that of 55.51 μg/ml of cisplatin (Figure 3).

Table 3. Absorbance value of cell and medium control groups.

| Group           | OD       | Average | % Inhibition |
|-----------------|----------|---------|--------------|
|                 | 1 | 2 | 3 | SD |                |
| Cell control    | 0.836 | 0.782 | 0.781 | 0.03 | 0.800 | 0 |
| Medium control  | 0.140 | 0.141 | 0.142 | 0.00 | 0.141 | - |
The graph above is a plot of the extract concentration (X-axis) and % inhibition at each concentration (Y-axis) of the black soybean crude extract on HCT-116 cells. The plot results were then analysed by linear regression to obtain the linear line equation that was used for determining the IC\textsubscript{50} value of the black soy crude extract in this study. Based on the linear line equation obtained, IC\textsubscript{50} of the black soy crude extract on HCT-116 cells is 97.56 μg/ml.

\[ y = 37.677x - 24.95, \quad R^2 = 0.9811 \]

**Figure 2.** Percentage inhibition of HCT-116 cell growth by the black soybean crude extract.

The graph above is the plot of cisplatin concentration (X-axis) and inhibition percentage at each concentration (Y-axis) of cisplatin on HCT-116 cells. The plot results were then analysed by linear regression to obtain the linear line equation that could be used for determining the IC\textsubscript{50} value of cisplatin as a positive control. Based on the linear line equation obtained, (\( y = 26.873x + 3.1233 \)) IC\textsubscript{50} of cisplatin on HCT-116 cells is 55.51 μg/ml.

\[ y = 26.873x + 3.1233, \quad R^2 = 0.9746 \]

**Figure 3.** Percentage inhibition of cisplatin on HCT-116 cell growth

4. **Discussion**

In Table 4, the % inhibition of HCT-116 cell growth after treatment with black soybean crude extract showed an increase with increasing concentrations. The lowest concentration (6.25 μg/ml)
had the lowest inhibition (9.2%), whereas the highest concentration (800 μg/ml) had the highest inhibition (85%). The IC\(_{50}\) of the black soybean crude extract on HCT-116 cells was 97.56 μg/ml. Interpretation of IC\(_{50}\) values is based on that of the National Cancer Institute.\(^\text{[13]}\) The value of IC\(_{50} ≤ 20 \)μg/ml indicated strong cytotoxic properties, IC\(_{50} 21–200 \)μg/ml indicated moderate cytotoxicity, IC\(_{50} \)201–500 μg/ml indicated weak cytotoxicity and IC\(_{50} ≥ 500 \)μg/ml indicated no cytotoxic properties.

Therefore, IC\(_{50}\) of the crude extract of black soybeans in this study showed moderate cytotoxic properties. Kim et al. determined IC\(_{50}\) values for individual fractions of black soybean extract against MCF-7 cells and determined IC\(_{50}\) values of 100–250 pg/ml. However, compared with the study described by Kim et al. (2008), cyanidin and delphinidin, active anticancer components of black soybean extract against HT-29 colorectal carcinoma cells, had IC\(_{50}\) values of approximately 14.4 and 17.0 pg/ml \(^\text{[9]}\), respectively these values indicate that active substances in black soybean have strong cytotoxic properties. Another study on anticancer effects of black soybean, conducted by Zou et al. showed a cytotoxic effect against AGS gastric cancer cells with IC\(_{50}\) of 3690 μg/ml, indicating no cytotoxic properties. This result may have been due to the use of black soybean extract concentrations in the range 1–5 μg/ml in the study.

The IC\(_{50}\) value for cisplatin in the current study was 55.51 μg/ml, indicating moderate cytotoxic properties; similar to the result for the black soybean crude extract. From the proven anticancer properties of cisplatin, we would have expected strong cytotoxic effects and a lower IC\(_{50}\) value. The relatively high IC\(_{50}\) value of cisplatin and the black soybean crude extract in this study may have occurred because of several reasons, such as undissolved formazan during the MTT assay. After reduction of MTT by living cells, formazan is formed as a purple insoluble compound. Formazan can be dissolved using various solvents. The solvent used in this study was DMSO. Although DMSO is known to be the best solvent for formazan, it has some disadvantages during the MTT assay, such as the loss of formazan crystals when the culture medium is removed and added to PBS because of complete dissolution of formazan crystals. The loss of some of the formazan crystals would result in an underestimate of the concentration of formazan. This error could be prevented using 10% SDS in HCl as a stopper solution or as a formazan solvent.

The other cause was the cells in each well less dense. The number of cells/ well affects the efficacy of absorbance, particularly in the control group. The control group is expected to have a 100% live cell percentage and hence a very high absorbance value because all living cells reduce MTT to formazan crystals. However, in this study, absorbance values of the control group were not significantly different from those of the lowest concentration treatment group (6.25 μg/ml). This may indicate that the number of cells per well used in this study, i.e. 10,000 cells/well did not reduce all the MTT to formazan. A previous study showed that the optimal value for the number of cells per well is approximately 200,000 cells/well \(^\text{[14]}\).

Next, high IC\(_{50}\) values of the black soybean crude extract and cisplatin in this study could also be caused by the selection of inappropriate wavelengths in the ELISA reader. The wavelength used for measuring absorbance in the well has an optimal value, related to the principle of absorbance, transmission and reflection of light. Wavelength selection errors may occur which result in inappropriate absorbance values. This is because a substance with a certain colour can absorb at several different wavelengths. In this case, the purplish-blue formazan absorbed the orange-yellow light wavelength (λ = 550–600 nm), but in the current study, the wavelength used was 515 nm, which corresponds to green light; therefore, the light from the well was partially absorbed and partly reflected. The most appropriate wavelength for achieving the optimal formazan absorbance value is 562 nm \(^\text{[15]}\).

The last part of the MTT performed is the significance test for comparing absorbance values in each group. The significance test used was the Kruskal–Wallis test, which is used for unpaired numerical comparative analysis of > 2 groups \(^\text{[16]}\). Results obtained from the Kruskal–Wallis significance test was that there was a significant difference in the absorbance value between the control group and each treatment group and between treatment groups. The Mann–Whitney post-
hoc test was performed on each group to identify significant differences between groups. Of the concentration groups tested, the concentrations of 12.5 and 800 μg/ml showed significant differences with the control group and with other tested concentrations.

5. Conclusion
The black soybean ethanolic extract (or crude extract) gave positive results for the presence of alkaloids, flavonoids, tannins, saponins, triterpenoids and glycosides, which are molecules with demonstrated anticancer potency. This black soybean crude extract showed moderate cytotoxic levels.

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